Development and validation of a UPLC-MS/MS method for studying the degradation kinetics of ofloxacin and trimethoprim during the application of solar Fenton process in secondary treated sewage
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ABSTRACT
In this work, a sensitive and highly selective method was developed and validated to study the degradation of two antibiotic compounds (ofloxacin (OFX) and trimethoprim (TMP)), spiked in secondary treated domestic effluents, by the solar Fenton process. Three different chromatographic columns were tested on a ultra performance liquid chromatography-tandem mass spectrometric (UPLC-MS/MS) instrument working in the electrospray ionization (ESI) mode with twelve combinations of eluting solvents. Samples were enriched prior to the analysis by solid phase extraction using the hydrophilic–lipophilic balanced (HLB) reversed phase polymeric sorbent. The method was optimized and showed very good performance characteristics and was successfully applied to study the degradation kinetics of the selected antibiotics during the solar Fenton process applied. The degradation was found to follow a pseudo first-order kinetics for both compounds at initial concentration of 100 μg L⁻¹ with \( k = 0.0345 \text{ min}^{-1} \) for OFX and \( k = 0.0768 \text{ min}^{-1} \) for TMP, whereas the complete removal was achieved after 120 min of treatment for both compounds.

Key words | antibiotics, ofloxacin, solar Fenton, trimethoprim, UPLC-MS/MS, wastewater

INTRODUCTION
The occurrence of pharmaceuticals in various compartments of the aquatic environment worldwide has received considerable attention in recent years as these compounds have been implicated for negative effects on flora and fauna (Loganathan et al. 2009). Within the large group of pharmaceuticals, antibiotic compounds are of special interest because of their potential to accelerate widespread bacterial resistance to antibiotics. Wastewater treatment plants (WWTPs) are considered as the major contributors of these compounds in the environment (Fatta-Kassinos et al. 2011).

Conventional wastewater treatment processes are not specifically designed to remove antibiotic residues, and the degree at which they are removed appears to be highly variable with performance apparently being dependent upon specific operational conditions (McArdell et al. 2005). Reusable treated wastewater (especially for irrigation purposes in countries with dry weather conditions) should be free of these compounds and therefore an effective advanced treatment is required to remove these substances completely. Advanced oxidation processes (AOPs), through the generation of hydroxyl radicals (HO·) are capable of oxidizing various organic compounds. Among the various AOPs, photo-Fenton is one of the most widely used advanced technologies for wastewater treatment due to its environmentally-friendly application. Fenton’s reagent oxidation is a homogeneous catalytic oxidation process using a mixture of hydrogen peroxide (H₂O₂) and ferrous ions (Fe²⁺) in acidic medium. This process can be made more efficient in the presence of light (photo-Fenton process) by reducing the Fe³⁺ produced during the process to Fe²⁺ and by the generation of additional hydroxyl radicals (Andreozzi et al. 1999).

A growing number of analytical methods for the determination of pharmaceuticals in the aquatic environment can be found in the literature (Petrovic et al. 2006; Ibanez et al. 2009). Liquid chromatography in combination with tandem mass spectrometry (LC-MS/MS) has been widely used during the last few years to analyse antibiotics and other drugs at trace concentration levels in complex...
environmental samples (Fatta et al. 2007). The recently introduced improvements of LC, like the use of ultra-performance liquid chromatography (UPLC), has made this technology more attractive and powerful when combined with tandem MS. An ultra-high-pressure system operates using small particle-packed columns with small diameter (<2 μm), which allows for operation at high pressures (>15,000 psi), providing an increased chromatographic resolution compared with high-performance liquid chromatography (HPLC) (Jin et al. 2010).

Ofloxacin (OFX) belongs to the fluorinated quinolones (Okeri & Arhewoh 2008) while trimethoprim (TMP) is a broad-spectrum antimicrobial agent belonging to the 2,4-diaminopyrimidines frequently combined with sulphonamides (e.g. sulphamethoxazole) which acts as an inhibitor of bacterial dihydrofolate reductase (Barbarin et al. 2002). Investigations on the occurrence of OFX and TMP in wastewater effluents and natural waters have been conducted worldwide (Le-Minh et al. 2010).

The aim of this paper was: (i) to develop a fast and reliable UPLC-MS/MS method for the detection and quantification of OFX and TMP in secondary treated wastewaters at the ng L\(^{-1}\) levels, and (ii) to study the degradation kinetics for both antibiotics during the solar Fenton process with the UPLC-MS/MS method which is more sensitive and reliable than the HPLC method regularly used to study the degradation kinetics of pharmaceuticals in wastewaters.

**MATERIALS AND METHODS**

**Chemicals**

The pharmaceutical standards (OFX, Sigma Aldrich, CAS 82419-36-1; TMP, Fluka, CAS number: 738-70-5) used were of high purity grade and were not subjected to any further purification. Solutions containing each compound separately at initial concentration of 100 μg L\(^{-1}\) were prepared by spiking the appropriate mass of the compound in the secondary treated effluent samples and then performing the necessary dilutions. The secondary treated wastewater samples were collected from a WWTP in Cyprus (pe ≈ 272,000). The steps through which the secondary wastewater is treated at the WWTP and its main quality characteristics including dissolved organic matter (DOM) are described in a previous study (Michael et al. 2010). Taking into consideration that typical environmental concentrations in the effluents are in the ng\_μg L\(^{-1}\) range, it was decided to work at 100 μg L\(^{-1}\), which is a compromise between: (i) a sufficiently high concentration to characterize the degradation kinetics using available analytical techniques, and (ii) low enough to simulate real environmental conditions. The pH of the spiked effluents was adjusted to 2.8–2.9 with 2N H\(_2\)SO\(_4\) solution. The solar Fenton experiments were performed using FeSO\(_4\)-7H\(_2\)O (Riedel-de Haen) and reagent-grade H\(_2\)O\(_2\) (35% w/w, Merck).

Stock solutions of each analyte (1 mg mL\(^{-1}\)) were prepared by dissolving the appropriate amount of standard in MeOH (Fluka) and stored in the dark at 4°C. Working standard solutions were prepared daily by diluting the appropriate volume of stock solution with 10:90 v/v MeOH:H\(_2\)O to give concentrations in the range of 0.01–0.1 and 0.1–100 μg L\(^{-1}\). OFX-d\(_3\) and TMP-d\(_3\) obtained from Dr Ehrenstorfer (Augsburg, Germany) were used as surrogate standards. LC/MS-grade methanol and acetonitrile (Sigma Aldrich) were used as the main solvents. Formic acid (98%) was purchased by Fluka. Ammonium acetate (98%) was supplied by Sigma Aldrich. Ultrapure water (milli-Q) was used throughout all experiments.

**Experimental set-up**

A bench-scale solar simulator (Newport type 91193, 1 KW Xenon lamp) in combination with an appropriate photochemical batch reactor of maximum capacity 300 mL was used for the photocatalytic experiments. The spectrum of the xenon lamp used differs from the solar spectrum because of the intense line output in the 800–1,100 nm region while the rest of its spectrum simulates very well the natural sunlight one. The reactor was connected to a thermostatic bath for maintaining the temperature at the selected value of 25 ± 0.1°C. The detailed experimental procedure is described elsewhere (Michael et al. 2010). Samples from the reactor were taken at regular time intervals and the reaction was terminated by adding MeOH. Then the samples were filtered through a 0.22 μm filter (Milli-pore, Millex) and eluted through solid-phase extraction (SPE) (see below) before being analysed by UPLC-MS/MS.

**Solid-phase extraction (SPE)**

Oasis HLB cartridges (200 mg, Waters) were used for all SPE steps. The SPE cartridges were conditioned with 4 mL MeOH followed by 4 mL of deionized water (pH ≈ 3) at a flow rate of 1 mL min\(^{-1}\). After the conditioning step, 10 mL of sample were percolated through the cartridges at a flow rate of 1 mL min\(^{-1}\) followed by 3 mL of deionized water. The cartridges were then dried under
vacuum for 15–20 min. The final elution was performed with 2 × 2 mL of MeOH at 1 mL min⁻¹.

**Instrumentation**

All analyses were performed on an ACQUITY TQD UPLC-MS/MS system (Waters) equipped with auto sampler, binary solvent manager, and column manager. Electrospray ionization (ESI) in positive mode was used for all the experiments. Data acquisition was performed with MassLynx™.

Three different LC columns were tested for the analysis: (i) HSS C18 SB (1.8 μm; 2.1 × 50 mm), (ii) BEH Shield RP18 (1.7 μm; 2.1 × 50 mm), and (iii) BEH Phenyl (1.7 μm; 2.1 × 50 mm) (all provided by Waters). Four solvents were used for the binary elution: (i) A₁: 0.1 mM ammonium acetate in water + 0.01% formic acid, (ii) B₁: Methanol, (iii) A₂: 0.1 mM ammonium acetate in methanol + 0.01% formic acid, and (iv) B₂: Acetonitrile. Twelve combinations between columns and solvents were examined in order to optimize chromatographic separation (reduction of peak tailing and better resolution) and the ESI ionization. The column temperature was maintained at 60 °C, the solvent flow was 0.3 mL min⁻¹ and the sample injection volume 10 μL.

**Tandem mass spectroscopy (MS/MS)**

A tandem quadrupole mass spectrometer TQD (ACQUITY), was used for the detection of target analytes. In order to achieve sensitive target quantitative analysis, data acquisition was performed with ESI in multiple reaction monitoring mode (MRM), recording the transitions between the precursor ion and the most abundant fragment ions. Precursor and product ions with associated collision energies and retention times are summarized in Table 1, together with the operating MS/MS parameters. The choice of the two most abundant fragmentation products for each compound was done according to the intensity and the signal to noise ratio. The most abundant transition product ion was typically used for quantification of the target compound, while the second transition, together with the ratio of two transitions, was used for confirmation purposes.

MRM chromatograms of the standard solutions (10 μg L⁻¹) of the investigated analytes are shown in Figure 1.

**RESULTS AND DISCUSSION**

**Method optimization**

To optimize the chromatographic separation different mobile phases containing acidic additives were investigated. Retention time, peak shape and resolution were the main criteria for the method optimization. Column BEH Shield RP18 was found to be the most efficient for separation and determination of OFX and TMP using 0.1 mM ammonium acetate in water + 0.01% formic acid (A₁) and 0.1 mM ammonium acetate in methanol + 0.01% formic acid (A₂) as mobile phase. The other combinations caused broad peak shapes (higher retention times) and poor sensitivity for both compounds.

The formic acid applied into the mobile phase at concentration 0.01% was found to provide both good sensitivity and better peak shapes. Formic acid which is one of the most common acids used for chromatographic analysis has the ability to effectively mitigate the effect of residual silanols on the stationary phase and possibly even salvage residual metals (Zhu et al. 2001). Additionally, formic acid is known to promote positive ionization of acidic molecules and as a result an increase of signal in ESI source operating in positive mode takes place (Kasprzyk-Hordern et al. 2007). Li et al. (2009) also reported that

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Retention time (min)</th>
<th>Precursor ion (m/z)</th>
<th>Cone voltage (V)</th>
<th>Capillary voltage (kV)</th>
<th>Product ions (*) (m/z)</th>
<th>CE (eV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>OFX</td>
<td>2.59</td>
<td>362.1</td>
<td>28</td>
<td>3</td>
<td>58.1</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>261.2</td>
<td>35</td>
</tr>
<tr>
<td>TMP</td>
<td>2.67</td>
<td>291.1</td>
<td>38</td>
<td>2</td>
<td>261.1</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>250.2</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>123.1</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>110.1</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>81.1</td>
<td>46</td>
</tr>
</tbody>
</table>

*The most intensive fragment ion (in bold) from each precursor ion was selected for quantification. A less sensitive secondary transition (shown above the most intensive fragment ion) was used as the second criterion for confirmation purposes.*
formic acid dissociation affects the ionization efficiency thus providing good chromatographic separation.

Validation study

Linearity

Linearity was examined from five point calibration curves using a set of working standard solutions (in triplicate) at concentration levels in the range 0.01–0.1 and 0.1–100 μgL/L typically measured in wastewater effluents (data not shown). Calibration curves were generated by plotting the peak area versus the measured standard concentrations. Correlation coefficients ($R^2$) were greater than 0.98 and 0.99 for both analytes over the range 0.01–0.1 and 0.1–100 μgL/L respectively, demonstrating an excellent linearity.

Limit of detection (LOD) and limit of quantification (LOQ)

The LOD of the method was calculated according to ISO 11843–2 (Ternes et al. 2001), with a confidence interval of 99% using the standard deviation of a linear regression curve for a concentration range from 0.01 to 100 μgL/L. Stock solutions of TMP and OFX were diluted serially to low, mid and high concentrations and used to determine the LOD and the LOQ. LOD of the target analytes was found to be 4.6 ng L$^{-1}$ for OFX and 5.5 ng L$^{-1}$ for TMP, whereas the LOQ was 8.8 and 9.5 ng L$^{-1}$ for OFX and TMP, respectively. These values were in the same order of magnitude as the values in the literature (Chen et al. 2012).

Accuracy/recovery

Recovery experiments were performed to examine the possible effect of the environmental matrix. Recovery was assessed by extracting spiked wastewater samples with deuterated internal standards (OFX-d$_3$; TMP-d$_3$) at concentration levels of 0.01, 0.1, 50 and 100 μgL/L for each individual compound. The samples in triplicate were eluted through separate cartridges. As shown in Table 2 recoveries were, at all ranges of concentrations examined, higher than 90%. The recovery values are in agreement with those reported by other research groups (Lindberg et al. 2005; Xiao et al. 2008).

Precision

Method precision data were obtained by measuring multiple samples at each concentration (0.01–100 μgL/L) for each analyte. The precision (or reproducibility), expressed as the relative standard deviation (RSD) of independent multiple measurements.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (μgL/L)</th>
<th>Recovery (%) ± RSD (%) ($n=3$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMP ($m/z$ 230.2)</td>
<td>0.01</td>
<td>91.87 ± 2.66</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>92.48 ± 4.14</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>91.25 ± 1.79</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>96.47 ± 1.78</td>
</tr>
<tr>
<td>OFX ($m/z$ 261.2)</td>
<td>0.01</td>
<td>91.70 ± 5.14</td>
</tr>
<tr>
<td></td>
<td>0.1</td>
<td>93.52 ± 14.32</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>91.38 ± 1.74</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>98.59 ± 3.05</td>
</tr>
</tbody>
</table>

Figure 1 | MRM chromatograms of the target analytes in a standard solution of 10 μgL$^{-1}$ (for the applied MRM transitions see Table 1).
analyses \((n = 3)\) was typically found in the range of 0.98% to 1.54% for OFX and 0.85–5.54% for TMP in the range of 0.01–1.0 \(\mu\)g L\(^{-1}\). For the higher concentration level of 10–100 \(\mu\)g L\(^{-1}\), RSD was 2.36–14.65% and 2.59–5.54% for OFX and TMP, respectively.

**Identification**

Positive confirmation of the analytes was made using the intensity ratio \((Q/q)\) of the two MS transitions in the samples. The \(Q/q\) ratio is defined as the ratio between the intensity of the quantification ion \((Q)\) and the confirmation ion \((q)\) (Cervera et al. 2010). The experimental average \(Q/q\) value for each compound was calculated as the mean value obtained from standard solutions at different concentration levels in the range of 0.01–0.1 and 1–100 \(\mu\)g L\(^{-1}\) injected in triplicate. The average \(Q/q\) ratios were less than 1.88 for TMP and less than 4.70 for OFX.

A ratio tolerance of ±20% \(Q/q\) ratio lower than 2) and ±25% \(Q/q\) ratio between 2 and 5) was accepted for TMP and OFX respectively to confirm a finding as positive according to the European Commission Decision (2002/657/CE). Despite the fact that this decision was originally defined for the determination of organic contaminants in food samples, it is being increasingly used for the confirmation of positive findings in other matrices such as environmental and biological samples. The \(Q/q\) ratio for each analyte in each treated sample was calculated and it was found to be within the above tolerance levels. Furthermore, an agreement in the retention time between reference standard and sample was also required to characterize a detection as positive.

**Solar Fenton process**

The developed analytical method was successfully applied to the analysis of secondary wastewater samples which were treated by the solar Fenton process. The optimization of the oxidation process for OFX at 10 mg L\(^{-1}\) is described in a previous study (Michael et al. 2010). The optimum concentration of peroxide used was twice of the peroxide stoichiometric concentration for each analyte \([\text{H}_2\text{O}_2]_{\text{OFX}} = 2.714 \text{ mmol L}^{-1}; [\text{H}_2\text{O}_2]_{\text{TMP}} = 3.062 \text{ mmol L}^{-1}\) whereas the ferrous concentration was 5 mg L\(^{-1}\) for both cases.

It was found that OFX and TMP degradation proceeded more slowly at 100 \(\mu\)g L\(^{-1}\) compared with that at 10 mg L\(^{-1}\). The complete degradation of the compounds was achieved within 120 and 30 min of solar illumination at 100 and 10 mg L\(^{-1}\), respectively (data not shown). Although many studies (e.g. Lucas & Peres 2006; Malato et al. 2009) reported that degradation of substrates contained in distilled water at levels of mg L\(^{-1}\) during photo-Fenton process increases with decreasing the initial substrate concentration, it seems that in our case when substrate concentration is in the \(\mu\)g L\(^{-1}\) level in real effluent the degradation is slower. The possible explanation for such results is that at very low substrate concentration, the generated HO\(^-\) cannot easily attack the substrate molecules contained in a complex wastewater matrix and thereby lowering the degradation rates.

**Degradation kinetics**

Previous studies have indicated that the kinetics of drugs degradation by the photo-Fenton process is a pseudo first-order reaction (Radjenovic et al. 2009). Therefore, the concentration of the examined compounds can be given by the kinetic expression (Equation (1)):

\[
C = C_0e^{-kt}
\]

where \(C\) and \(C_0\) are the time-dependent concentration and the initial concentration respectively, and \(k\) is the apparent pseudo first-order rate constant.

The experimental data appear to fit well in the linear kinetic equation with regression coefficients \((R^2)\) higher than 99% (data not shown). In Table 3 the kinetic constants are summarized (rate constants \((k)\), half-life times \((t_{1/2})\) and initial rates of degradation \((r_0)\)) for each compound which

<table>
<thead>
<tr>
<th>Analyte/concentration</th>
<th>([\text{Fe}^{2+}]_{\text{BS}}) (mg L(^{-1}))</th>
<th>([\text{H}_2\text{O}<em>2]</em>{\text{BS}}) (mmol L(^{-1}))</th>
<th>(k) (min(^{-1}))</th>
<th>(t_{1/2}) (min)</th>
<th>(r_0) (mg L(^{-1}) min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>TMP (10 mg L(^{-1}))</td>
<td>5</td>
<td>3.062</td>
<td>0.1165</td>
<td>5.95</td>
<td>0.830</td>
</tr>
<tr>
<td>TMP (100 (\mu)gL(^{-1}))</td>
<td>0.0768</td>
<td>0.0027</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OFX (10 mg L(^{-1}))</td>
<td>5</td>
<td>2.714</td>
<td>0.1128</td>
<td>6.14</td>
<td>0.538</td>
</tr>
<tr>
<td>OFX (100 (\mu)gL(^{-1}))</td>
<td>0.0345</td>
<td>0.0018</td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>
were calculated for both drugs at concentration levels of 10 mg L\(^{-1}\) and 100 μg L\(^{-1}\). From the kinetic data in Table 3 it can be seen that the degradation kinetic constants for both compounds are higher at substrate concentration of 10 mg L\(^{-1}\) compared with substrate concentration of 100 μg L\(^{-1}\).

This kinetic Equation (1) (general form: \(y = Ae^{-kt}\)) may be acceptable for a series of time-concentration data at early reaction times (usually 15–20 min). However, for wider reaction spans, this equation is unsuitable for characterizing the behaviour of the irradiated system and despite the acceptable values for the correlation coefficient (\(R^2\)) this may lead to the estimation of negative concentrations and inexisten concentration minimum values (Navarro et al. 2011). Also, Equation (1) cannot be used for the experimental data above 15–20 min presumably due to the competitive effects of the matrix, formation of by-products, pH changes, etc. When the linear approach fails, other equations can be employed. A modification of the first-order kinetic expression (Equation (2)) was proposed and has been successfully used to explain the degradation kinetics of the compounds during the solar Fenton treatment.

\[
y = Ae^{-k_1t} + Ae^{-k_2t}
\]  

(2)

Figures 2(a) and (b) depict the adjustment of the experimental data for TMP and OFX to the above equations. The experimental data fit better kinetic Equation (2) for both compounds while the mean square error (MSE) of the fitting was lower in Equation (2) \((1.35 \times 10^{-3} - 3.30 \times 10^{-3})\) compared with Equation (1) \((0.072 - 0.032)\).

The two terms of Equation (2) represent the parallel conduction of two oxidation mechanism reactions during the solar Fenton process possibly due to the presence of different hydroxyl radicals concentration areas in solution contained in a reactor volume dV: (i) one area \((k_1)\) with high hydroxyl radicals concentration due to the direct photon absorption from iron; and (ii) a second area \((k_2)\) with lower hydroxyl radicals concentration due to the filter inner effects or to the presence of natural DOM in the wastewater matrix (where \(k_1 > k_2\)). The inner filter effects are the competitive absorption of photons by other light absorbing species, usually the various organic contaminants present in the wastewater matrix (Malato et al. 2009). Furthermore, the presence of natural organic compounds occurring in the wastewater matrix can affect the degradation rate of the target compounds since they exhibit significant iron complexation (or can alter the redox cycling of iron) and thereby change the formation rate of hydroxyl radicals (Lindsey & Tarr 2000).

**CONCLUSIONS**

- The method developed is quick and reliable, suitable for the identification and quantification of OFX and TMP in secondary treated municipal effluents down to low μg L\(^{-1}\) levels.
• The method was optimized and showed very good performance characteristics ($R^2 > 99\%$ and $R^2 > 98\%$ in the range of 0.1–100 and 0.01–0.1 $\mu$g L$^{-1}$ respectively, RSD in the range of 0.98–1.54\% for 0.01–1.0 $\mu$g L$^{-1}$ and 2.36–14.65\% for 10–100 $\mu$g L$^{-1}$ for OFX and in the range of 0.85–3.66\% for 0.01–1.0 $\mu$g L$^{-1}$ and 2.59–5.54\% for 10–100 $\mu$g L$^{-1}$ for TMP, LOD 4.6 and 5.5 $\mu$g L$^{-1}$ for OFX and TMP respectively, LOQ 8.8 and 9.5 $\mu$g L$^{-1}$ for OFX and TMP respectively, overall recovery $\geq 90\%$).

• The degradation rate of both compounds is lower at $100 \mu$g L$^{-1}$ compared with $10 \mu$g L$^{-1}$ possibly due to the fact that the generated hydroxyl radicals cannot easily attack the substrate molecules (at very low concentration level) in the presence of natural DOM contained in the complex wastewater matrix.

• OFX and TMP degradation follows a first-order kinetic law during the first 20 min of reaction while parameters such as pH, matrix complexity, formation of oxidation by-products affect the experimental fitting on the first-order kinetic equation above 20 min. However, with proper processing of the experimental data using a mathematical model, the degradation rate constants derived from it were more accurate.

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