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**Regular oscillatory behavior of aqueous solutions of Cu<sup>II</sup> salts related to effects on equilibrium dynamics of ortho/para hydrogen spin isomers of water**

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## Abstract

Cell surface and growth-related NADH oxidases with protein disulfide-thiol interchange activity, ECTO-NOX, exhibit copper-dependent, clock-related, temperature-independent and entrainable patterns of regular oscillations in the rate of oxidation of NAD(P)H as do aqueous solutions of copper salts. Because of time scale similarities, a basis for the oscillatory patterns in nuclear spin orientations of the hydrogen atoms of the copper-associated water was sought. Extended X-ray Absorption Fine Structure (EXAFS) measurements at 9302 eV on pure water were periodic with a ca. 3.5 min peak to peak separation. Decomposition fits revealed 5 unequally spaced maxima similar to those observed previously for  $\text{Cu}^{\text{II}}\text{Cl}_2$  to generate a period length of about 18 min. With  $\text{D}_2\text{O}$ , the period length was proportionately increased by 30% to 24 min. The redox potential of water and of  $\text{D}_2\text{O}$  also oscillated with 18 and 24 min period lengths, respectively. Measurements in the middle infrared spectral region above a water sample surface revealed apparent oscillations in the two alternative orientations of the nuclear spins (ortho and para) of the hydrogen atoms of the water or  $\text{D}_2\text{O}$  with 5 unequally spaced maxima and respective period lengths of 18 and 24 min. Thus, the time-keeping oscillations of ECTO-NOX proteins appear to reflect the equilibrium dynamics of ortho-para hydrogen atom spin ratios of water where the presence of metal cations such as  $\text{Cu}^{\text{II}}$  in solution determine period length.

**Keywords:** Copper<sup>II</sup>; ECTO-NOX hydroquinone (NADH) oxidase; EXAFS (extended x-ray absorption fine structure spectroscopy); molecular and biological time keeping; redox potential; para-H<sub>2</sub>O/ortho-H<sub>2</sub>O ratio; infrared spectroscopy; water.

## I. Introduction

Our work has described a family of growth and redox-related ECTO-NOX proteins some of which appear to function as core oscillators of the cells' biological clock [1]. The unique feature of the ECTO-NOX proteins is that the two enzymatic activities they catalyze, hydroquinone (NADH) oxidation and disulfide-thiol interchange, alternate within a 24 min period [2]. The ECTO-NOX proteins carry out hydroquinone (NADH) oxidation for 12 min and then that activity rests. While the hydroquinone (NADH) oxidative activity rests, the proteins engage in disulfide-thiol interchange activity for 12 min. That activity then rests as the cycle repeats. Within the hydroquinone oxidation part of the cycle, there are two maxima separated by 6 min and within the protein disulfide-thiol interchange portion of the cycle, there are three maxima separated by intervals of 4.5 min. The 24 min periodic behavior is exhibited by pure recombinant tNOX protein and is accompanied by a recurring pattern of amide I/amide II FTIR and CD spectral changes suggestive of  $\alpha$ -helix- $\beta$ -sheet transformations [2].

The periodic oscillations associated with the time keeping activities of ECTO-NOX proteins not only require copper but appear to be inherent in the molecular structure of the  $\text{Cu}^{\text{II}}$  aqua ion [3] itself. Solvated  $\text{Cu}^{\text{II}}$  as the chloride or other salts alone exhibit the property of catalysis of NADH oxidation. With  $\text{Cu}^{\text{II}}\text{Cl}_2$ , the pattern also consists of 5 maxima, two of which are separated by an interval of 6 min and three which are separated by an interval of 4.5 min. The total period length is 24 min and the period length is independent of temperature and pH [3].

In a previous report [4], a possible underlying pattern of distortion in the four equatorial oxygens of the Cu<sup>II</sup> aqua ion at a close distance relative to the two axial oxygen atoms at a longer distance was sought from extended X-ray absorption fine structure (EXAFS) studies that might correlate with changes in redox potential sufficient to catalyze NADH oxidation in solution. The findings were consistent with changes in the coordinated water molecules of the Cu<sup>II</sup> aqua ion possibly related to a metastable equilibrium condition in the ratio of ortho to para nuclear spin orientation of the water-associated hydrogen atoms.

In this report we show that oscillatory patterns similar to those observed with aqueous copper solutions or with the ECTO-NXO proteins are seen to be inherent in the properties of pure water or of D<sub>2</sub>O. With D<sub>2</sub>O, the period length is extended in proportion to the length of the circadian day which is increased from 24 h to 30 h in organisms grown in D<sub>2</sub>O [5]. D<sub>2</sub>O is the only substance known to so affect both the length of an ultradian biorhythm [3] and the length of the circadian day [5].

## 2. Materials and methods

The X-ray absorbance studies were conducted at the Pacific Northwest Consortium Collaborative Access Team (PNC-CAT) at the Advance Photon Source (APS) of Argonne National Laboratories as described [4]. The experiments were at 25° C and at intrinsic pH around 4.2. The measurements were taken at the sector 20 bending magnet beam line (PNC-CAT) using a 3 mm thick sample cell constructed of Teflon with Teflon coated kapton windows. Measurements were recorded at a fixed energy of 9302 eV ( $k = 9.3$ ) using an integration time of 10 s. Data were collected for several successive time periods of about 90 min each.

A copper foil spectrum was taken to calibrate the energy. The optical setup consisted of a double crystal monochromator with Si 111 crystals and a 5 milliradian rhodium-harmonic-rejection mirror. The crystals were detuned resulting in a 15% reduction in intensity in order to reduce harmonics. The radiation exiting the monochromator passed through a 1 mm high by 8 mm wide slot and all ionization chambers were filled with nitrogen.

Infrared spectra were recorded on a Nexus 470 E.S.P. FTIR spectrometer equipped with a MCT/B detector and KBr beam splitter at the wave numbers and conditions indicated with the figures.

Results were analyzed using fast Fourier transform and decomposition fits [6] to verify the reproducibility of the signals. To determine the period length of the NADH oxidase activity, the fast Fourier transforms were performed by means of a user-defined transform in SigmaPlot 8.0. Decomposition fits on both the enzyme activity and the growth determinations are used to predict patterns based on the period length determined from Fourier analysis. The decomposition fits used Minitab 14, a statistical package. Three measured values, average percentage error (MAPE), a measure of the periodic oscillation, mean average deviation (MAD), a measure of the absolute average deviations from the fitted values, and mean standard deviation (MSD), the measure of standard deviation from the fitted values plus R-squared and the Henriksson-Merton p value were used to evaluate accuracy [6].

Oscillations in redox potential were determined using a platinum electrode and a CH Instruments (Austin, TX), Model 600 B Series Electrochemical Analyzer/Workstation system as a function of time. Baseline corrections used a standard frequency filtering algorithm in combination with Fourier analysis to generate a moving average for subtraction where the averaging area was the measured period of the signal.

### 3. Results

The hypothesis under investigation is that oscillations in redox potential and oxidation of NADH of hydrated  $\text{Cu}^{\text{II}}$  both in solution and associated with protein [1-4] were inherent in the properties of the associated water. To test the hypothesis, EXAFS measurements previously made on  $\text{Cu}^{\text{II}}\text{Cl}_2$  solution [4] were extended to double distilled water at 9302 eV with continuous measurements (absorbance recordings every 10 s). A total of 5 individual measurement sets of 90 min duration each were analyzed with similar results. Figure 1 is a moving average of a typical data set after baseline subtraction. A pattern of oscillations similar to that reported for  $\text{Cu}^{\text{II}}\text{Cl}_2$  in solution [4] and unrelated to beam top off was observed. Although the pattern was similar, the period length was not as long. Fourier analysis indicated an average peak to peak separation of about 3.5 min. For 5 decomposition fits the average recapitulated the asymmetric pattern (Fig. 2). Previously observed with  $\text{Cu}^{\text{II}}\text{Cl}_2$  in solution or associated with protein, there were 7 maxima within 24 min instead of the 5 maxima observed with  $\text{Cu}^{\text{II}}\text{Cl}_2$ . The maxima were not equally spaced however. The first and the second and the sixth and seventh were farther apart than the others suggesting a period length of about 18 min with 5 maxima, two of which were unequally spaced. Decomposition fits assuming a period length of 18 yielded the predicted 5 maxima pattern (Fig. 3). Maxima ① and ② were more widely separated than the others.

For comparison with the EXAFS measurements, NADH oxidation with pure water was measured at 340 nm. The only substances present were water and NADH. The NADH



was oxidized spontaneously in water and generated an oscillatory pattern similar to that observed by EXAFS with maxima spaced at intervals of about 3.5 min (Fig. 4a). The maxima labeled ① and ② again were more widely separated to suggest a period length of about 18 min corresponding to the pattern for EXAFS measurements of Figure 3. With D<sub>2</sub>O, the pattern was similar except that the period length was increased by about 30% to 24 min in length (Fig. 4b).

In addition to catalysis of NADH oxidation, cyclic changes in redox potential also were observed both for deionized distilled water (Fig. 5a) and for D<sub>2</sub>O (Fig. 4b). Redox potential oscillated with a pattern consistent with an 18 min period for pure water (Fig. 5a). With D<sub>2</sub>O a similar pattern of oscillations was observed except that the period length was increased to 24 min (Fig. 5b). These changes correlated closely with the ability of NADH oxidation to occur spontaneously in water (Fig. 4a) or D<sub>2</sub>O (Fig. 4b). Both exhibited the characteristic 2 + 3 patterns of oscillations.

Further analyses indicating that water could give rise to the 2 + 5 patterns of oscillations was provided from infra red spectral measurements in the mid-region of the water absorption spectrum. Decomposition fits of measurements with absorption at 1645 cm<sup>-1</sup> with reference at 2500 cm<sup>-1</sup> close to the  $\nu_2$  bend at 1640 revealed a clear oscillatory pattern of 5 unequal maxima within an 18 min period (Fig. 6). Also revealing such a pattern were measurement of  $\nu_3$  asymmetric stretch at 3600 and 3400 cm<sup>-1</sup> and  $\nu_1$  symmetric stretch measured at 3200 cm<sup>-1</sup> (not shown).

For D<sub>2</sub>O, measurements were made in the range of 2600 to 2650 cm<sup>-1</sup> or 2425 to 2475 cm<sup>-1</sup>. The asymmetric 5-peak pattern of D<sub>2</sub>O was extended to a 24 min period length with measurements yielding 5 peaks in decomposition fits using a 24 min period (Fig. 7). Results were similar for data collected at 2600, 2625 or 2650 cm<sup>-1</sup> or at 2425, 2450 or 2475 cm<sup>-1</sup>.

Further infrared measurements were made to determine if the oscillations were due to periodic variations in the relative abundance of the two alternative orientations of the nuclear spins (ortho and para) of the hydrogen atoms of water (see Discussion). Infrared measurements at 3801 cm<sup>-1</sup> and 3779 cm<sup>-1</sup> of water vapor oscillated around the theoretical ratio of 3 for A<sub>3801</sub>/A<sub>3779</sub> (Fig. 8). Fourier analyses yielded a maximum at a frequency of about 0.055 min<sup>-1</sup> to yield a period length of 18.3 min. Decomposition fits gave 5 unequally-spaced maxima.

#### 4. Discussion

The oscillations with maxima separated by intervals of 4.5 or 6 min are slow by comparison even to Jahn-Teller equilibria [7]. The only atomic phenomenon that occurs on a similar time scale is the equilibrium of the two alternative orientations of the nuclear spins (ortho and para) of the hydrogen atoms of water [8]. The relaxation time to convert spin-modified ice to the 3:1 ortho:para equilibrium ratio has been estimated to require several months. Liquid lifetimes were measured as  $26 \pm 5$  min for para water and  $55 \pm 5$  min for ortho water. Metals and other impurities can result in ortho:para imbalance and conceivably an oscillatory non-equilibrium condition that would translate into a regular periodic pattern of distortion in the four equatorial oxygens of the copper aqua ion and concomitant redox changes ultimately affecting rates of NADH oxidation [4]. A similar contribution from the two equatorial oxygens might account for the 2 + 3 asymmetry [4].

The hydrogen atom nuclear spins may either be parallel (total spin is 1) in ortho molecules or antiparallel (total spin is 0) in para molecules [8]. Each spin isomer has its own system of rotational levels. Optical transitions between levels of different spin isomers are not permitted. The number of ortho isomer molecules is three times greater than the number of para isomer molecules in the thermal equilibrium state at room temperature.

Ortho and para water isomers have been successfully separated [8]. The method of ortho/para ratio measurement used was based on microwave absorption spectroscopy.

The middle infrared spectral region has been reported as suitable as an indirect method to study liquid water states [9, 10]. Infrared spectroscopic measurement of the para-H<sub>2</sub>O/ortho-H<sub>2</sub>O ratio above the water sample surface is based on the fact that the vibrational infrared spectra of many molecules depend on their nuclear spin state. For a narrow spectral range including two close lines belonging to the different water molecule modifications within the same quantum transition, the relative heights of lines will, on average, equal the natural ortho to para composition ratio of water of 3:1 [9].

Because of spectral differences comparing water and D<sub>2</sub>O, potential oscillations in the ortho-para hydrogen equilibrium of water and D<sub>2</sub>O were recorded at 3801 and 3779 cm<sup>-1</sup> for water and in the range of 2600 to 2650 cm<sup>-1</sup> or 2425 to 2475 cm<sup>-1</sup>, for D<sub>2</sub>O where the oscillatory changes above baseline differed approximately 3-fold. Repeating patterns of 5 maxima at approximately 18 min intervals was obtained for water (Fig. 8b) which, when analyzed by decomposition fits (Figs. 8 a-c), indicated 2 + 3 patterns of oscillations similar to those observed previously [3, 4]. Results with D<sub>2</sub>O were similar except that the period length was increased to 24 min. Why the ortho/para hydrogen compositions should exhibit a regular pattern of oscillation from their equilibrium ratio of 3:1 remains unexplained but such variation would account for the oscillatory behavior of the redox potential and catalytic activity of aqueous solutions in relation to our time keeping model [1].

The explanation as to why the period length of the ortho-para transition would increase from 18 to 24 min in the presence of  $\text{Cu}^{\text{II}}$  appears to reside in the previously observed response to the ionic environment of the water [8]. We have measured period lengths of the oscillatory patterns for a series of ionic solutions and find proportionality of the period length of the associated water to the ionic radius with  $\text{Cu}^{\text{II}}$  uniquely giving rise to a 24 min period. This observation provides a basis for the potential importance of protein bound copper hexahydrate to biological time keeping at the molecular level [1].

Several mechanisms for the ortho/para-conversion have been discussed in the literature. A paramagnetic mechanism for dihydrogen has been developed where the spin conversion is caused by the magnetic interaction of the hydrogen spins with paramagnetic centers such as the unpaired electron spins themselves [11]. Normally the ortho/para-conversion is a slow process but paramagnetic impurities, i.e., unpaired electron spins or electrons with orbital momentum such as provided by metallic cations including copper would serve as catalytic centers for the interconversion process [12]. In a recent model [13], ortho/para- $\text{H}_2$  molecules in one particular spin state were suggested to react with a suitable catalyst to create an  $\text{X-H}_2$  complex. Within the complex a postulated evolution of the initial density matrix of the dihydrogen would initiate a partial conversion of one rotational state to the other. Upon decay of the bound state, the final density matrix would then be transferred to the free dihydrogen state.

Water molecules take on the two different spin states in a manner analogous to hydrogen molecules and the net interconversion of the two forms might occur by a similar mechanism even within the Cu<sup>II</sup> hexahydrate. However, the oscillations we observe with water are apparently not due to the net final equilibrium position determining ortho/para conversions. Net ortho/para conversions are temperature dependent [14] and reduced in ice [15]. While amplitudes of the oscillatory phenomena reported here are temperature dependent, the period length by contrast is temperature independent [3] and unchanged even at liquid nitrogen temperatures [Results unpublished].

Thus, as with previous correlative studies and measurements [1-4], it is the period length of the ortho-para conversion rather than amplitude that we anticipate will serve as the basis for the temperature independence of the period length of the biological clock [16, 17]. With pure water, the measured period length of the ortho/para interconversion is 18 min (24 min for D<sub>2</sub>O). However, in the presence of Cu<sup>II</sup>, the period length increases to 24 min (30 min for D<sub>2</sub>O). As such, relevance of ortho-para water driven oscillations may have relevance to clock-related phenomena only in the presence of copper.

The differences in energies of the different spin isomers of water are small (less than 10<sup>-24</sup> erg) [16] and are much lower than the energy of thermal motion. Therefore, a spin-only interaction would not be expected to affect intermolecular interactions [18]. On the other hand, the absorption rates of ortho and para water from water vapor to

various organic and inorganic sorbents have been observed to differ markedly. The binding of the para isomer with such preparations is distinctly faster and with greater avidity than the binding of the ortho isomer. Binding avidity is most likely determined from differences in quantum statistics for the two different spin isomers as noted by Potekhin and Khusainova [19]. Estimates of the energy barriers that determine rates of absorption suggested to these authors that the difference in free energy barriers may exceed the energy of spin-spin and spin-orbit interaction by many orders [19]. This raises the possibility that the spin state of water may substantially influence physical, chemical and biological phenomena including redox potential through differences in binding avidity.

## **Abbreviations**

ECTO-NOX, cell-surface and growth-related NADH oxidase with protein disulfide-thiol interchange activity.

ECTO, prefix meaning outside or external

EXAFS, extended x-ray absorption fine structure

FTIR, Fourier transform infrared

MAD, mean average deviation

MAPE, mean average percentage error

MSD, mean standard deviation

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## List of Figures

**Fig. 1.** Moving average of EXAFS measurements recorded at intervals of 10 s at a constant  $k$  ( $\text{\AA}^{-1}$ ) of 9.3 as described [4]. An oscillatory pattern was observed with a recurring pattern of 5 maxima (arrows).

**Fig. 2.** Average of 5 decomposition fits from the analysis of the data of Figure 1 with an imposed period length of 24 min as used previously for copper. Seven maxima were obtained instead of the 5 observed for copper. However, the maxima were not equally spaced. The first and second and the sixth and seventh were more widely separated than the others suggesting a period length consisting of 5 maxima or of 18 min.

**Fig. 3.** Average of 5 decomposition fits of data of Figures 1 and 2 with standard deviations assuming a period length of 18 min. The predicted 5 maxima pattern was obtained. Maxima ① and ② were more widely separated than the others.

**Fig. 4.** Periodic catalysis of the oxidation of NADH by deionized, distilled water (a) with a period length of 18 min and  $\text{D}_2\text{O}$  (b) with a period length of 24 min. The final NADH concentration was  $150 \mu\text{M}$ . The solution was unbuffered. Within each period of 18 min maxima labeled ① and ② are separated by 4.5 min for  $\text{H}_2\text{O}$  and by 5.8 min for  $\text{D}_2\text{O}$ . The remaining maxima designated ③, ④ and ⑤ are separated by 3.4 min for  $\text{H}_2\text{O}$  and

by 4.4 min for D<sub>2</sub>O. The pattern repeats after the fifth maximum to generate the 18 min period for H<sub>2</sub>O or 24 min period for D<sub>2</sub>O.

**Fig. 5.** Oscillations in redox potential of deionized, distilled water (a) and D<sub>2</sub>O (b) as determined using a platinum electrode and a CH Instruments (Austin, TX), Model 600 B Series Electrochemical Analyzer/Workstation system as a function of time. Maxima ① and ② are separated by 4.5 min as in Fig. 4.

**Fig. 6.** Fast Fourier and decomposition analyses of FTIR data for distilled water collected at 1433 cm<sup>-1</sup> (a). Maxima marked by arrows are separated by ca 18 min. Fast Fourier analysis revealed a period length of 18 min corresponding to the measured frequencies of 0.055 min<sup>-1</sup> (b). The decomposition fit of the data utilized the period length of 18 min determined by Fast Fourier analysis (arrow) (c). The pattern is that of 5 recurrent maxima, two of which, labeled ① and ②, are separated in time by 4.5 min and 3 additional maxima separated in time by 3.4 min labeled ③, ④ and ⑤. The accuracy measures, average percent error (MAPE), mean average deviation (MAD) and mean standard deviation (MSD) are indicative of a close fit between the original and the fitted data.

**Fig. 7.** Fast Fourier and decomposition analyses of FTIR data for D<sub>2</sub>O collected at 2450 cm<sup>-1</sup> (a). Maxima marked by arrows are separated by ca 24 min. Fast Fourier analysis revealed a period length of 24 min corresponding to the measured frequency of 0.042 min<sup>-1</sup> (arrow) (b). The decomposition fit of the data utilized the period length of 24 min

determined by Fast Fourier analysis (c). The pattern is that of 5 recurrent maxima, two of which, labeled ① and ②, are separated in time by about 6 min and 3 additional maxima separated in time by about 4.4 min labeled ③, ④ and ⑤. The accuracy measures, average percent error (MAPE), mean average deviation (MAD) and mean standard deviation (MSD) are indicative of a close fit between the original and the fitted data.

**Fig. 8.** FTIR spectroscopic measurement of the ratio of para-H<sub>2</sub>O/ortho-H<sub>2</sub>O above a water sample surface determined at 3801 and 3779 cm<sup>-1</sup> respectively (a). The ratio of the two wavelengths exhibited a repeating pattern of oscillations of 5 maxima at intervals of about 18 min (arrows) (b). Decomposition fits using an imposed period length of 18 min of data collected at 3801 cm<sup>-1</sup> (c), and at 3779 cm<sup>-1</sup> (d) as well as the ratio of the two (e) revealed the oscillatory pattern typical of water with 5 recurrent maxima, two of which, labeled ① and ②, were separated in time by about 4.5 min and 3 additional maxima separated in time by 3.4 min labeled ③, ④ and ⑤. The accuracy measures, average percent error (MAPE), mean average deviation (MAD) and mean standard deviation (MSD) are indicative of a close fit between the original and the fitted data.

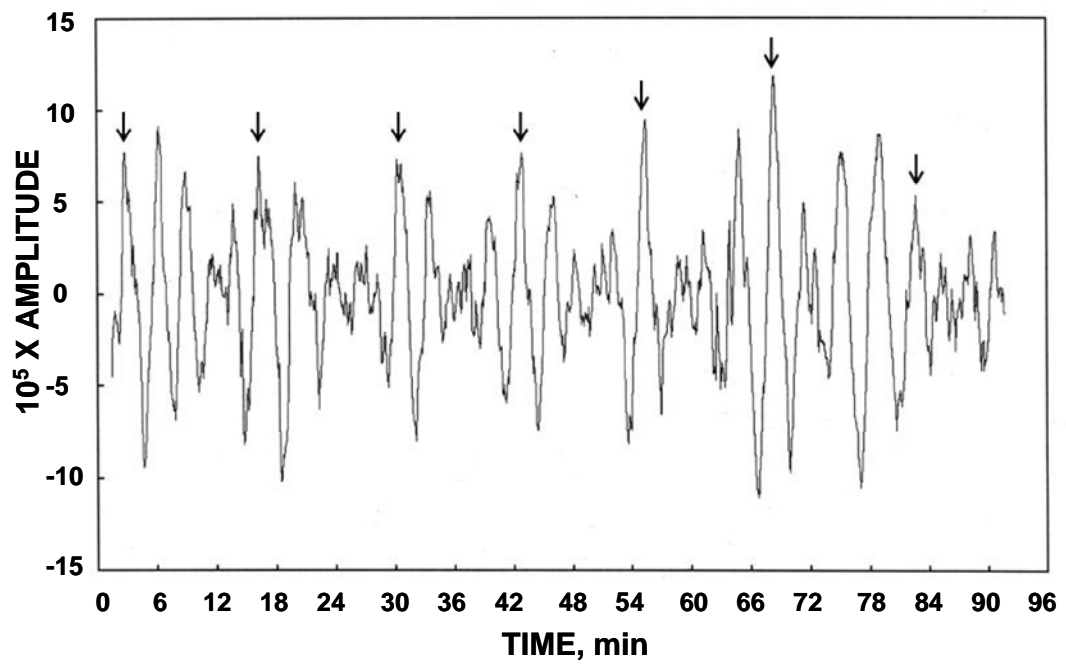


Figure 1

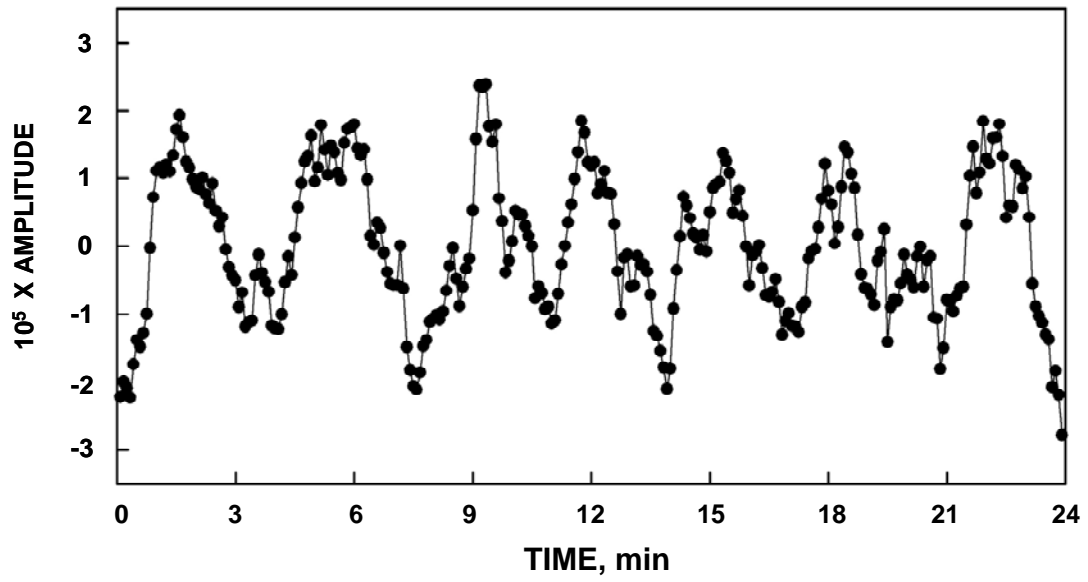


Figure 2

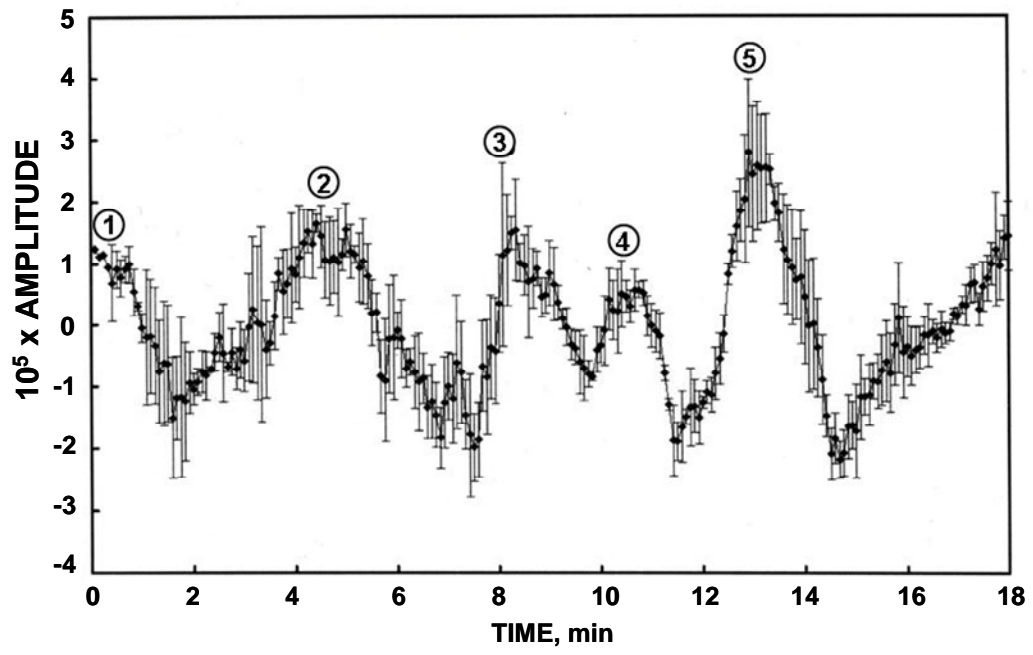


Figure 3



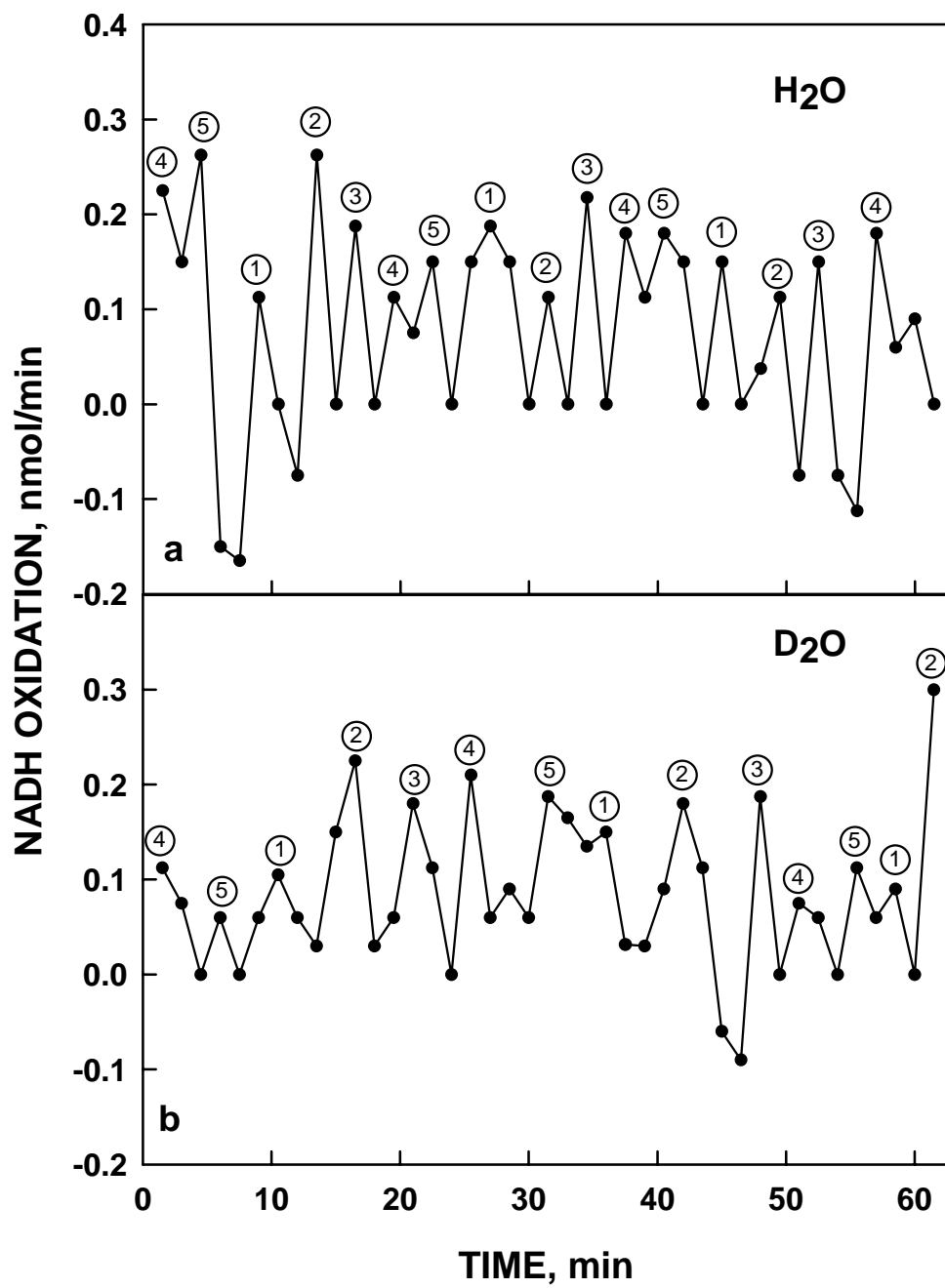


Figure 4

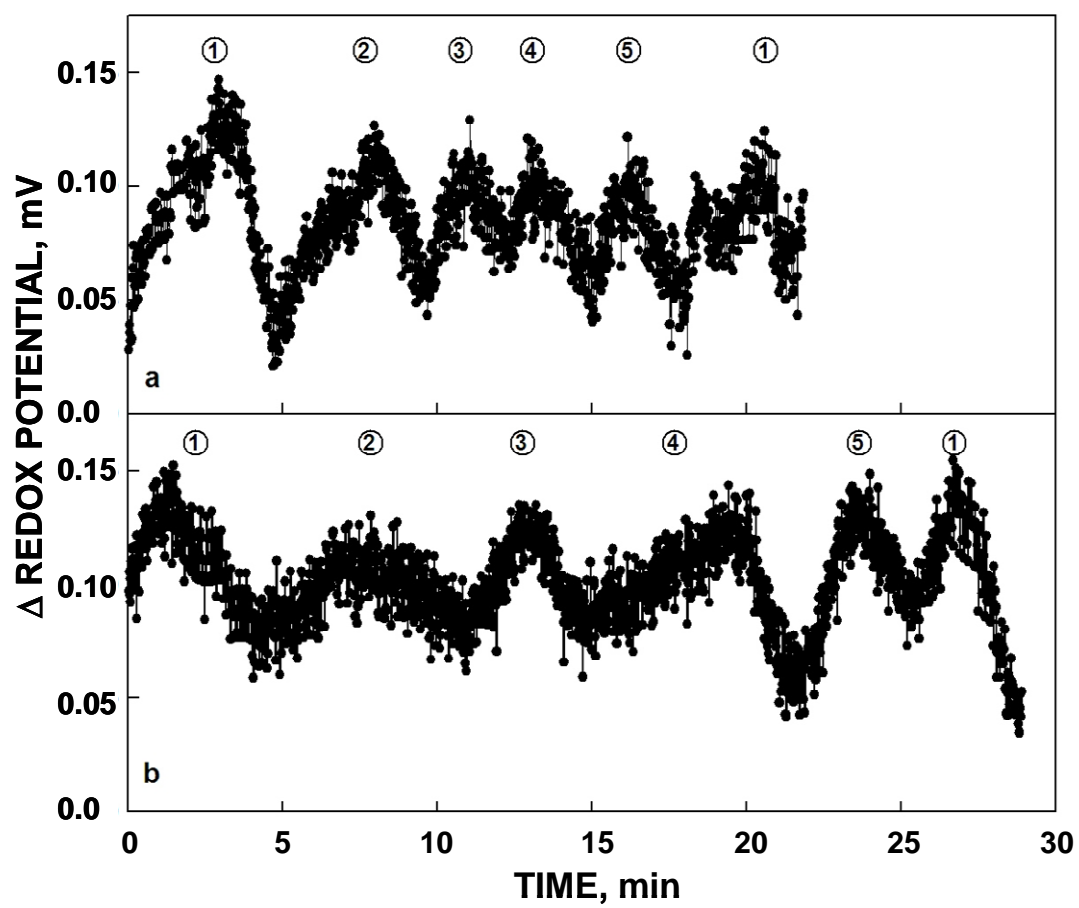


Figure 5

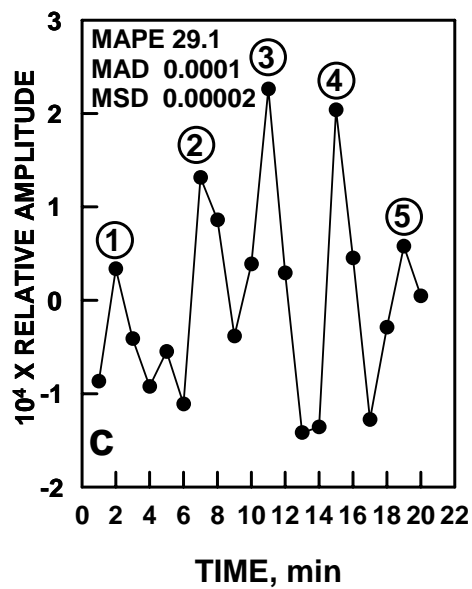
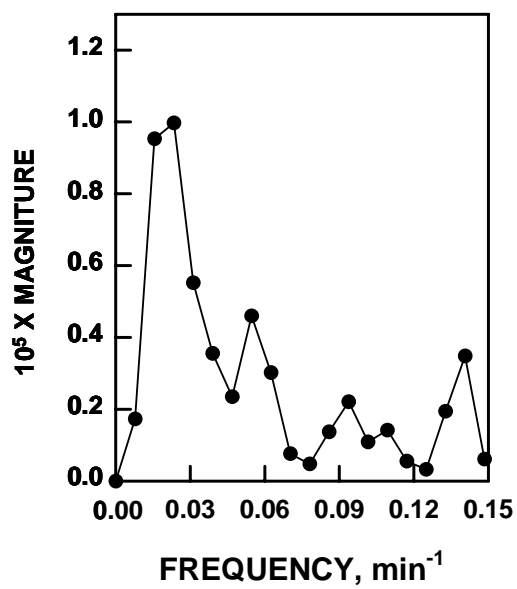
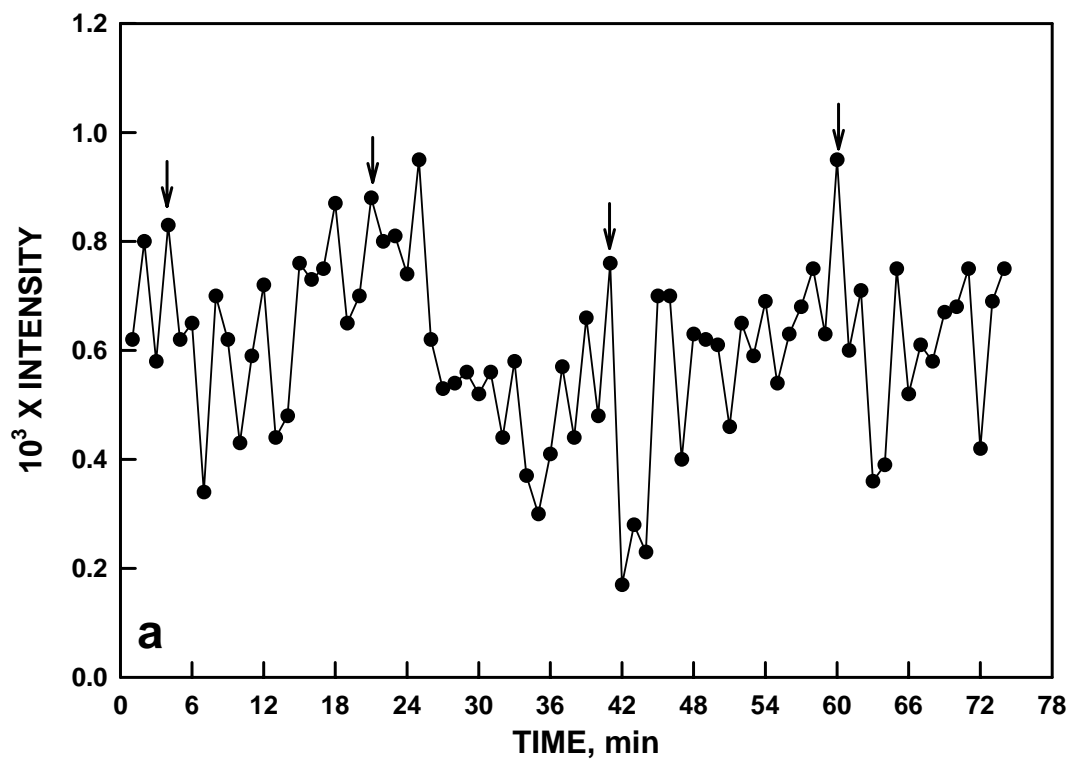


Figure 6

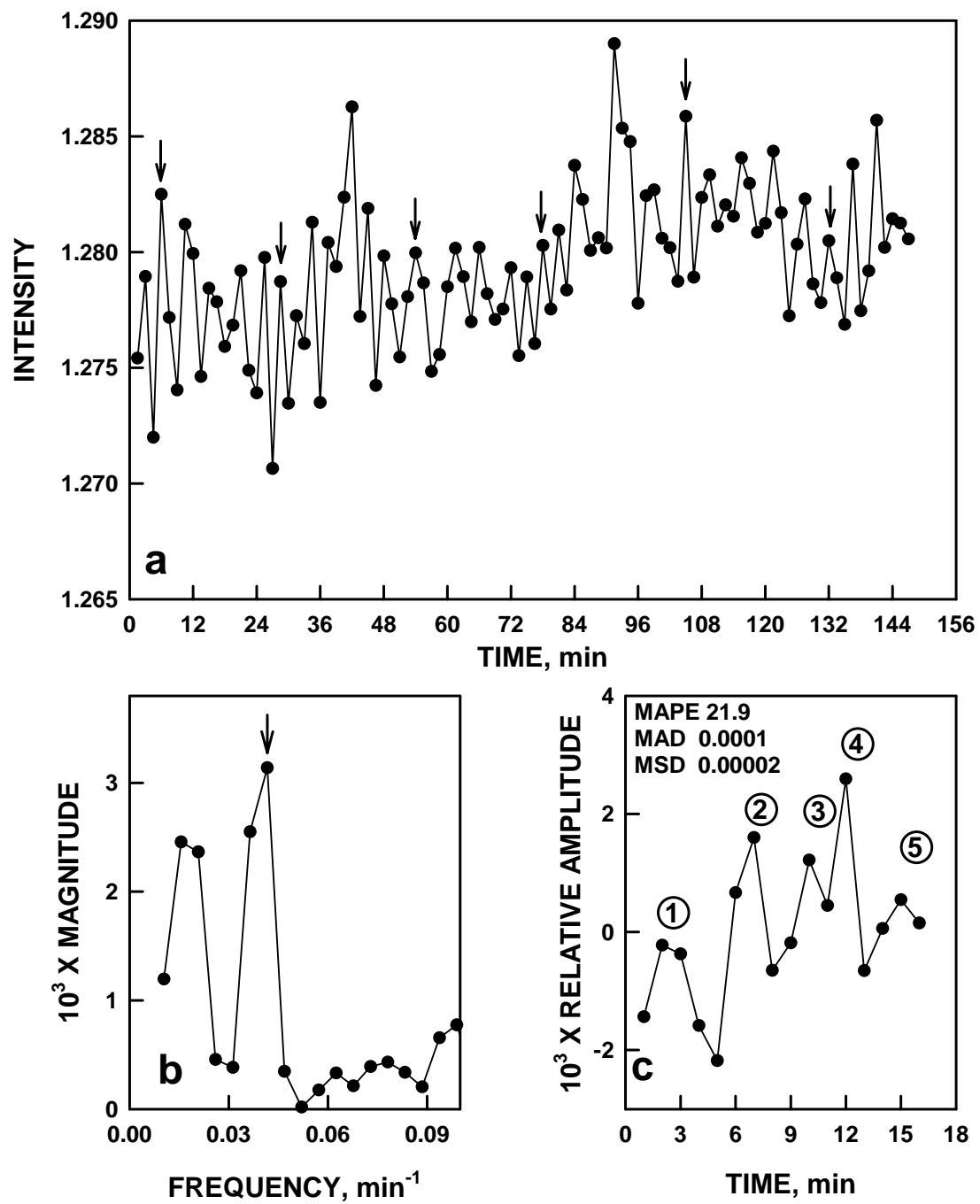


Figure 7

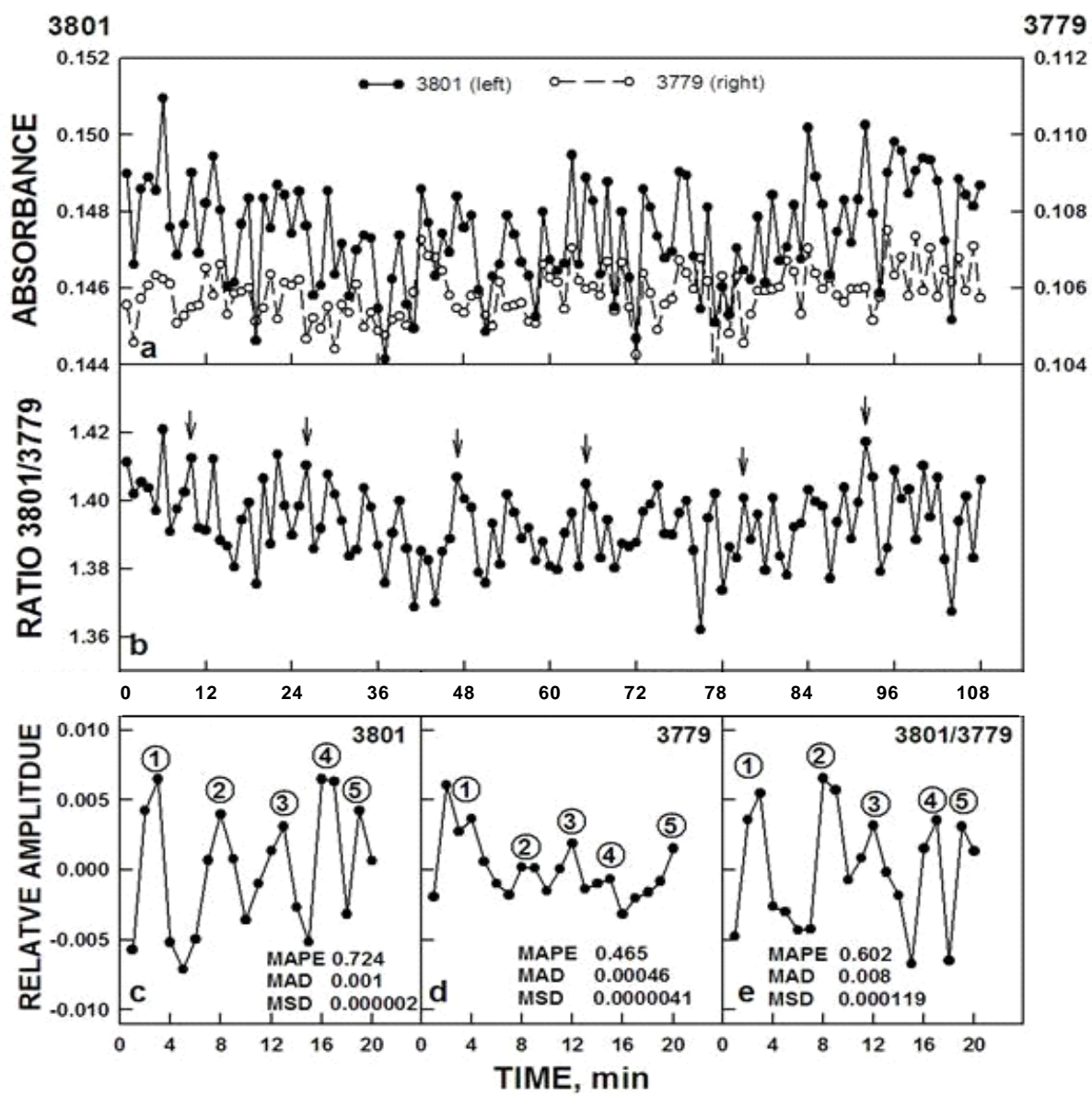


Figure 8