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Pentacyanidoferrate(III) complex of metformin provides a simple colorimetric test for this important anti-hypoglycemic medicament

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ABSTRACT

Metformin (MF) is one of the most important medicaments in the market. It has been extensively employed in the treatment of type-2 diabetes, but its analytical detection is rather complicated, requiring, for instance, high-performance chromatographic methods. Here, we report that metformin reacts with the pentacyanidoferrate (II) complex, generating a deep red product in the presence of sodium percarbonate, $Na_2CO_3{\cdot}1.5H_2O_2$. A colorimetric assay has been performed by measuring the absorption band at 520 nm, with a limit of detection (LOD) of 0.018 mmol/L. The reaction can also be probed by Raman spectroscopy, since the $[Fe^{III}(CN)_5MF]^{3-}$ complex, exhibits a strong resonance effect which enhances the vibrational peaks of the iron(III)-guanidine chromophore. The Raman spectral features are associated with the Fe(III)-N═C-bonds and the metal-to-ligand charge-transfer transition, as confirmed by DFT calculations.

Introduction

Metformin (MF) is a worldwide-used drug for treating type 2 diabetes mellitus [1,2]. It has been used for more than a century as an anti-hypoglycemic medicament. Among the other existing pharmaceutical products for hypoglycemia, metformin has been recognized as the lead medicine in most treatments. Metformin promotes increased peripheral glucose uptake by increasing the biological efficiency of available exogenous/endogenous insulin. As a plant-based drug, it is well-accepted in medicine for the management of non-insulin-dependent diabetes mellitus. Metformin has also attracted attention in other medical applications, such as in weight loss treatment [3]. However, recently, metformin has raised [4] concerns in wastewater management, because of its increasing release from domestic sources and hospitals.

The chemical composition of metformin encompasses a biguanide structure (Fig. 1) which has attracted the attention of bioinorganic chemists as a possible metal-chelating agent, especially for biological purposes [5,6]. Its analytical detection can be performed in many ways, including chromatographic, electrochemical, and spectrophotometric methods [4,7,8]. The most used ones are based on expensive high-performance liquid chromatography, thin-layer chromatography, and liquid chromatography-mass spectrometry methods [2]. In this sense, the development of simple and accessible methods can be important for the small-scale industry, and public health [4].

Among the most accessible methods, the colorimetric ones are particularly convenient and readily available in the laboratory. In this sense, the direct spectrophotometric detection of metformin has been performed [9,10] by monitoring its absorption band in the UV region, around 250 nm. However, it should be noticed that most organic compounds displaying aromatic and conjugated bonds also absorb in this region and seriously interfere in the analysis. Since metformin does not absorb in the visible region, to the best of our knowledge, its colorimetric detection has never been performed up to the present time.

In this work, we have observed that metformin reacts selectively with pentacyanidoferrate(II) complexes yielding a deep red product, in the presence of sodium percarbonate, $Na_2CO_3{\cdot}1.5H_2O_2$. This reaction is rather novel and its discovery has prompted the investigation of the red compound, aiming for a possible application in the colorimetric detection of metformin.

Pentacyanidoferrate(II) ions are typical low-spin iron(II) complexes, exhibiting great affinity for π-receptor ligands (L) such as pyridine and pyrazine species [11–14]. Such substituted $[Fe^{II}(CN)_5L]^{3-}$ complexes display strong charge-transfer bands in the visible, responsible for their characteristic colors. In this series of complexes, the $[Fe^{II}(CN)_5]^{3-}$

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moiety is kinetically inert to substitution, while the ligands L are relatively labile, depending upon their specific electronic affinity to the iron (II) center. Such affinity increases with the π-receptor properties of the ligands and modulates the kinetic and redox properties of the complexes. In contrast, in the oxidized form, the pentacyanidoferrate(III) ions exhibit a preference for σ and π-donor ligands, such as amines and thiols. Metformin is a good candidate, since it is a biguanide ligand, exhibiting a strong basic character, with pKa = 13.25 and 3.07. Accordingly, we have isolated and characterized the red product by electronic and Raman spectroscopy and developed a quick colorimetric assay for metformin, which may be useful for many different purposes.

Experimental section

Materials

Metformin was obtained from Sigma-Aldrich with high purity and employed as supplied. Sodium percarbonate, $Na_2CO_3{\cdot}1.5H_2O_2$, and all other reagents were obtained from Sigma-Aldrich, Labsynth, or Merck, with analytical purity, and were employed as supplied. The reagent is a convenient solid source of hydrogen peroxide, and the presence of carbonate ions is important for turning the medium alkaline enough for the metformin test.

Sodium aminopentacyanidoferrate(II) was synthesized as previously reported in the literature [15–17], starting from sodium nitroprusside, $Na_2[Fe(CN)_5NO].2H_2O$, and reacting with an ammonium hydroxide solution (7 M), for three days. The reaction should be carried out at low temperatures, e.g. in an ice/water bath, in the hood, using an Erlenmeyer equipped with a glass stopper for gas release. Nitrogen is produced from the reaction of the nitrosyl group and ammonia. Its release increases the internal pressure of the flask but helps to keep an inert atmosphere preventing the oxidation of the iron complex. By using concentrated solutions, the $Na_3[Fe(CN)_5NH_3].3H_2O$ complex crystallizes very slowly, yielding a yellow solid that can be collected on a glass-sintered filter. The solubilized complex can also be precipitated as a fine yellow powder, by adding ethanol. After washing the solids with ethanol, they can be dried under a vacuum for a few minutes, but they should be stored in the dark, in closed bottles or ampules. Long exposition to vacuum can promote their dehydration and release of some coordinated ammonia, imparting a green or brown color. They can be safely used at room temperature but can get oxidized after several days of exposition to the atmosphere and light.

The metformin complex with pentacyanidoferrate(II) ions has also been isolated by the following procedure: 0.325 g of $Na_3[Fe(CN)_5NH_3].3H_2O$ (1 mmol) was mixed with 0.330 g of MF.HCl (2 mmol) in 10 mL of water, and treated with 0.320 g of $Na_2CO_3{\cdot}1.5H_2O_2$ (2 mmol) and kept under stirring for 30 min. During this period the initial yellow solution changed gradually into a deep red color, with the evolution of O_2 bubbles. Then 30 mL of ethanol was added, precipitating a white-yellow solid and yielding a deep red solution containing the product. The solution was filtered and left overnight in a Petri dish, kept in the hood, for the slow evaporation of the ethanol solvent. The black residue was collected on a filter and washed many times with pure ethanol. The final product was kept under vacuum, in a desiccator with $CaCl_2$. Anal. Calcd (Experimental) for $Na_2[Fe(CN)_5MF].6H_2O$, $C_9H_{24}FeN_{10}Na_2O_6$: C = 23.00 (23.23), N = 29.79 (30.03), H = 5.15 (5.15).

Measurements

The electronic spectra were recorded on a Hewlett Packard model 8453-A diode-array spectrophotometer, with deuterium and tungsten lamps using rectangular cuvettes with a 1 cm optical path.

The colorimetric tests were performed using a freshly prepared 5.00 mM solution of $Na_3[Fe(CN)_5NH_3].3H_2O$, and an aqueous stock solution of metformin (50.0 mM). After mixing the reagents, the metformin concentration varied from 0.1 mM to 5 mM. Then, sodium percarbonate was added, yielding a final concentration of 7.5 mM hydrogen peroxide, and a 5 mM solution of carbonate ions. The last ones are relevant for providing a basic solution required for the test. The yellow solution turned rapidly into a deep red color, monitored colorimetrically at 520 nm.

Raman measurements were carried out with a WITec 300-R confocal microscope [18]. The samples were monitored with a laser spot area of 1 μm^2, using a 10× lens and 0.25 numerical aperture, 600 grating, and a CCD detector of 1600 × 200 pixels. The laser wavelength, power, and integration time were and 532 nm, 1mW, 3 s respectively.

Magnetic susceptibility measurements were carried out using an analytical balance adapted to the Gouy method [19,20], with the nickel tris(ethylenediamine) thiosulfate complex as standard.

Theoretical calculations were carried out using Orca 5.0.3 DFT with PBE0 functional, and the def2-TZVP base for all the electrons. Water was implicit as the solvent in C-PCM calculations. The electronic spectra were simulated with TD-DFT, encompassing 100 energy states. Similar calculations were also carried out using B3LYP instead of PBE0, with consistent results.

Results and discussion

In our preliminary experiments we have observed that metformin reacts with the $[Fe^{II}(CN)_5NH_3]^{3-}$ complex, yielding a yellow product, with minor color changes. It should be noted that the reacting species is the $Fe(CN)_5H_2O]^{3-}$ ion generated from the dissociation of the aminopentacyanidoferrate(II) complex [11,12], however, in the presence of an oxidizing agent such as hydrogen peroxide, a deep red color appears, with dramatic spectral changes, as shown in Figs. 2 and 3.

A characteristic absorption band is observed at 520 nm, increasing systematically with the amount of metformin, in the presence of the pentacyanidoferrate(II) complex in excess (5.0 mM) and hydrogen peroxide (from $Na_2CO_3{\cdot}1.5H_2O_2$ = 7.5 mM).

The red color is associated with the $[Fe^{III}(CN)_5(MF)]^{3-}$ complex, and the following scheme can be proposed:

$$Na_2CO_3.(3/2)H_2O_2 + H_2O \rightarrow 2Na^+(aq) + CO_3^{2-}(aq) + (3/2)\ H_2O_2$$

$$[Fe^{II}(CN)_5NH_3]^{3-} + MF \rightleftharpoons [Fe^{II}(CN)_5MF]^{3-} + NH_3$$

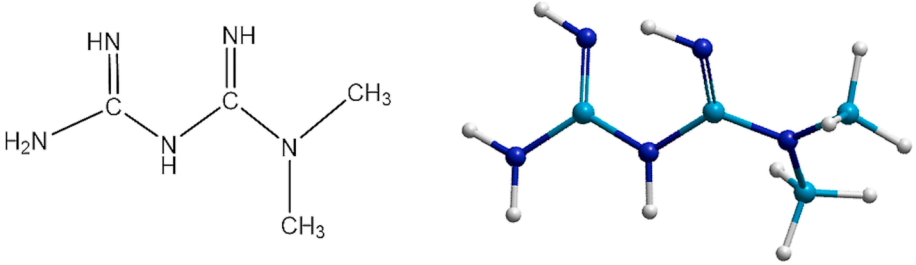

Fig. 1. The chemical and structural formula of metformin.

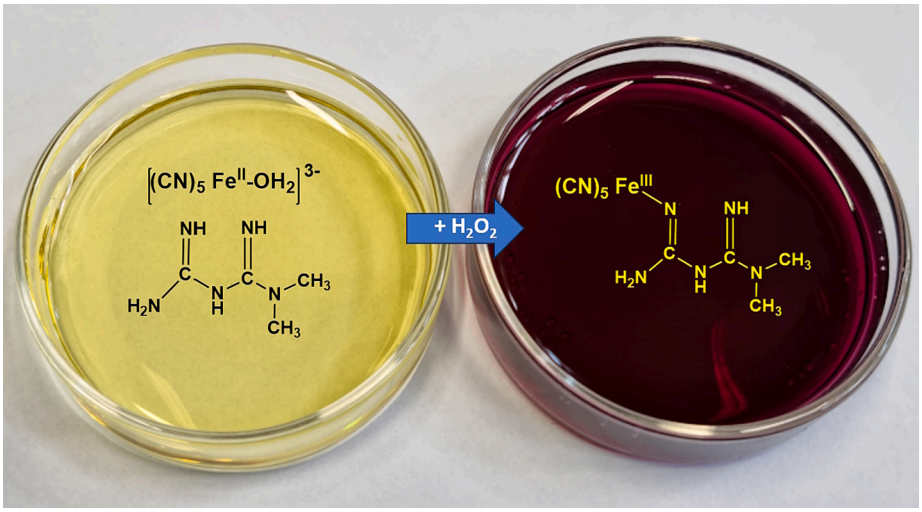

Fig. 2. Illustration of the colorimetric reaction between the pentacyanidoferrate(II) complex and metformin, in the presence of hydrogen peroxide.

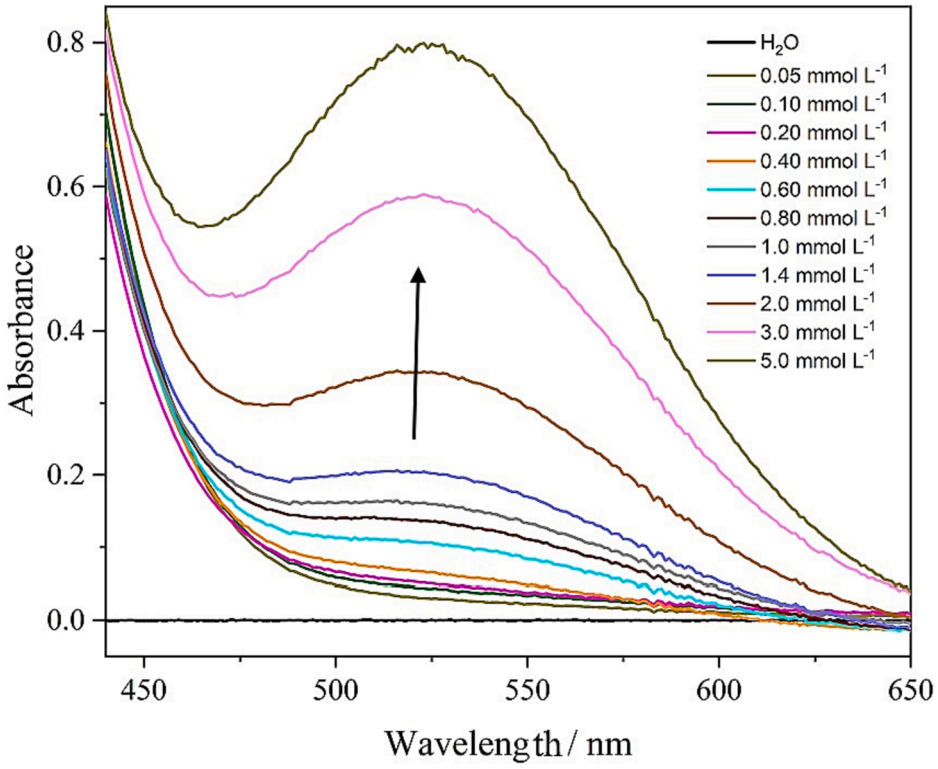

Fig. 3. Absorption spectra of the red product generated from the reaction of the aminopentacyanidoferrate(II) (5.0 mM) and variable amounts of metformin, in the presence of hydrogen peroxide (7.5 mM). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

$2[Fe^{II}(CN)_5MF]^{3-} + H_2O_2 \rightarrow 2[Fe^{III}(CN)_5MF]^{2-} + 2OH^-$

H_2O_2 (excess) $\rightarrow H_2O + (1/2)O_2$ (Fe^{III}/Fe^{II} catalysis)

The red $[Fe^{III}(CN)_5MF]^{2-}$ complex has been isolated in solid form by adding ethanol to the aqueous solution. In general, pentacyanidoferrate (II) complexes are rather insoluble in 3:1 ethanol: water mixtures, in contrast with the pentacyanidoferrates(III) species. This difference allows the selective extraction of the $[Fe^{III}(CN)_5MF]^{3-}$ species from the aqueous solution. In this process, the ethanol addition also leads to the precipitation of Na_2CO_3, which can be removed by filtration. After the solvent evaporation, the resulting dark residue was washed many times with ethanol. This leaves the final product, $Na_2[Fe(CN)_5MF].6H_2O$, in pure form, since it is insoluble in pure ethanol.

To understand the electronic spectra, DFT and TD-DFT theoretical calculations were carried out for the metformin molecule and the pentacyanidoferrate complex. Accordingly, the electronic structure (Fig. 4) was consistent with the metal ion in the Fe(III) oxidation state. It was found that the coordination of the Fe(III) ion stabilizes the guanidine moiety in the deprotonated form by 15 kcal mol^{-1}. In this form, the ligand is a better π-donor, stabilizing the Fe(III) oxidation state. This point can be seen in the TD-DFT results (Fig. 4) where the ligand π-donor orbitals appear red and the Fe(III) π-acceptor orbitals in blue color. Therefore, the strong visible band arises from the $\pi \rightarrow \pi^*$ charge-transfer transition from the guanidino group (π-donor) to the Fe(III) d_π-orbital in the $[Fe^{III}(CN)_5]^{3-}$ complex (π-acceptor). In support of this conclusion, it

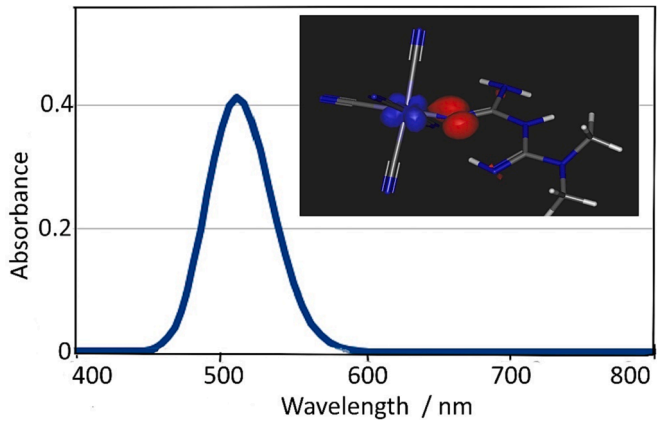

Fig. 4. TD-DFT theoretical spectrum of the [FeIII(CN)$_5$(MF)]3- complex, with the donor (red) – acceptor (blue) orbitals involved in the ligand-to-metal charge transfer band (inset). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

should be noticed that the red color completely fades in fades in the presence of ascorbate ions, which promote the reduction of the complex.

Magnetic susceptibility measurements have indicated an effective magnetic moment for the iron complex of 1.46 BM, at room temperature, consistent with the Fe(III) oxidation state.

For the quantitative evaluation of metformin using the pentacyanidoferrate(II)/H_2O_2 reaction, a colorimetric calibration plot has been elaborated. As one can see in Fig. 5, the linear correlation was quite good, leading to a limit of detection (LOD) of 0.018 mmol/L and a limit of quantification (LOQ) of 0.05957 mmol/L [21].

Although these values are not competitive with the existing chromatographic methods in terms of sensitivity, the colorimetric method can be interesting considering its facility and cost. It should be noted that colorimetric and spectroscopic methods provide the real analytical fingerprints of the species, because of their unique electronic and vibrational characteristics.

We have validated the detection method by performing a comparative analysis of commercial samples from three different suppliers in Brazil, namely Glifage, Prati, and Merck generics. About the nominal contents of the samples, our analysis indicated 99 and 98 % purity, in agreement for the first two suppliers and 96 % for the last one, with excellent reproducibility (>98 %) under ambient conditions.

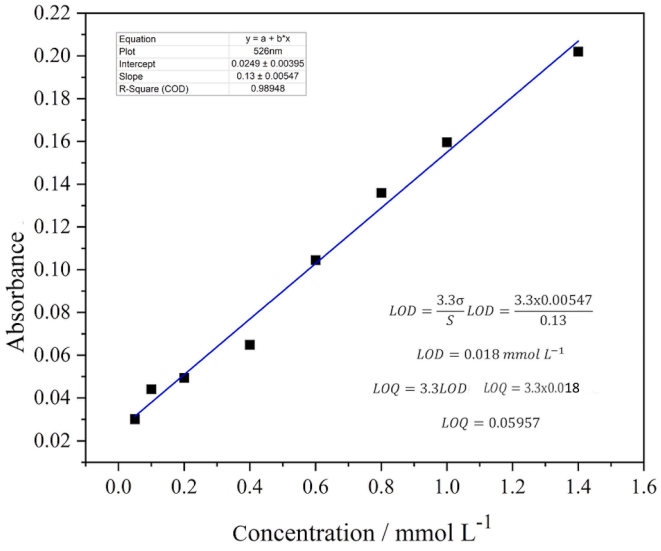

Fig. 5. Calibration plot for the colorimetric reaction, and the corresponding LOD and LOQ values.

Resonance Raman spectra

The red product can also be probed with Raman spectroscopy. It should be mentioned that normal Raman spectroscopy exhibits a poor intensity due to the small scattering efficiency of the molecules, thus requiring very concentrated solutions to be detected, e.g. 1 M. However, when the energy of the exciting radiation coincides with the maximum absorption of a strong band of the molecule, a resonance Raman effect takes place. This effect increases the scattering cross-section of the molecule, and can enhance the Raman signals up to 6 orders of magnitude, allowing monitoring of very diluted solutions, e.g. 10^{-4} M or below. The most important aspect of the resonance Raman mechanisms [22,23] is their chromophore selectivity, which enhances only the vibrational peaks of the groups of atoms involved in the electronic transition. In this way, Raman spectroscopy can be very useful for monitoring the reaction of metformin with the pentacyanidoferrate(III) complex.

The red product exhibited strong and well-defined Raman signals, as shown in Fig. 6. The Raman spectra are compatible with the proposed composition, indicating the presence of the $[Fe^{III}(CN)_5]^{3-}$ moiety (ν_{CN} = 2103 cm^{-1}, $\delta_{Fe\text{-}CN}$ = 514, 563 cm^{-1}), $\nu_{Fe\text{-}N,\ Fe\text{-}C}$ = 366, 422 cm^{-1} and metformin ($\nu_{N\text{-}H}$ = 3193, 3378 cm^{-1}, $\nu_{C\text{-}H}$ = 2940, 2977 cm^{-1}, $\nu_{C=C,C=N}$ = 1424, 1472, 1508, 1579 cm^{-1}), δ_{NH2} = 1649 cm^{-1}, $\nu_{C\text{-}C,\ C\text{-}N}$ = 936, 1038, 1080, 1166, 1281 cm^{-1}, ω_{NH2} = 737, 800 cm^{-1} [24–26]. Their high intensity and vibrational peak profiles are consistent with the involvement of a charge-transfer excitation from the guanidine π-orbitals to the Fe(III) dπ* orbitals, as shown by the theoretical calculations in Fig. 4. The Raman profiles in Fig. 6 are associated with this major chromophore group in the $[Fe^{III}(CN)_5MF]^{3-}$ complex.

As reported by Gul et al. organic ligands can act as effective sensors for coordination [27], and our findings are in harmony with this expectation. In addition, for curiosity, Smith et al. [28] have mentioned that pentacyanidoferrates are possible carriers of extraterrestrial cyanide from primitive meteorites, and could be involved in the abiotic synthesis of numerous organic compounds participating in the origin of life on Earth [28]. In this sense, the biguanide reaction reported in this work can also inspire new interesting ideas, since the molecule can be produced from prebiotic species such as cyanamide and ammonia [29].

Conclusion

Since metformin is a colorless species, the colorimetric detection of this important anti-hypoglycemic drug has never been performed up to the present time. In this work, we have shown that this can be done through the formation of a deep red compound, after reacting with pentacyanidoferrate(II) complexes in the presence of sodium percarbonate, $Na_2CO_3{\cdot}1.5H_2O_2$. The reaction can be easily monitored by probing the absorption band at 520 nm. The red color is associated with a new pentacyanidoferrate(III)-guanidine chromophore, which can also be accessed and characterized using Raman spectroscopy.

Because of its great simplicity, the colorimetric assay provides a useful alternative method for the routine detection of metformin in commercial drugs. Although it does not compete with the existing chromatographic methods in terms of sensibility, it is very simple and practical, yielding a unequivocal fingerprint response for MF through its characteristic electronic and vibrational profiles.

Author contributions

M.D. Ramos Jr, A.L. Hennemann, and L.M. Sinh performed the analytical and spectrophotometric measurements, M. Nakamura carried out the resonance Raman study, A.L.B. Formiga performed the theoretical calculations and H.E. Toma was responsible for conceptualization, investigation, and writing.

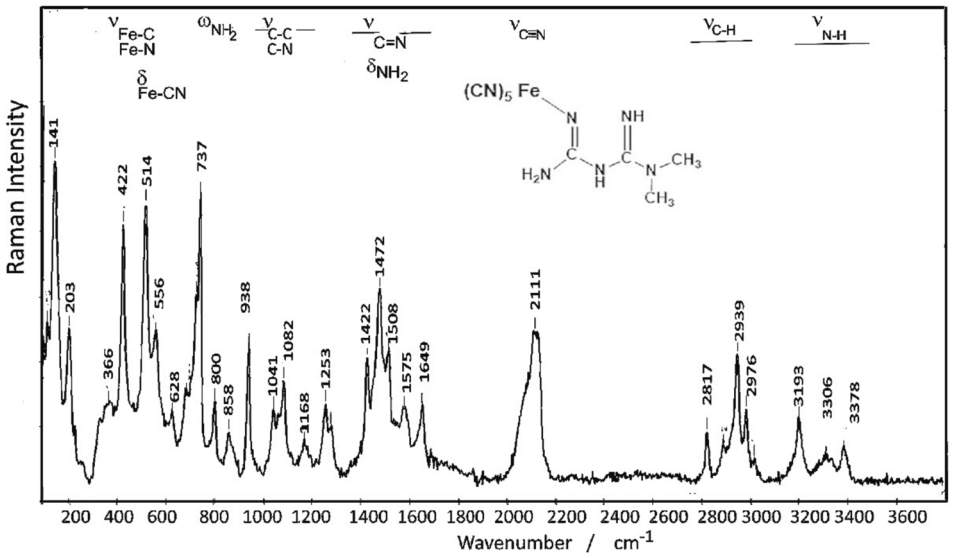

Fig. 6. Resonance Raman spectra of the $Na_2[Fe^{III}(CN)_5MF].6H_2O$ complex, at $\lambda_{exc} = 532$ nm.

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CRediT authorship contribution statement

Artur L. Hennemann: Investigation, Formal analysis. **Miguel D. Ramos-Jr:** Methodology, Formal analysis. **Luca M. Sihn:** Methodology, Investigation. **Marcelo Nakamura:** Methodology, Formal analysis. **André L.B. Formiga:** . **Henrique E. Toma:** Writing – review & editing, Supervision, Investigation, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: HENRIQUE EISI TOMA reports financial support was provided by University of São Paulo. HENRIQUE EISI TOMA reports financial support was provided by State of Sao Paulo Research Foundation. HENRIQUE EISI TOA reports a relationship with University of São Paulo that includes: employment. Nothing to declare If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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