



STUDYING NEUROMELANIN SYNTHESIS BY PCA

A deeper knowledge on neuromelanin synthesis from dopamine oxidation is mandatory to clarify the role of neuromelanin in neurological disorders pathogenesis. The present work focuses on defining the role of temperature, buffer and metal ions on both dopamine oxidation rate and oligomer size by monitoring the reaction by UV-Vis spectroscopy and applying Principal Component Analysis.

Neuromelanin in brain: good or naughty?

The name melanin indicates a large and very different class of polymeric pigments, present in almost all living species and characterized by their dark colour [1]; among the various melanin types, neuromelanin (NM) is the dark pigment observed in the brain, composed of covalently linked melanic, lipid, and peptide moieties, with coordinated iron and copper ions [2]. NM biosynthesis begins in the cytosol, upon accumulation of catecholamines [3] with DA being the main component but NM is also rich in iron and copper ions [2], organized in both multinuclear clusters and mononuclear centers: the metal composition is different in NMs from the various brain areas, with iron always the most abundant metal ion.

The main reason behind the increasing interest in the study of NM comes from its involvement in the pathogenesis of Parkinson's and Alzheimer's diseases: in fact, NM accumulates during aging in catecholaminergic neurons of the substantia nigra pars compacta (SN) and locus coeruleus (LC) but the loss of NM following neuronal degeneration and depigmentation of the brain regions mostly triggered by these diseases is a common phenomenon, at least in early events [4]. Going into more detail, NM plays two basic roles: on one hand, NM is associated with neuroinflammation as its release from dying neurons promotes the formation of reactive oxygen species causing neuroglia activation [5]; on the other hand, NM is also considered a protective component of the neurons since it acts as a scavenger of redox-active metal ions and neurotoxic species [5].

How can we study neuromelanin's related processes?

The study of NM activities is complicated by its large heterogeneity, insolubility and very low availability [2]. Thus it is of paramount importance to set up a reliable preparation procedure to get synthetic neuromelanin as a model compound for studying the properties of the natural pigment. To develop protocols that allow the preparation of synthetic NM, it is necessary to evaluate the effects of all the parameters involved in the process on both DA polymerization rates and the resulting molecular structures. Typically, activity studies are performed by changing one variable at the time, holding all the others at a fixed value, and observing how this single change affects the results but, having clear in mind that all the parameters are interconnected in the synthesis of NM models, it glaringly appears that a similar approach should not be applied to the characterization of complex systems. Furthermore, these investigations are usually conducted in conditions far from those in vivo and require laborious and complex result interpretation.

In the present work, we attempted to overcome the previously listed disadvantages by following a chemometric approach to rationalizing the effect exerted by the parameters involved in DA oxidation and consequent NM synthesis, mainly focusing on the temperature, buffering agent, Fe^{II} and Cu^{II} concentrations. Specifically, the activating, inhibitor or non-influencing effect of the listed parameters was observed by allowing the simultaneous modification of multiple parameters, following the changes in the entire UV-

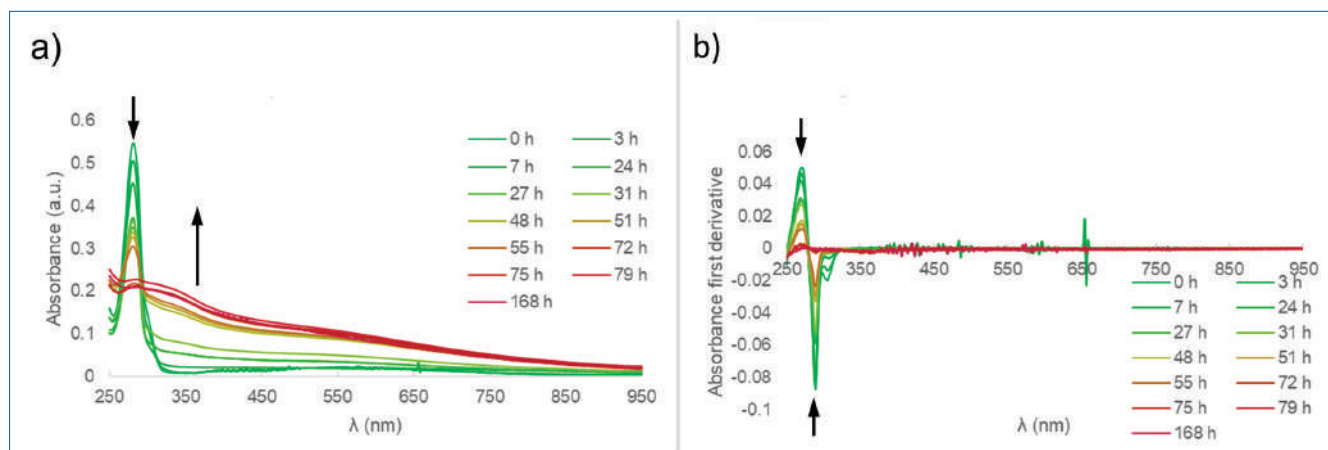


Fig. 1 - UV-Vis spectra (a) and spectra first derivative (b) acquired at different times during DA oxidation kinetic experiments at 25 °C in 50 mM MOPS buffer ($I \approx 20$ mM) at pH 7.0 for the samples containing both Cu^{II} and Fe^{II}

Vis spectra of the reaction mixture by means of the Principal Component Analysis (PCA) [6] applied both on the raw spectra and their first derivative to jointly monitor NM formation and DA consumption.

Chemometric-assisted approach to NM synthesis investigation

The chemometric-assisted approach here applied allowed to extract valuable information from apparently noisy and disturbed signals acquired during DA oxidation; to better clarify this aspect, in Fig. 1a an example of the UV-Vis spectra acquired during DA oxidation kinetics in the presence of both Cu^{II} and Fe^{II} is reported. The progress of the oxidation results in two main phenomena: (i) the decrease of the DA absorption band ($\lambda_{\text{max}}=280$ nm) with time together with (ii) an increase of the spectra baseline, resulting in the characteristic decreasing monotonic curve, caused by the scattering of light by DA oligomers [7]. In addition, a shoulder slightly above 300 nm is present, probably related to the fraction of DA coordinated to the metal ions [8]. It is worth noting that, by taking all these spectral features together, extracting valuable information from such experimental data is hard, especially relying on classical univariate data treatment. To avoid extensive data manipulation, ranging from subtracting the background absorption to applying some kind of operator-biased baseline correction or other techniques that may lead to data misinterpretation, we opted for a multivariate approach relying on PCA [6]. This technique allows considering the entire UV-Vis spectra to characterize the system and extract the information contained in the raw data from the background noise. In particular, from multivariate analysis on UV-Vis spectra, we extracted information on background absorption, thus on the formation of small oligomers of DA, which

were not separated from the solution by centrifugation. On the other hand, from the analysis of UV-Vis spectra first derivative (d1), reported in Fig. 1b for the reference dataset, we could investigate DA consumption from the decrease in the DA absorption band ($\lambda=280$ nm). UV-Vis spectra or d1 for each set of samples are firstly submitted to PCA to reduce the dimensionality; then, if the first principal component PC1 accounts for a sufficiently high percentage of explained variance and can be associated with the physical or chemical phenomena of interest, the score value on PC1 for each original spectra at a given time is plotted vs. time to obtain the trend we defined “Multivariate Kinetic Curves” (MKCs); this final output summarizes the ongoing process and allows to visualize the kinetics of NM formation and DA consumption. For the sake of brevity, here only the first case will be presented while further details are available at [9].

Case study 1: DA oxidation in MOPS buffer at 25 °C

The first screening experiment was performed in 50 mM MOPS buffer ($I \approx 20$ mM) at pH 7.0, monitoring six different samples, containing only DA or DA+ Fe^{II} , DA+ Fe^{III} , DA+ Cu^{II} , DA+ $\text{Fe}^{\text{III}}+\text{Cu}^{\text{II}}$, for 8 days, acquiring three spectra per day. Consequently, the UV-Vis spectra first derivative is calculated per each spectrum acquired and both the UV-Vis spectra and spectra first derivative (d1) were exploited to build MKCs. Fig. 2 shows the MKCs obtained plotting the average score value on PC1 vs. time for both spectra (Fig. 2a) and d1 (Fig. 2b): starting from the spectra analysis, the loadings plot (here omitted but reported in [9]) suggested that the score value on PC1 increases when background absorption increases, thus when the DA oligomers are characterized by small dimensions and cannot be removed by centrifugation.

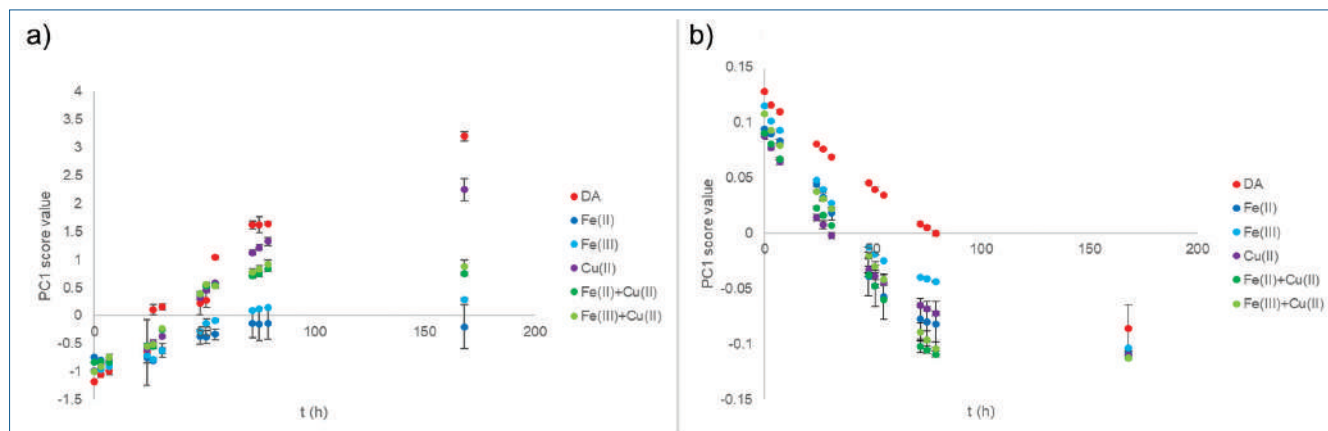


Fig. 2 - MKCs for DA oxidation in MOPS buffer at 25 °C, obtained plotting the average score value on PC1, calculated for the three replicates, vs. time for spectra (a) and spectra first derivative d1 (b) for DA-only (red), Fe(II) (blue), Fe(III) (light blue), Cu(II) (violet), Fe(II) + Cu(II) (green) and Fe(III) + Cu(II) (light green)

Having this in mind, Fig. 2a suggests that all the samples started from a similar background absorption which underwent a steady increase in the first five days, up to 79 h, while the increase between 79 and 168 h (Day 8) is negligible. Secondly, DA autoxidation, in the absence of metal ions, led to the formation of the smallest DA oligomers, thus resulting in the highest background absorption while Fe^{III}-containing samples are the ones with biggest DA oligomers, almost completely removed by centrifugation. Last but not least, when both Cu^{II} and Fe^{III} were added, no cooperation was observed between the two metal ions but the samples showed a behaviour in between the ones containing either copper or iron.

Moving to the analysis of spectra first derivatives, the loadings plot clearly highlighted that PC1 score value decreases together with DA consumption thus the MKCs (Fig. 2b) suggests that DA oxidation mainly occurred within the first five days. As for the effect of sample composition, (i) DA oxidation is slower when no metal ions were added; (ii) iron-containing samples, especially in the case of Fe^{II}, presented a sort of linear behaviour within the first five days; (iii) Cu^{II}-containing samples showed the highest oxidation rate in the first two days and then the reaction slowed down; (iv) mixed samples again present an intermediate behaviour. We could conclude that the difference in copper-containing and iron-containing samples relies on the balance between nucleation and growth of DA oligomers: in the case of Cu^{II}, the higher initial DA oxidation rate strongly encouraged nucleation, which resulted in the formation of a higher number of seeds that, on one hand, could not reach large dimensions and remained in solution after centrifugation, and on the other hand, might sequester the metal ion, reducing its catalytic activity towards DA oxidation. In the case of iron, the

slower initial kinetics led to the formation of fewer seeds exhibiting constant growth during time, not affecting iron catalytic activity. This explanation was consistent with the results obtained for both copper and iron-containing samples in which we could assume that no cooperation occurred between the two metal ions.

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Come studiare la sintesi di neuromelanina tramite PCA

Una miglior conoscenza della sintesi della neuromelanina dall'ossidazione di dopamina è fondamentale per chiarire il ruolo della neuromelanina nella patogenesi di disturbi neurodegenerativi. Questo lavoro punta a definire il ruolo di temperatura, agente tamponante e cationi metallici sulla velocità di ossidazione e sulla dimensione degli oligomeri di dopamina grazie al monitoraggio della reazione con spettroscopia UV-Vis e all'applicazione dell'analisi delle componenti principali.