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ORIGINAL ARTICLE

Qualitative evaluation of ferritin in serum samples by Raman spectroscopy and principal component analysis

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Abstract
Iron molecule is of great importance in the synthesis of hemoglobin which is essential for oxygen transport. Iron levels are quantified by accurately high sensitivity tests, such as serum ferritin (SF). However, common studies to quantify SF are long and strenuous (~ 5 h), for example enzyme-linked immunosorbent assay (ELISA). In this paper, blood serum samples were analyzed by Raman spectroscopy (RS), and a computational analysis of spectra is proposed to detect differences in SF as an alternative procedure. Serum samples were obtained from 22 patients, 9 who were clinically diagnosed with anemia and 13 controls. Patients with anemia had low levels of SF (< 30 ng/ml), and a control group had levels between 30 and 500 ng/ml. The spectra obtained were conditioned with a baseline correction and smoothing, then evaluated by principal component analysis (PCA), and a predictive model was estimated by lineal discrimination analysis (LDA). The results showed a clear differentiation of the study groups by PCA, also 99.69% sensitivity and 100% specificity by LDA. This study suggest that Raman spectroscopy is a fast (~ 5 min) and a powerful tool capable to qualitative differentiate ferritin concentrations.

Keywords Iron levels · Serum ferritin · Raman spectroscopy · Principal component analysis

Introduction

Assessment of iron levels in human body is important because it is involved in many biochemical processes for example hemoglobin production and oxygen transport. The excess of iron in the body can generate free radicals causing lipid peroxidation and DNA chain breakdown as well as degradation of other biomolecules leading to chronic degenerative diseases or even cancer [1]. The most common indicators for iron titration are tests of mean corpuscular volume, mean corpuscular hemoglobin, transferrin saturation, and SF [2]. Ferritin is an iron deposition protein in mammals composed of 24 peptide subunits types [3]; there are two forms to storage iron: as ferritin and as hemosiderin. Ferritin is found in high concentrations in hepatocytes, cells of the endothelial reticulum system of the liver, spleen, and bone marrow. This protein is found naturally in blood circulation with similar characteristics of reactivity, molecular size, and isoelectric point as

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compared in the liver and spleen [4]. The main function of SF is to regulate the iron metabolism, which in turn contributes to the synthesis of hemoglobin and the oxidation of Fe (II) (highly toxic) to Fe (III). Another important function is as an indicator of iron levels in the body; low levels of SF are found in patients with iron deficiency as anemia, while high levels of SF represent iron overload or hemochromatosis. Normal iron levels in men and women are ~ 103 and ~ 35.6 μg/L respectively [5–7].

There are different methods for the quantification of serum ferritin, for example, enzyme-linked immunosorbent assay (ELISA), chemiluminescence, celectrochemiluminescence, and SDS-PAGE electrophoresis [8, 9]. Because of the long time that these techniques require, other alternatives are sought.

Other techniques have been used to evaluate biological systems, such as RS, which is an optical technique based on the study and classification of molecular vibrations generated by the interaction of light (laser) with matter (sample). This provides information about chemical composition, molecular structure, symmetry, and bonds, allowing quantitative and qualitative analysis [10–12]. In recent years, there have been substantial studies using RS for cancer diagnosis [13, 14], blood analyte test [15, 16], and cell differentiation [17]. Also, RS allows easy handling and preparation of samples, using a minimum amount of sample to perform the analysis, due to the high sensitivity of the technique that allows to detect low concentration of molecules [18, 19]. So RS can be used to evaluate iron levels in the body SF.

Recently, the spectrum of characteristic ferritin peaks is reported by Szybowicz et al. [20] where ferritin presents a Raman spectrum with nine characteristic peaks ranging from 200 to 1000 cm^{-1}, and ordered from the lowest to the highest, those are found at 269, 332, 389, 455, 550, 642, 761, 853, and 950 cm^{-1}. In this work, a qualitative analysis in blood serum was carried out by RS, analyzing the region of the spectrum that comprises the ferritin peaks to differentiate levels of ferritin in anemic and control patients, also, developing a predictive model to differentiate spectra and create a fast an accurate alternative analysis to test iron levels in the body.

Material and methods

Blood serum samples

The present study was conducted in association with the Mexican Social Security Institute (IMSS)- Clinical Research Unit No.1. A cross-sectional study was developed using BS collected from anemic patients with low ferritin levels (< 30 ng/ml), and control patients with levels between 30 and 500 ng/ml, whom previously signed an informed consent. Nine blood serum samples from anemia group and 13 from the control group were stored at − 70 °C. The samples were transported at − 70 °C to the university laboratory to perform the measurements.

Raman spectra acquisition

All blood samples were thawed at room temperature, then 20 μL were placed on an aluminum-coated slide and allowed to dry; and for each sample, a 25-point mapping was performed, generating 25 spectra, as shown in Fig. 1. A total of 23 samples were processed (13 control samples and 9 anemia

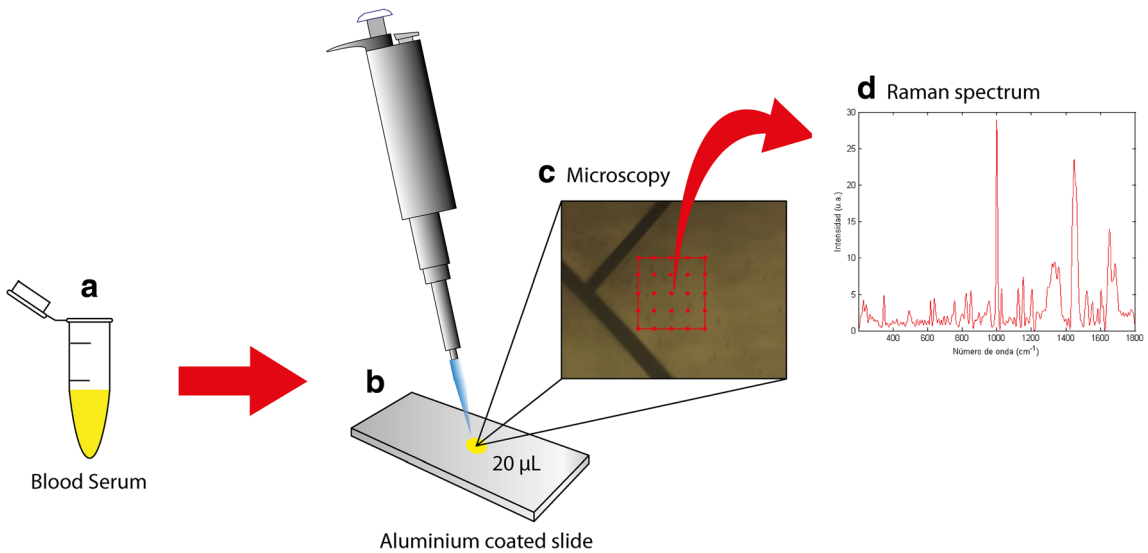

Fig. 1 Schematic process of sample handling and spectrum acquisition. **a** Blood serum thawed at room temperature. **b** 20 μL placed on aluminum coated slide. **c** Microscope focus with × 50 objective. **d** Point-to-point obtained spectra measurement

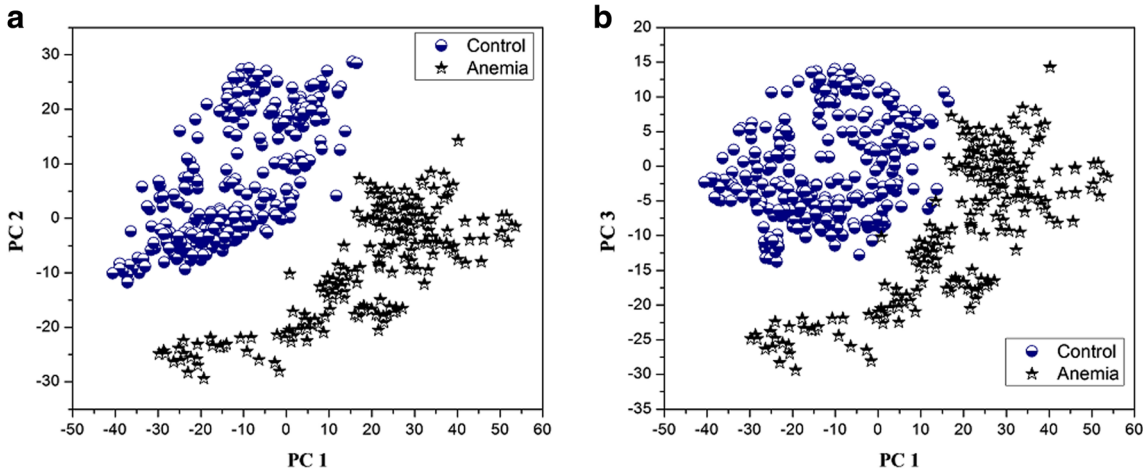

Fig. 2 PCA of the Raman spectra. Score plots take two principal components of both, control group and anemia group. (**a**) Principal component 1 vs. principal component 2. (**b**) Principal component 1 vs. principal component 3

samples), for each one 25 spectra were taken, having a universe of study of 550 spectra (325 control spectra and 225 anemia spectra). For data acquisition, a Thermo scientific Raman microscope DXR with 780 nm excitation laser diode was used. All spectra were taken using laser power of 24 mW and 60 s of exposure time. Samples were focused through a × 50 objective. The spectral resolution was ~3.5 cm^{-1}, and all measurements were carried out in range 200 to 1800 cm^{-1}.

The spectra were processed and analyzed by different steps using MATLAB v. 2014 functions and scripts. The purpose of pre-process data is to reduce the error inherent in sample and equipment handling [21], performing a baseline correction and smoothing of each spectrum.

Spectra analysis

Two-sample *t* test was made to the average spectra of each group as the first analysis of differentiation. *T* test assumes that all samples have normal distribution, same meaning, and same variances with 95% confidence interval.

Moreover, PCA was used as a second analysis of differentiation. PCA is normally used in spectroscopy analysis as a dimensionality reduction technique for a data set by extracting the eigenvalues and eigenvectors of the covariance matrix. The eigenvectors become the new set of axis, and the eigenvalues describe the variability of the data associated to each eigenvector [22].

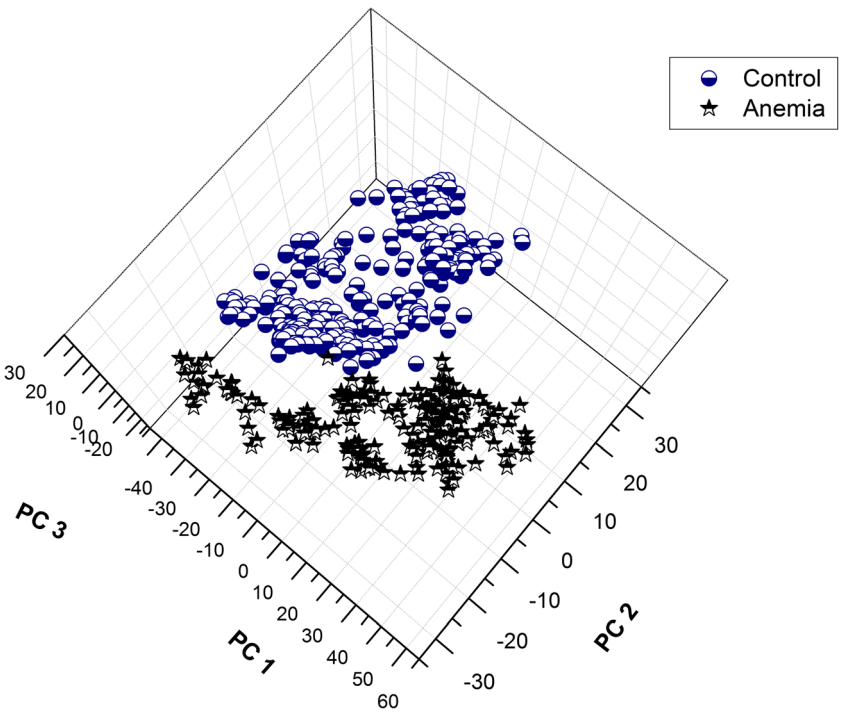

Fig. 3 PCA of the Raman spectra. Score plots take three principal components for both, anemia group and control group (principal component 1 vs. principal component 2 vs. principal component 3)

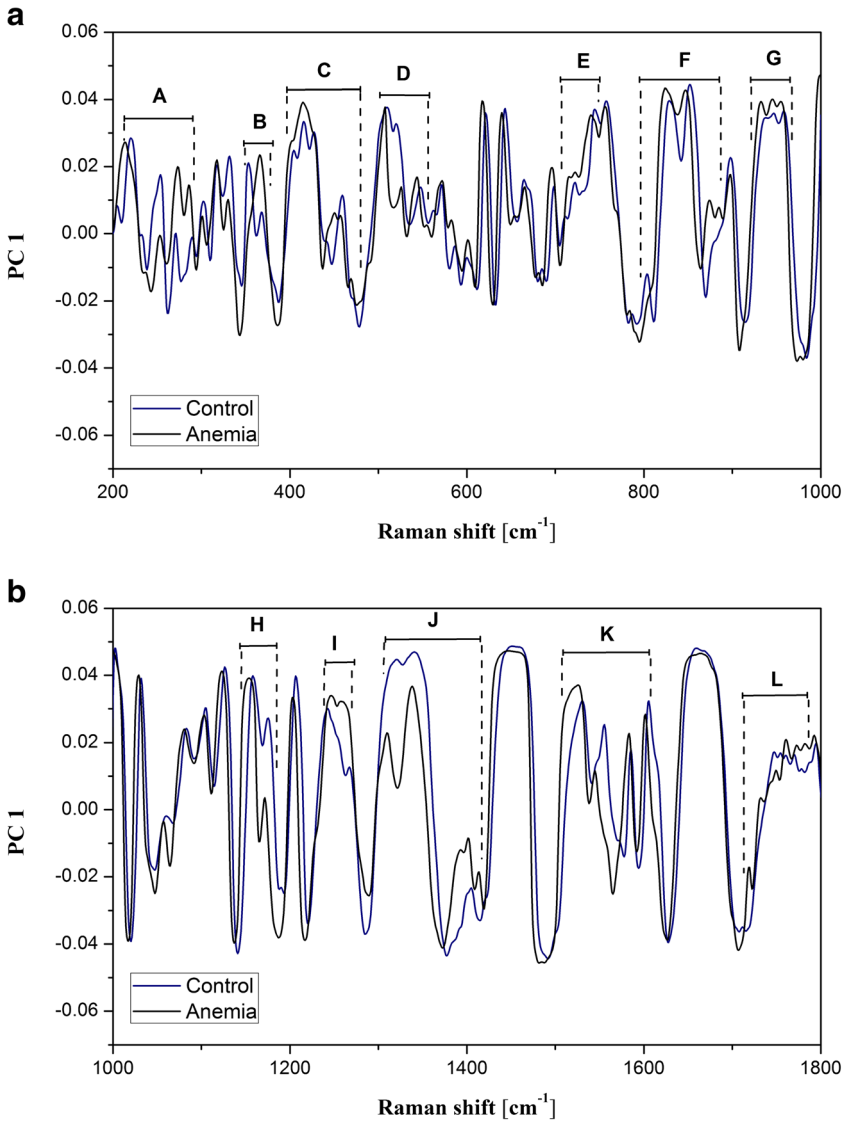

Fig. 4 Plots of the first principal component as a function of the wavenumber

All the spectral block (500 spectra) was taken to a baseline correction and smoothed to remove the noise and fluorescence caused by the samples and their subsequent evaluation through an analysis by PCA. The first three components

Table 1 Main bands where differences were observed in the first main component

Region	Frequency range	Tentative molecular vibrations and biomolecules assignments
A	220–290	Xmetal-O, Ferritin
B	349–376	Xmetal-O, Phy
C	396–479	Xmetal-O, Ferritin, Phy, Tyr, Trp
D	500–555	Ferritin, Phy, Tyr, Trp
E	708–750	Phy, Tyr, Trp
F	795–888	C-O-C, Ferritin, Tyr, Trp
G	921–967	C-O-C, Ferritin, Phy
H	1145–1185	Phy, Tyr
I	1239–1270	Tyr
J	1303–1416	Phy, Tyr, Trp
K	1508–1607	Phy, Trp
L	1713–1786	Carboxilic acid

(PC1, PC2, and PC3) were plotted to verify the significant differences between the control group and the anemia group. In order to observe the biochemical differences between both groups evaluated, PC1 was plotted as a function of the Raman frequency. Derived from this analysis, the regions that presented significant changes were selected and labeled from letters A to L.

Finally, LDA was used to create a prediction model of spectra classification. This method is useful to obtain a linear combination that best separates the known classes using a lineal equation [23]. The results were evaluated by a receiver operating characteristic (ROC) curve model.

Results and discussion

Student's *T* test

Using a 95% confidence interval a two-sample *t* test, we obtain a probabilistic $p < < 0.05$. Therefore, the null hypothesis was rejected, indicating that the study groups did not come from the same population.

Principal component analysis

The spectra were analyzed in the range of 200–1800 cm^{-1}. Taking two principal components (Fig. 2) and three principal components (Fig. 3), the PCA compares anemia group with control group, where separation between groups was observed.

By plotting the first main component of anemia group and control group, the intensities of certain vibration regions with differences were observed (Fig. 4). In our study, 12 regions of vibration were observed with differences in intensity, associated with their corresponding biomolecules (Table 1) [24], including five characteristic peaks of ferritin in these regions.

Lineal discriminant analysis (LDA)

We confirmed the difference of spectra by the test carried out; therefore, a model for prediction pattern was developed (Table 2). After the dimensionality reduction by PCA, the coefficients of the linear model were calculated. This linear combination of variables projects the data, that generates the best class separation. The LDA predictive model presented a sensitivity of 99.69% and a specificity of 100%. The results were computed and plotted by a ROC curve (Fig. 5) showed an area under the curve (AUC) of 0.996.

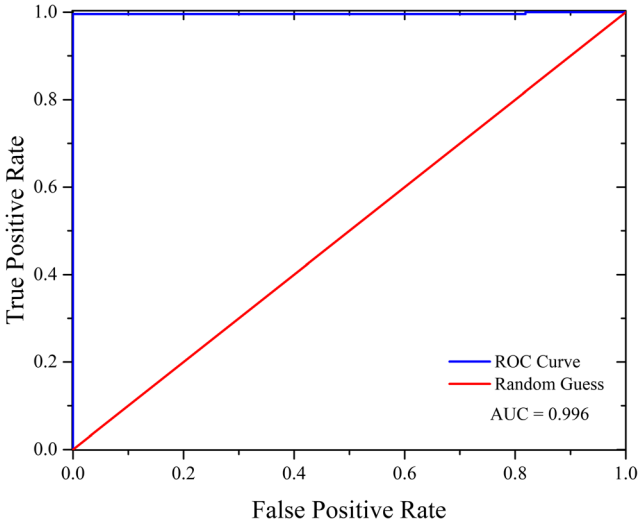

Fig. 5 ROC curve of the LDA model presenting an AUC of 0.996

Table 2 Model predictions by LDA, sensitivity, and specificity for study groups

Samples LDA	Anemia	Control
Predicted anemia	325	0
Predicted control	1	224
Total	326	224
Sensitivity	100%	
Specificity	99.56%	

Conclusions

In this work, the main objective was to evaluate different concentrations of ferritin in blood serum by RS to estimate the iron levels in the human body through the acquisition and computational analysis of Raman spectra. The methodology is divided into several stages including detection, differentiation, and estimation by a linear model. As a first step, signal conditioning is performed to obtain an improved signal quality followed by more accurate comparative statistical analyses.

As a first differentiation study, the two-sample *t* test performed showed differences between study groups. To confirm the observed differences in the previous analyses, PCA was used. Differences among groups were found by the formation of two well-differentiated clusters into the three principal components axis. By means of the graph of principal components against the Raman shift, 12 regions with marked differences were observed, each of them related to specific molecular vibrations, including five regions of ferritins; therefore, it can be assumed that the differences found between the study groups are primarily due to ferritin concentration.

To finish, a linear model was elaborated from LDA where high sensitivity and specificity was obtained. Furthermore, the ROC analysis showed a good behavior of the model, that can be used to classify spectra for future analysis. As future work, a calibration curve will be performed to obtain quantitative

data on ferritin and iron levels in the body and compare with the standard tests.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Additional informed consent was obtained from all individual participants for whom identifying information is included in this article.

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