Rapid diagnosis of malaria by chemometric peak-free LIBS of trace biometals in blood

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Research Article

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Abstract

Background

Laser Induced Breakdown Spectroscopy (LIBS) trace atomic species of diseased biofluids are very subtle (peak-free) in complex spectra. Trace analysis requires a considerable push in analytical strategy. Enabling LIBS with chemometrics can help identify, extract, analyze and interpret the species' spectral signatures to give an insight on the biophysiological status of the bodies from which the biofluids originate.

Methods

We report on the trace quantitative performance of peak-free LIBS enabled by chemometrics modeling using principal components analysis (PCA) for direct artificial neural network (ANN)–based analysis of Cu, Zn, Fe and Mg in *plasmodium falciparum*-infected blood in the context of rapid spectral diagnosis of malaria utilizing the biometals as the disease biomarkers. Only one standard is required in this method - to delineate the analyte spectral regions (feature selection) and to test for accuracy.

Results

Based on the alteration of the biometal levels and their multivariate and correlational patterns in cultured blood, peripheral finger blood drops dried directly on Nucleopore membrane filters was accurately discriminated as either malaria-infected or healthy utilizing principal components analysis (PCA) modelling. Further the morphological evolution of *plasmodium* was accurately predicted using spectral features of the biometals wherein high negative correlations between Fe (-0.775) and Zn (-0.881) and high positive correlations between Cu (0.892) and Mg (0.805) with parasitemia was observed. During the first 96 hours of malaria infection Cu increases profoundly (from 328 to 1,999 ppb) while Fe, Zn and Mg decrease (from 1,206 to 674 ppb), (from 1,523 to 499 ppb) and (from 23,880 to 19,573 ppb) respectively. Compared with healthy, *plasmodium falciparum*-infected blood has high Cu but low levels of Fe, Zn and Mg. Cu and Zn are highly (≥0.9) positively correlated while Fe and Cu as well as Zn and Cu are highly (≥ 0.9) negatively correlated.

Conclusion

Chemometric peak-free LIBS has demonstrated the potential for direct rapid malaria diagnostics in blood based on the levels, alterations and multivariate associations of analyzed trace biometals which are used as biomarkers of the disease.

Background

Body fluids have characteristic biochemical compositions but which change in response to pathological stimuli [1]. This leads to impairment of biological functions resulting to abnormalities in body metabolism [2–4] as well as structural changes and functionality of essential body components [5–7]. Accurate analysis, therefore of trace biometals in body fluids and tissue, particularly of those biometals involved in regulation of immunity is of interest since their levels, alterations and multivariate associations can be metrics for disease diagnosis utilizing the biometals as biomarkers. Metals and metalloids are crucial for a series of biopathological processes: up to a third of all proteins require metals to carry out their functions.

Nonetheless sequential changes in trace biometals in fluids harboring infections are hardly studied. Blood trace biometals have a role in both the protection as well as exacerbation of malaria, which is now considered to be perhaps the most important parasitic disease of man [8]. Malaria is a major problem especially in the Tropics where it is a leading cause of mortality [9]. The disease arises from infestation of the red blood cells by *plasmodium* parasites, the most common species in Africa [10, 11]. The infestation causes major changes in the metabolism and transport of important cellular components: a host's body normal response to infection includes increased synthesis of metal-binding proteins and a concomitant flux of trace elements in tissues.

For which reason the relationship of biometal levels in blood with cellular disorders linked to pathogenesis has been increasingly receiving attention [12–17]. Dogan *et al*. (1993) [18] used total reflection X-ray fluorescence (TXRF) to study behcet disease in serum. Using Möussbauer spectroscopy Bauminger *et al*. (1998) [19] determined the levels of Fe in serum and red blood cells in several blood diseases including malaria. Huszank *et al.* (2017) [20] analyzed Na, P, S, Cl, K, Ca, Fe, Cu, Zn, and Br directly in ~10 μL of blood. A dried blood spot method based on direct sampling by laser ablation-inductively coupled plasma-mass spectrometry (LA-ICP-MS) was reported to provide low LOD and LOQ of 3.5 $\mu g \cdot L^{-1}$ and 11.6 $\mu g \cdot L^{-1}$, respectively [21]. Se, Cu, Zn, Br and Rb were determined in very small (0.75 μL) human serum and mice whole blood by X-ray fluorescence (XRF) [22] wherein accurate results were obtained without the need of exact volume measurement because the backscatter correction method was used. Most of the above techniques however require sophisticated sample preparations including addition of chemical precursors and acid digestions.

A technique which is rapid and could be used directly, needing only one drop of blood without special preparation, would be advantageous in practice. Because most malaria occurs in resource-constrained regions of the world, the technique should preferably be portable, easy to use and inexpensive. LIBS presents unique advantages in these respects as it requires little or no sample preparation and therefore saves on time; it is ideal for remote applications especially when analyzing biohazardous samples; its spectral range has many lines available for analysis; and lastly it is minimally invasive and can perform real-time, stand-off spectroscopy [22, 23]. LIBS has also the advantages of small sample requirement (0.1 μg – 1 mg) converted to plasma [24, 25], and high sensitivity for determination of low-Z metals for which only a few analytical methods are available. Further hand-held LIBS systems now exist for field/ clinical deployment.

In LIBS a high energy, pulsed laser beam is focused onto the analysed sample to create a high temperature microplasma wherein the sample is ablated into atoms and ions. The subsequent emission becomes the analytical signal containing quantitative information from which the elemental composition can be determined. Since the precursor biochemicals in body fluids are in very low concentrations, their quantitative analysis requires to be made sensitive and accurate. The complexity of such samples (and data interpretation) constitutes a multivariate analysis problem; so chemometrics techniques may be used to overcome the challenge as they have ability to reduce dimensionality of the spectral data and to extract the subtle biomarker information from interfering spectrawithout altering the crucial analytical information [26, 27]. This work aimed at exploring chemometric peak-free LIBS for diagnostic analysis of trace biometal (Zn, Fe, Mg, Cu) concentrations and their multivariate alterations in human blood (embedded onto Nucleopore membrane filters) in relation to malaria onset and pathogenesis. The potential of the dried blood spot analysis has been recognized in diverse fields [28–39]. Its advantages over liquid blood analysis lie in the minimally invasive sampling, the sufficiency of small blood volumes as well as the simple transport and storage requirements [40–42]. The study focused on these biometals since they influence the cellular and molecular immunological functions, which are important in the host of *plasmodium* parasite. Fe, Cu, Mg and Zn are also antioxidants or antioxidant enzyme co-factors fighting against abnormal elevation of oxidative stress both from host and the parasite [43–46]. Most studies on *plasmodium falciparum* infection for malaria diagnosis have focused on the morphological aspect of the parasite, the structure of the red blood cells and how it changes in optical environment, as well as on the by-product of red blood cells (hemozoin) ingestion by the malaria parasites [47–50]. Common methods of malaria diagnosis (optical microscopy ('Gold Standard'), rapid diagnostic tests, molecular diagnosis, and syymptoms-based (which are not specific in malaria)) seek to determine the presence of *plasmodium* in the body [51]. These are labour-intensive, tedious, and require skilled personnel.

Methods

Preparation of parasite culture

Plasmodium falciparum parasites were cultured in a complete medium at 1% haematocrit at 37 °C in a 5% CO_2/3% O_2/balanced N_2 gas mixture. The complete media, a mixture of healthy O+ human blood and RPMI 1640 culture media, were used as the control samples. Infected human red blood cells were washed three times (to remove any undesired components such as bacteria) and stored at between 4–8 °C. They were added to the complete media to initiate the culture process. Whole blood samples were taken from the consenting 20 malaria patients as well as 23 healthy volunteers at the Kenyatta National Referral Hospital, Kenya. To take part in the study, all of the participants were well briefed and agreed to provide informed consent. They all also agreed willingly and signed a written consent form that was part of the Ethics Approval forms. The study was approved by the Kenyatta National Hospital-University of Nairobi Ethics and Research Committee (ERC certificate number: P112 / 03 /2018). All the methods that were used to handle and prepare samples in this study were performed in accordance with the relevant

guidelines provided in the Ethics Research Committee guidelines and regulations. The Haematocrit (initial parasitemia) was measured from a packed red blood cells volume that had been centrifuged in a swinging bucket rotor at 2000xg for 5 minutes at room temperature. Schizonts were isolated on Percoll cushions. For RS parasite analysis, aliquots were layered on top of 70% Percoll in 1.6 mL tubes and centrifuged at 4,000X g for 5 minutes. Red blood cell pellets were then washed once with RPMI 1640 medium and immediately frozen at -20 °C until they were used. The pellets were added to the complete media and kept in the CO_2 incubator at 37 ^{0}C and thereafter harvested after every 24 hours for four days [52].

Staining and examination of thick and thin smears

Each time the media was harvested both thick (6 µL) and thin (2 µL) blood smears were prepared on a glass slides. The slides were immersed in absolute methanol for fixation then dried. Ten percent (1:9 mL) for 10 minute and 3% (3: 97 mL) for 45–60 minute fresh, working Giemsa stains were prepared. The stained blood smears were washed with water to remove excess stain, dried and observed under a microscope. The thick blood smear was used to microscopically confirm infection by *plasmodium falciparum*; while the thin smears were used to determine the parasite morphology. The smear was first screened at a low magnification (10X × 40X objective lens) to detect suitable fields for analysis then later examined using X100 oil immersion. Parasitemia and morphology were determined at each harvested stage. The percent of infected red blood cells was determined by enumerating the number of infected in relation to the number of uninfected cells. This method required the preliminary determination of the number of erythrocytes present in the average microscopic field. Although in this work sub-culturing was not done and therefore the media and pellets were not renewed hence the parasitemia should be higher, the parasite load, expressed as a percentage parasitemia was in agreement with other studies where *plasmodium falciparum* was cultured without change of media [53, 54].

LIBS instrumentation and sample analysis

The LIBS system used in this study is the Ocean Optics LIBS2500plus with a laser wavelength of 1064 nm. This is a broad-band, high resolution system which allows spectral analysis in the wavelength range 200 - 980 nm with a resolution of 0.06 nm. The system has seven spectrometers with a specified wavelength range for each channel. Each spectrometer has a 2048 pixel linear silicon charge coupled detector (CCD) array with an optical resolution of 0.065 nm. These spectrometers are synchronized to acquire data using the OOILIBS software and store it in a PC. The laser fitted in this system is a 50 mJ maximum energy CFR Nd:YAG from Big Sky Laser Technologies emitting laser pulses of 10 ns width and 10 Hz fixed pulse frequency. Analysis samples are placed on a manually controlled X-Y stage and a laser beam focused on them to produce plasma.

Trace biometal analysis using artificial neural networks (ANN) calibration models

Multivariate ANN calibration models that had been developed and successfully tested for accuracy using a standard reference material (SRM)-1598a (inorganic constituents in animal serum) [52] were applied to

analyze the biometals in the *plasmodium falciparum* infected blood samples and the controls. SRM-1598 is a serum sample derived from a mixture of serum from healthy bovine and porcine animals and is used to evaluate the accuracy of analytical methods for selected elements in biological fluids similar to blood serum and plasma.

ANN is one of the computational ways of mapping non-linear input data to a target space. ANN is capable of performing different function such as curve fitting, pattern recognition as well as clustering. The most common of the network architectures is the multilayer feed-forward system in which the input data proceeds forward only (to the hidden layer and then to the output layer) and never makes loops as opposed to other architectures like the recurrent neural network system. The power of the network depends on the transfer function and the learning rule [42]. Prior to analysis the spectral data was pre-processed in order to eliminate data that was irrelevant to the study. The data was transformed in order to make the distribution of given variables and samples more suitable for a powerful analysis and for a more relevant analysis. Smoothing of the data was done by the moving average technique where the segment size was set to three. The degree of smoothing is usually determined by the width of the smoothing window i.e. the number of data points averaged. The preprocessed spectral databases were compressed into a smaller matrix for each element by obtaining data at different spectral regions for each element at emission wavelengths where LIBS has a spectral response. The output data (targets) were developed with the same number of columns as the input data. The datasets were imported to the neural network platform in MATLAB for analysis. The network was trained using the Levenberg-Marquardt training algorithm which randomly divides the input and output data set into three categories, that is, 60% training, 20% validation and 20% test set.

Table 1 shows the analytical accuracy of the developed ANN multivariate calibration models on a SRM-1598a. The obtained results demonstrate the good accuracy of the ANN calibration strategies that were developed. The Shapiro-Wilks normality test for the ANN model-determined concentrations in the blood indicated that the spectral data used for analysis was normally distributed; that is, Fe-0.8773, Cu-0.8412, Zn-0.8571 and Mg-0.9048. Therefore multivariate exploratory modeling for malaria diagnostics utilizing the determined biometal concentrations may be trusted.

Table 1 Comparison of Cu, Fe, Zn, Mg by ANN and SRM-1598a concentration (Source: [52])

	Cu	Fe	Zn	Mg
NIST/SRM-1598a [Actual value – (ppm)]	1.58±0.09	1.68±0.06	0.88±0.02	Not given
ANN model (ppm)	1.14±0.74	1.14±0.32	0.88±0.56	17.90±3.63

Spectral data modeling by principal components analysis (PCA)

PCA is a powerful tool for exploratory analysis as it performs a projection of the original data which allows for the visualization of the natural clustering of the data, evaluation of class similarities as well as reasons behind the observed classes or patterns. The technique is based on the evaluation of the total variance within data such that the greatest variance by any projection of the data lies on the first coordinate, the first principal component, the second greatest variance on the second coordinate, and so on.

The original data matrix is denoted as ***X***, with *n* rows, termed 'objects', which correspond to the samples, and *p* columns, termed 'variables', which comprise the measurements made on the objects. PCA will provide an approximation of ***X*** in terms of the product of two small matrices ***T*** and ***P*** as in Eqn. 1 which captures the essential data patterns of ***X*** such that

X=T.P+ E…… (1)

where **T** represents the scores matrix, calculated as (*n* x ***A***), and **P**, the loading matrix, obtained as (*A* x *p*). ***A*** is the intrinsic dimension; that is, the number of principal components necessary to describe all the information in the data set. The scores matrix expresses the relation among the samples and shows the sample coordinates in the new system of axes. The loading matrix **P** shows the relations among the variables where in this case are the intensities at different wavelengths. **E** represents a matrix of residuals. This procedure applied to spectra helps to visualize and extract information from the data and can also be applied to show clustering of similar groups [55]. The PCA modeling used both entire spectral region and spectral features.

Results And Discussion

Determination of trace biometals in malarial blood

Table 2 shows the results of applying the developed ANN models to determine and compare the concentrations of the biometals in malaria in non-infected and malaria infected blood. The levels of Fe and Zn decrease sharply with malaria severity in agreement with the earlier observations that *falciparum* malaria decreases the serum Fe significantly at high parasitemia [55, 56]. Fe is required for the formation of blood in the body as well as for the transport of oxygen from the lungs. Although the interaction between Fe and malaria is more complex and controversial [57], as a large quantity of Fe in blood is in the form of hemoglobin, decreased Fe may be attributed to the digestion of hemoglobin by the malaria parasites. The decrease noted in Table 2 in the levels of Mg with malaria severity was also observed by Baloch *et al*. [3] and Maitland *et al*. [58].

Compared to their absolute concentrations the ratio of Cu/Zn has diagnostic significance: The Cu/Zn ratio increases progressively with parasitemia; at extreme malaria severity it is >10 times higher than at infection. Although Ginsburg *et al.* [7] observed that while the concentration of Fe remained constant throughout the parasite cell cycle, that of Zn increased in parallel with parasite maturation than that of uninfected red blood, this increase in Cu/Zn ratio has also been reported earlier and may be taken to be a

biomarker of malaria and perhaps other diseases as well [59]. The immunomodulatory and enzyme activity simulating properties of Zn are known [3, 60].

The absolute concentration of Cu in contrast increases profoundly with degree of *plasmodium falciparum* infection. The evidence for this was also shown by monitoring Cu concentration changes in the parasite itself where total copper content decreases by 34% in infected erythrocytes [61]. The importance of Cu homeostasis for the developmental progression of *plasmodium falciparum* has been confirmed by inhibition of Cu-binding proteins that regulate Cu physiology and function by associating with Cu ions [62], providing strong evidence for a link between healthy Cu homeostasis progressions of *plasmodium falciparum*.

Table 2
LIBS determined concentrations of Cu, Zn, Mg and Fe in malarial blood

Hours	Fe ± 32 ppb	Cu ± 78 ppb	Zn ± 43 ppb	Mg ± 189 ppb	Cu/Zn
0	1206	328	1523	23880	0.215
24 hours	1206	351	1521	21843	0.231
48 hours	805	862	849	19548	1.015
72 hours	514	1846	766	21317	2.410
96 hours	674	1999	499	19573	4.006

Parasite Density For Culture Samples

From Table 3, parasitic growth rate is observed to be exponential. In *falciparum* malaria there is sequestration of erythrocytes containing mature parasites in the microcirculation. This causes microvascular obstruction and accounts for much of the pathology of severe disease [63, 64]. Thus, the parasites causing pathology in severe infections are not represented directly by those counted in the peripheral blood smear. Patients can have the majority of their parasites circulating, or sequestered. In the latter case, the peripheral parasitemia can be low (depending on stage of development and synchronicity).

Table 3
Parasite density for blood culture samples

Sample type	Hours	Stage	Parasitemia
Whole blood	(Control)	No infection	N/A
Whole blood	24	Rings / Merozoites	15%
Whole blood	48	Merozoites / Trophozoites	18%
Whole blood	72	Trophozoites /Schizonts	26%
Whole blood	96	Trophozoites /Schizonts	40%

Pearson correlation was performed on the biometal concentrations and the results which show the relationship between the individual biometals as well as their relationship with the level of infection (parasitemia) are summarized in Table 4. It will be noted that Fe and Cu as well as Zn and Cu have a very high negative correlation (0.9). On the other hand, as expected, there is a very high positive correlation between Zn and Cu (0.9). With respect to parasitemia there is a high negative correlation with Fe and Zn (-0.775, -0.881) and high positive correlation with Cu and Mg (0.892, 0.805).

Table 4
Pearson correlation between Fe, Cu, Zn and Mg and parasitemia

	Fe	Cu	Zn	Mg	Parasitemia
Fe	1				
Cu	**-0.928**	1			
Zn	**0.923**	**-0.922**	1		
Mg	0.663	-0.611	**0.825**	1	
Parasitemia	-0.775	**0.892**	**-0.881**	**0.805**	1

Malaria Diagnostics Based On Pca Of Trace Biometal Spectral Signatures

Figure 1 shows the PCA score plot for the LIBS analysis of cultured blood infected with *plasmodium falciparum* parasite for different degrees of infection as well as the healthy blood sample (taken here as the controls - blank). 4 PCs were observed with the first 2 PCs explaining a total variance of 94%; that is, 83% by the first PC and 11% by the second PC. It is interesting to note the clustering based on the levels of malaria infection. The trace biometal signatures classify progressively from the initial Ring Stage of the *plasmodium falciparum* parasite to the last stage which is characterized by Schizonts.

The samples of each group (day of harvesting) are clearly separated from each other. The early Trophozoites stage is often referred to as 'Ring Form' because of its morphology [65]. This enlargement is accompanied by an active metabolism that involves ingestion of the host cytoplasm and proteolysis of hemoglobin into amino acids. The end of the trophic period results to formation of Schizont, an intra-erythrocyte parasitic stage that is due to repetitive nuclear division. Merozoites bud from the mature Schizont is released following rupture of the infected erythrocyte and hence attack healthy red blood cells. Ring Stage persists for the first 18 hours and thereafter the rings change to Trophozoites and Schizonts 24 hours later [53].

Figure 2 shows the score plot for cultured blood samples as well as the controls based [now] on the analyzed concentrations of the bimetals as opposed to their corresponding spectral signatures. It will be noted the separation of the clusters is not as distinct as that based on biometal spectra (feature selection). Although the concentration of the biometal is the main variable in the spectral feature, LIBS spectra contain a lot of biochemical information that is also correlated to the state of disease which when appropriately extracted, analyzed and interpreted can give an insight on the biophysiological status of the sample. We may therefore refer to these as trace biometal- mediated biochemical alterations in malaria pathogenesis. The score plot has a total explained variance of 96% with PC1 describing 92% of the total data and PC2 describing 4% of the remaining data. The corresponding loadings plot is shown in Fig. 3 which explained the importance of each biometal in the scores groupings. For instance information after 24 hours is characterized by high levels of Fe and Zn while information after 72 and 96 hours is characterized by high levels of Cu. Khoshmanesh *et al.* [66] also detected different stages of the malaria parasite, including Ring and Gametocyt based on trace element concentrations. The earlier observed high negative correlations of Cu with Fe and Zn (Table 2) are clearly also seen on the scores plot. The associated loadings plot is shown in Fig. 2 from where it is noted that the controls and the spectral data after 24 hours of infection with *plasmodium falciparum* had predominantly high levels of Zn and Fe. The levels of Cu are rather high after 72 hours and 96 hours with comparatively low levels of Zn.

In the flow chart (Fig. 4) it is proposed a proof-of-concept model for malaria diagnostics by chemometric peak-free LIBS of blood. Prospects are envisaged towards improvements and adaptability as well as integration of the model scheme into small, automated micro-total analysis systems. The advantage of sampling only small blood volumes could be preserved by combining the use of capillary sampling systems with dried spots of blood.

Towards this however further studies based on clinical samples are warranted to determine the malarial stage sensitivity and the influence of genotype on the accuracy of diagnosis. A good diagnostic modality must have high sensitivity and be easily affordable in the tropics. The model should be applicable to all species of malaria by identifying which species are present, provide no false positives, and it should be independent of by previous malarial infections [67].

Conclusions

We have explored and demonstrated the utility of chemometric peak-free LIBS to perform direct trace quantitative and explorative analysis of Cu, Zn, Fe and Mg in *plasmodium falciparum* infected blood in a manner that is applicable to rapid spectral diagnosis of malaria utilizing the biometals as disease biomarkers. In this technique there is neither cell counting nor chemical treatment; and sample preparation is minimal. Since the determined trace biometal concentrations were very low and analyte spectral peaks could not easily be observed, oyster tissue spectrum was used to identify the spectral regions of interest with which multivariate chemometrics (ANN) calibration models were developed and applied for trace biometal analysis. Using chemometrics peak-free LIBS, quantitative analysis of the biometals directly in tiny (~μL) blood dried spots was shown to be feasible. The results indicate that the concentrations of Cu, Fe, Zn and Mg in blood alter predictably during malaria pathogenesis (onset and progression). Cu/Zn ratio was shown to be an indicator of oxidative stress during *falciparum* malaria. PCA classified the biometal profile data based on the degree of malaria infection as well as the stages of the parasites. The morphological evolution of *plasmodium falciparum* was also accurately predicted using PCA of LIBS spectral features of the biometals. Chemometric peak–free LIBS is therefore a potentially robust technique for direct rapid malaria diagnostics in blood, a tool in which there is neither cell counting nor chemical treatment, sample preparation is minimal, and the analysis time is reduced to < 2 min/ sample.

Abbreviations

LIBS
Laser Induced Breakdown Spectroscopy. LA-ICP-MS:Laser Ablation Inductively Coupled Plasma–Mass Spectrometry. XRF:X-Ray Fluorescence. CCD:Charge Coupled Detector. ANN:Artificial Neural Network. SRM:Standard Reference Material. PCA:Principal Components Analysis. TXRF:Total Reflection X-Ray Fluorescence.

Declarations

Ethics approval and consent to participate

Whole blood samples were taken from the consenting 20 malaria patients as well as 23 healthy volunteers at the Kenyatta National Referral Hospital, Kenya. To take part in the study, all of the participants signed a written consent form. This study was approved by the Kenyatta National Hospital-University of Nairobi Ethics and Research Committee (ERC certificate number: P112 / 03 /2018).

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

DWM prepared the simulate samples, collected spectral data, performed statistical and chemometrics analysis of the samples and prepared the initial draft of the manuscript. HKA, AKD and KAK conceived the project. HKA and AKD supervised data collection, statistical and chemometric analysis, provided feedback and suggestions on the manuscript before submission. All authors read and approved the final manuscript.

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Figures

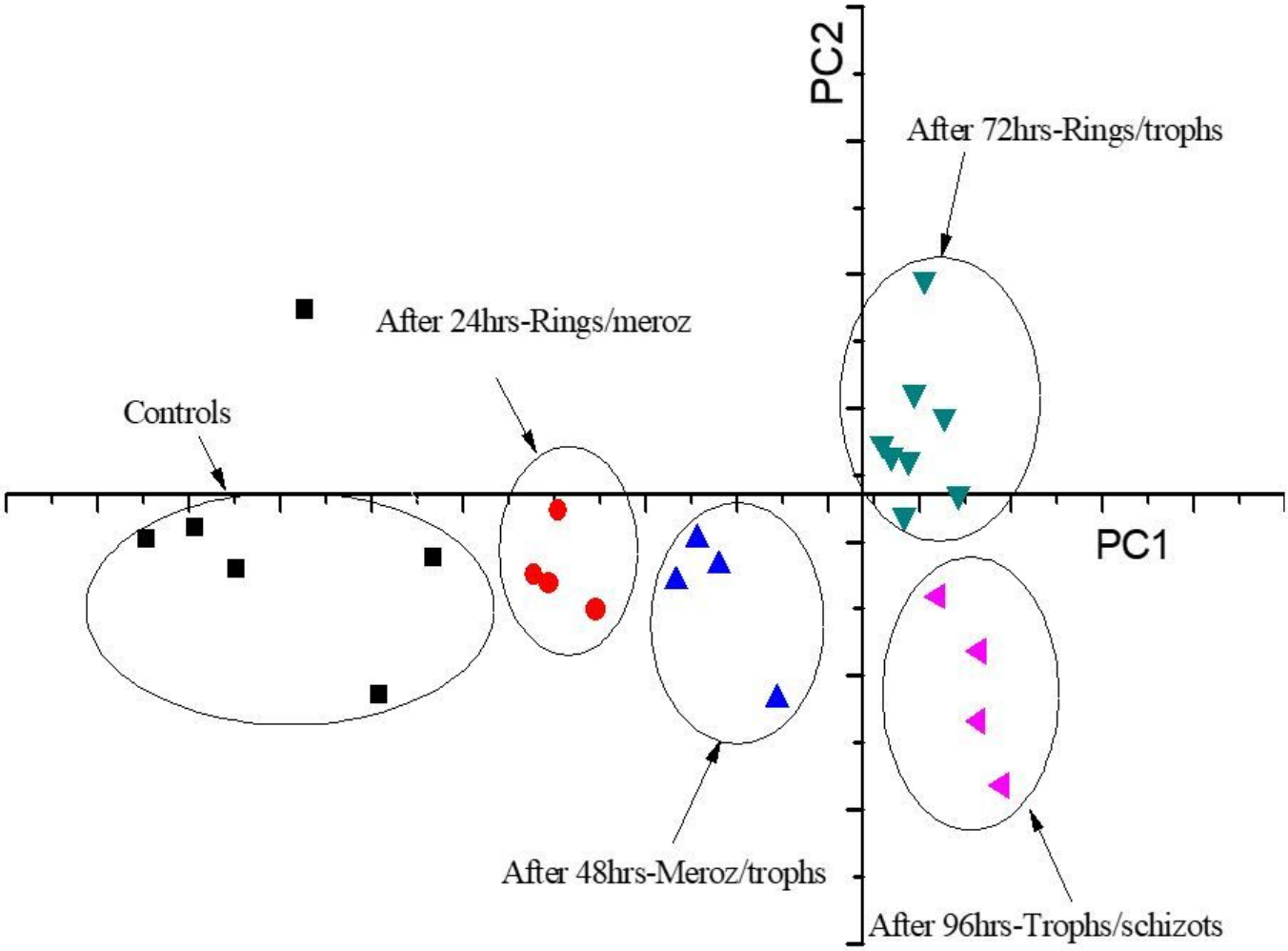

Figure 1

PCA score plot for cultured blood based on spectral features of the analyzed biometals.

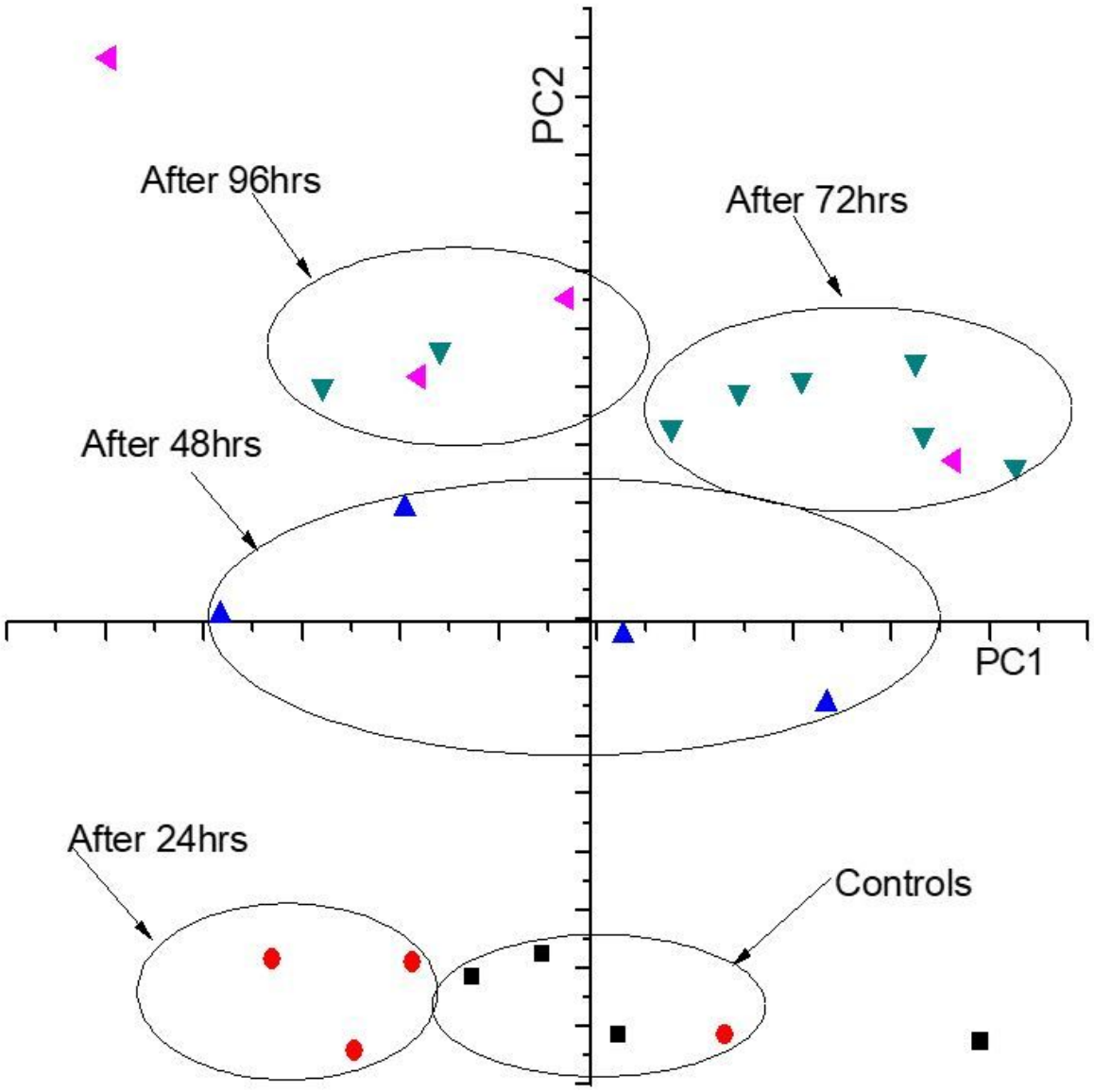

Figure 2

PCA score for cultured blood based on absolute concentrations of the analyzed biometals.

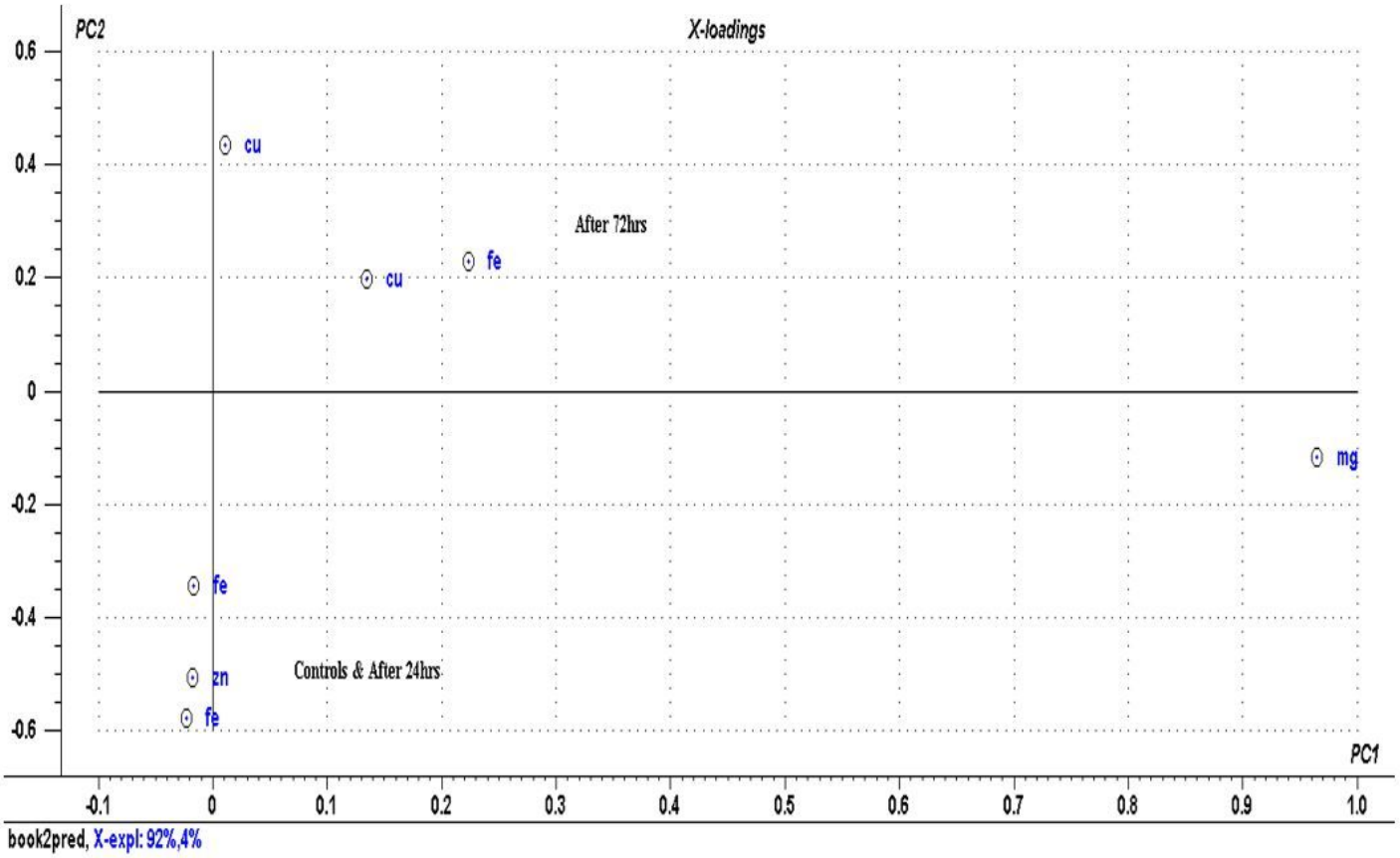

Figure 3

PCA loadings plot for cultured blood samples based on predicted biometal concentrations.

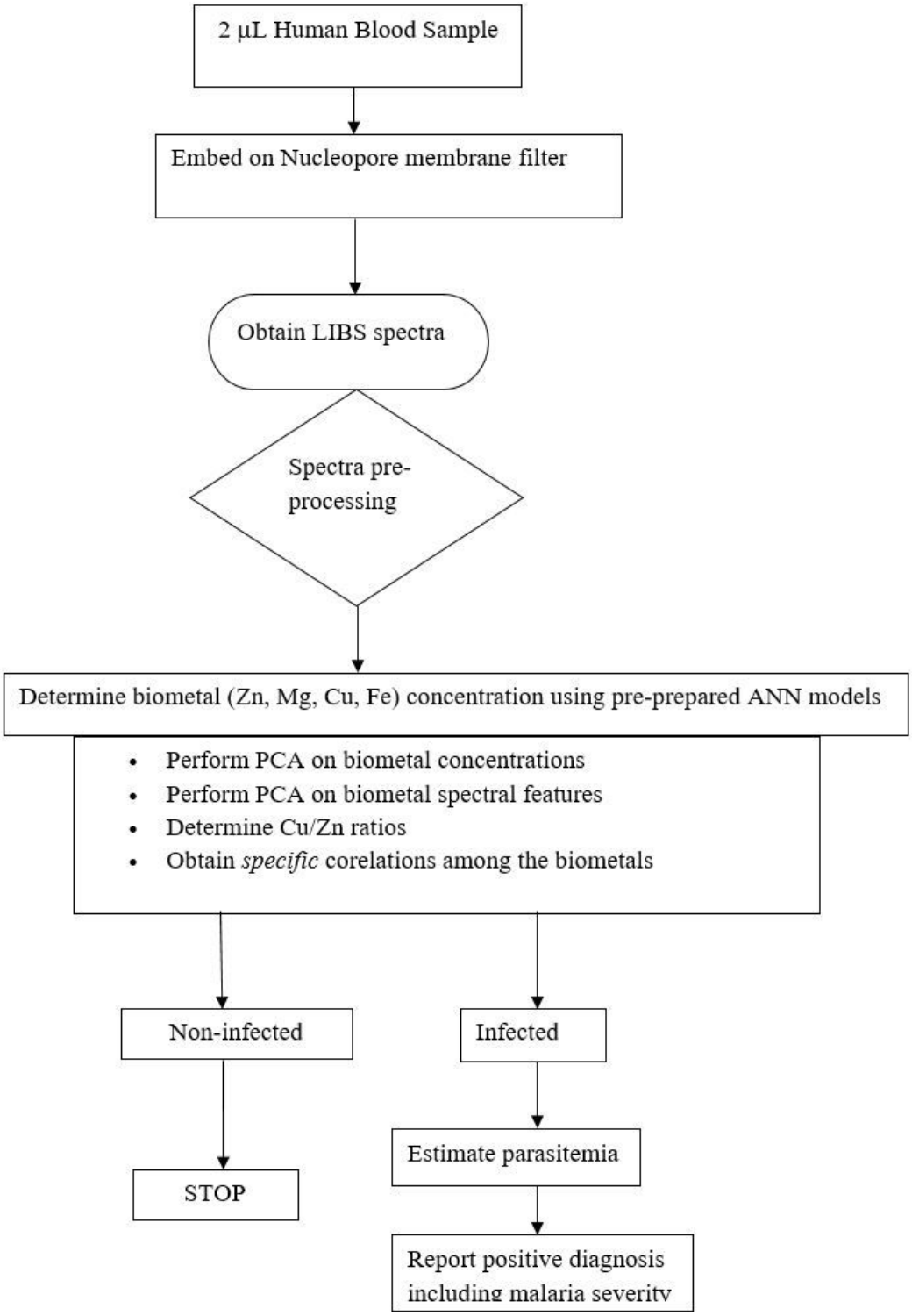

Figure 4

Flow chart of malaria diagnostics by chemometric peak-free LIBS.

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