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Rapid detection of aspergillosis in immunocompromised patients using DIMS and chemometric analysis

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Running Title: Rapid detection of aspergillosis using DIMS-PCA

Abstract

Rapid diagnosis of aspergillosis is one of the most important aspects for disease control and treatment. The purpose of this study was to develop a metabolic fingerprint of serum from immunocompromised patients that can enable rapid detection of aspergillosis. Serum samples from immunocompromised patients with and without aspergillosis were analyzed by direct infusion electrospray ionization mass spectrometry (DIMS). Data from the mass spectra were subjected to chemometric analysis using principal component analysis and partial least squares-discriminant analysis. Our method was able to efficiently distinguish patients with and without aspergillosis and predict the presence or absence of aspergillosis in unknown samples. Therefore, DIMS coupled with chemometric analysis seems to be a promising technique for rapid diagnosis of aspergillosis.

Keywords: DIMS; aspergillosis; chemometric; metabolic fingerprint; metabolomics

1. INTRODUCTION

Invasive aspergillosis (IA) is a severe and often fatal infection in immunocompromised patients, especially following bone marrow transplantation [1]. It is a fungal disease primarily caused by *Aspergillus fumigatus* and is known to cause considerable morbidity and mortality, typically ranging between 30% and 70% in transplant recipients [2, 3]. Therefore, early detection of aspergillosis will allow better prognosis and treatment of the disease thus increasing patient survival.

Current diagnostic tools for detection of IA typically include time-consuming techniques such as histopathology and culture analysis of blood or tissues from biopsies. An enzyme immunoassay (ELISA) that detects galactomannan, a cell-wall polysaccharide component released by *Aspergillus* can also be performed. However, enzyme immunoassays are only moderately accurate and have lower specificity owing to false-positive results caused by the concomitant use of antibiotics or infection of fungi other than *Aspergillus*[4-6]. Other methods for aspergillosis diagnosis include (1→3)-β-D-glucan assay, polymerase chain reaction (PCR), and gliotoxin detection by HPLC-MS/MS [1]. Nevertheless, their drawbacks include requirement of expensive certified glucan-free materials for the β-glucan assay and lack of inter-laboratory reproducibility of the PCR-based methods due to difference in material and protocol[7].

To date, metabolomics has been employed in various research areas including metabolic disease, cancer, infectious diseases and regenerative medicine because it quickly provides reliable information in biological systems [8,9], enabling us to comprehend the role of pathways and metabolites [10,11,12]. For example, global metabolic responses of mice to parasites such as *Trypanosoma brucei*[13] and viruses such as simian immunodeficiency virus [14], cytomegalovirus [15] and hepatitis B virus [16] have been studied using a metabolomics approach. An increasing number of published methods have

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proven that different analytical techniques can be applied in metabolomic analyses [17, 18, 19,20, 21, 22]. Nuclear magnetic resonance (NMR) and mass spectrometry (MS) are the most common techniques utilized since both can detect a broad range of metabolites with relatively high specificity and reproducibility [23].

One powerful MS-based technique used in metabolomics analysis is direct infusion mass spectrometry (DIMS), which provides quick processing, high reproducibility and rapid characterization of metabolites in complex matrices[24, 25]. In addition, DIMS does not require chromatographic separation or sample derivatization and hence there is no loss of metabolites during sample analysis[26]. Some relevant applications involving DIMS include analysis of amino acids for neonatal screening[27] and quantitative metabolic analyses to detect inherited metabolic disorders during the neonatal period[28, 29]. In clinical settings, DIMS offers great potential to be developed into a fast prognostic or diagnostic method for kidney cancer and early-stage human lung cancer with high accuracy, classification and prediction capabilities comparable to LC-MS[26, 30]. A methodology, based on ESI-MS/MS, provides an ideal tool for evaluation of lysophosphatidylcholine as diagnostic marker [31]. Particularly, DIMS coupled with chemometric analysis has been used for early cancer diagnosis in mice, thus indicating its potential as a promising technique[32].

Chemometric methods are being widely used to extract information from the large amount of multivariate chemical data generated in metabolomics analyses. Among these methods, principal component analysis (PCA) is employed to reduce the dimensionality of the data without loss of relevant information, whereas partial least squares-discriminant analysis (PLS-DA) is for data modeling and sample prediction[33-35].

This study aimed to evaluate the use of DIMS along with chemometric analysis for rapid detection of aspergillosis in serum samples from immunocompromised patients

owing to its potential to become a simple, low-cost and robust alternative to existing methods.

2. EXPERIMENTAL

2.1 Sample, Chemicals, and Reagents

Serum samples from immunocompromised bone marrow recipients with or without aspergillosis post-transplantation were provided by the Hospital de Clínicas da Universidade Federal do Paraná. The samples were identified according to the ELISA results for galactomannan (diagnostic method used for invasive aspergillosis in the hospital). Samples that tested positive and negative in the ELISA assay were grouped as those with and without aspergillosis, respectively. All serum samples were stored at -40 °C until analysis.

Acetonitrile (HPLC grade) and formic acid (88.0%) were purchased from J. T. Baker Chemicals B. V. (Deventer, The Netherlands). Trifluoroacetic acid (99.0%) was obtained from Sigma-Aldrich (St. Louis, USA), and ultrapure water was obtained using a Milli-Q purification system from Millipore (Bedford, USA).

2.2 Ethic statement regarding human samples

All studies involving human serum samples were approved by the Ethics Committee of the Universidade Federal do Paraná under number 10857012.0.0000.0102 of the Certificate of Presentation for Ethics Appreciation (CAAE). The volunteers were informed that their blood samples collected for routine analysis after bone marrow transplantation would be used for research purposes. All volunteers provided written informed consent for their participation in the study.

2.3 Serum sample preparation

The serum samples were thawed to room temperature before the preparation. For removal of high molecular weight proteins (HMWPs), to 50 μL of serum, 100 μL of acetonitrile containing 0.1% trifluoracetic acid (TFA) was added and the samples were incubated in an ice bath for 5 min. After cooling, the samples were vortexed for 10 min and centrifuged at 14.000 rpm for 5 min at 4°C using an Eppendorf centrifuge (model 5810R, Hamburg, Germany). The supernatant solutions were transferred to new tubes and evaporated at 40 °C in a sample concentrator (Labconco CentriVap, Kansas City, USA). These were then resuspended in 150 μL of acetonitrile/ water (98:2, *v/v*) with 0.1% formic acid and 1 mM ammonium formate before injection into the mass spectrometer. After each sample injection, 1 mL of methanol was injected using a syringe to clean the system followed by a blank run (diluent) before the consecutive sample to minimize any sample carryover and avoid cross contamination.

2.4 DIMS analysis

Mass spectrometry experiments were performed on an Applied Biosystems MDS Sciex API 3200 Triple Quadrupole Mass Spectrometer (Toronto, Canada) that was equipped with an ESI source and operated in the negative ion mode. A Harvard Model 22 Dual syringe pump (Harvard Apparatus, South Natick, USA) with a flow rate of 10 μL min^{-1} was used to infuse the samples directly into the mass spectrometer. The following ion-source parameters were used: ion spray voltage (IS), -4500 V; curtain gas (CUR), 10 psi; nebulizer gas (GS1), 15 psi; declustering potential (DP), -40 eV; and entrance potential (EP), -10 eV. High-purity nitrogen produced using a nitrogen generator (PEAK Scientific Instruments, Chicago, USA) was used as CUR and GS1. DIMS acquisition parameters were set at mass range 50–1400 by averaging 100 scans of 3 seconds each (5

min of acquisition). Data acquisition was performed using a MS Workstation with Analyst 1.4 software (ABI/Sciex).

2.5 Mass spectra processing

All mass spectra obtained from serum samples were adjusted to baseline by subtracting the corresponding blank injection values. The primary DIMS parameters were set as follows: a mass range of 200–800 Da, a mass window of 0.5, and noise filtering level of 5. Origin Pro 8 software (OriginLab) was used to convert the mass spectra into a variable tables displaying the mass and associated intensities as columns. Chemometric analysis using PCA and PLS-DA were performed using MATLAB software version 7.13 (The Math-Works, Natick, USA), and PLS Toolbox version 6.5 (Eigenvector Technologies, Manson, USA). PCA was used to verify the difference between the two tested groups and to identify the ions responsible for this difference. In addition, PLS-DA was used to develop a model from the available data and to obtain a prediction for samples for which the status of aspergillosis was unknown. Interval PLS (iPLS)[36] was used for range/variable identification and selection. The statistical measures of performance, sensitivity, specificity and accuracy, were obtained for iPLS experiment.

3. RESULTS AND DISCUSSION

In this study, serum samples from two groups of immunocompromised patients, with and without aspergillosis, were analyzed using DIMS. Classification of the groups as positive or negative for aspergillosis and possibility of rapid detection of the disease in unknown samples were assessed using chemometric analyses.

Prior to DIMS analysis, the serum samples were treated to remove HMWPs because they overshadow the lower weight proteins, which are commonly targeted as

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3 potential biomarkers [37, 38]. The use of acetonitrile with 0.1% TFA resulted in the rapid
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6 precipitation of HMWPs and could easily be removed by centrifugation [32].
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8 After HMWP depletion, DIMS analysis was conducted by directly infusing the
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10 samples into the mass spectrometer. The analytical variability between injections could
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13 be assessed through the intensity of the ions measured as a whole, which indicated greater
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15 reproducibility of the method. The data obtained from the mass spectra were pre-
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18 processed (subtraction of the respective blank injection values) and approximately 13,500
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20 negatively charged ions per sample were observed, which made it impossible to
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23 distinguish between the groups visually. A typical mass spectrum is shown in Fig. 1. The
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25 experiments using positive ion mode were performed. However, the obtained PLS-DA
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27 models failed to correctly predict unknown samples (data not shown).
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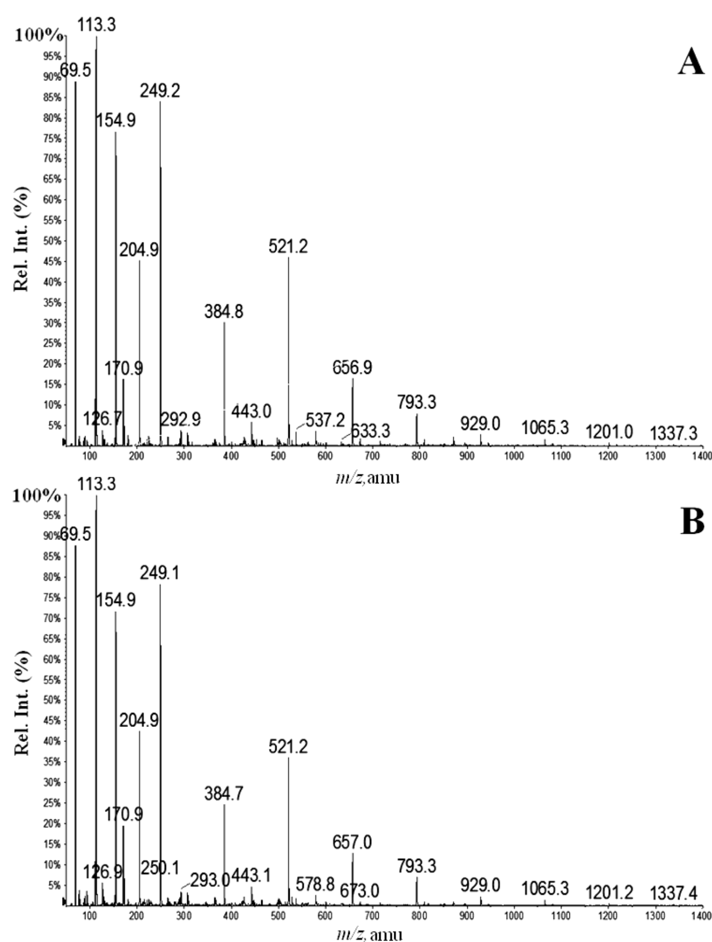

Fig. 1 DIMS (ESI^-) mass spectrum of blood serum samples treated from patient groups (A) with and (B) without aspergillosis.

Chemometric analyses were necessary for extracting useful information from the experimental data. PCA was performed on the metabolic fingerprints to interpret the intensity signals for each *m/z* value in the mass spectra. The PCA score plots obtained from the mass spectra data of patient groups with and without aspergillosis are presented in Fig. 2. Each data point refers to an individual sample (n=27).

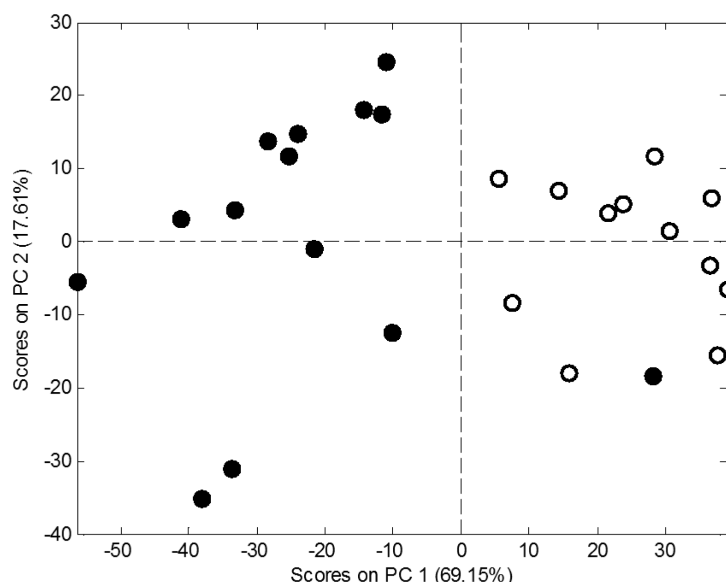

Fig. 2 Principal component analysis overview of blood serum samples from immunocompromised patients with (•) and without (○) aspergillosis.

As demonstrated in Fig. 2, the scores plots used for data classification showcase a clear differentiation between the tested groups. Four principal components (PCs) were responsible for 93.04% of the data variance. These results demonstrate that DIMS with chemometric analysis is highly efficient in data acquisition and sample classification, respectively.

After sample classification, loading plots were constructed for the PCs that differentiated the groups in order to identify the ions that were responsible for the differentiation. The negative loading data for PC1 shows that the most relevant ion in the group with aspergillosis was the ion of *m/z* 293.9 (Fig. 3), while the positive loading data of PC1 (for the group without aspergillosis) provides four high intensity ions of *m/z*

249.6, 385.4, 521.6, and 657.3 Da. Identification of metabolites in complex matrices requires chromatographic separation coupled to high-resolution mass spectrometers, and is being developed by the group. In another study published by Cerqueira and coworkers [39], the metabolite gliotoxin, one of the most toxic metabolites produced by several fungal species, was used as a tool for early diagnosis of aspergillosis. However, gliotoxin was not detected in DIMS analysis, probably due to its low concentration or because DIMS is not as selective as MRM used in their work.

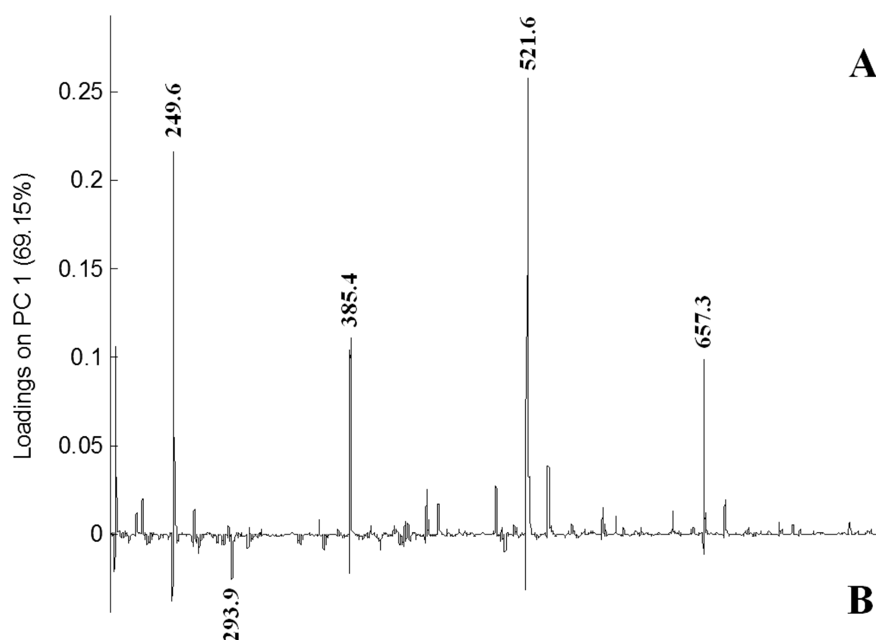

Fig. 3 Loading plots of serum samples obtained from immunocompromised patients. (A) Positive loading (group without aspergillosis) and (B) Negative loading (group with aspergillosis) data of PC1

Partial least squares discriminant analysis (PLS-DA) was performed to model the available data and obtain a qualitative prediction for the unknown samples. For this purpose, calibration sets (matrix X and vector y) were constructed. Initially, models were constructed with the entire spectrum ranging between *m/z* 50-1400 Da. However, an interval PLS (iPLS) method was employed, which served as an efficient tool for mass range selection. This is a variation of the normal PLS regression method aimed at

variable interval or mass range selection with a strong emphasis on a visual exploration of spectroscopic data [36, 40]. The mass range of *m/z* 200-800 Da was selected since a large amount of information was present in this region including the ions highlighted in the loading plots. Then, a selection of variables with multiple windows of interval was performed presenting the best results ranging between *m/z* 500-600. In this model, the matrix X for both groups was constructed using the maximum intensity data obtained for each sample at each *m/z* acquired by the mass spectrometer. The value of the vector y was 1 or 0 corresponding to the presence or absence of aspergillosis, respectively.

The calibration set was constructed with both the immunocompromised patient groups, without aspergillosis (n=13) and with aspergillosis (n=14). For preprocessing, the matrix X was built with mean-centered non-interactive minimum partial squares (NIPALS) and cross-validation leave-one-out. The graph plotted with the variance captured (%) against the LV showed that a major portion of the original data could be explained using 4 LVs in relation to minor errors found in the root mean square error of cross validation (RMSECV). The accumulated variance in the X and y matrices were 97.45% and 91.95%, respectively. In addition, the presence of anomalous samples (*outliers*) in the prediction model was determined by plotting a graph of the leverage *versus* the residues of student (Fig. 4). This demonstrated the presence of two outliers in the calibration set, but they did not compromise the robustness of the model because the student's residual of these samples remained within the allowable limit of +/- 2.5

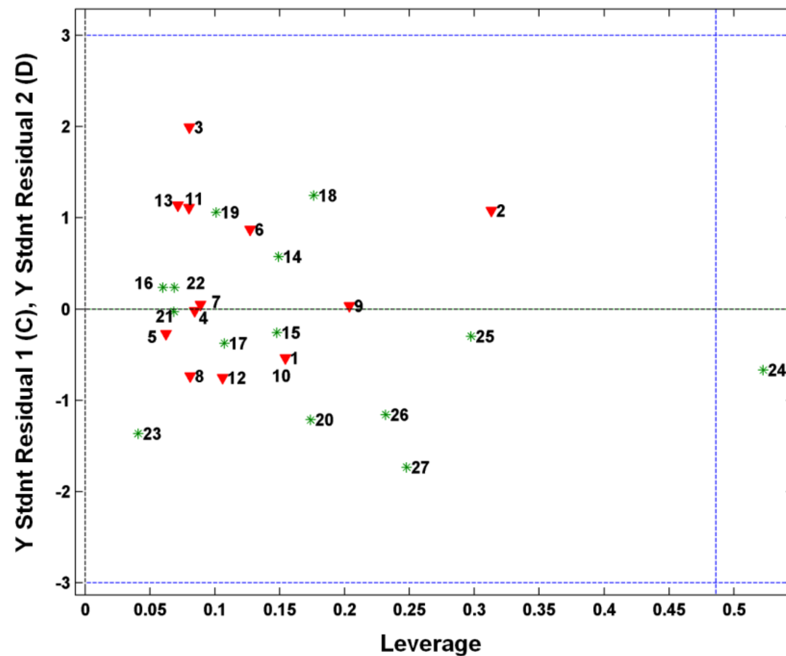

Fig. 4 The leverage *versus* student residual to check for of anomalous samples in the calibration set.(▼) group without aspergillosis (1 to 13); (*) group with aspergillosis (14 to 27)

Ten samples were used to test the model (validation set), being five samples from each group. The results of prediction are shown in Table 1 in form of a confusion matrix.

Table 1 – Confusion matrix of predicted results obtained with the model developed based on iPLS method.

	Actual Class	
Predicted Class	Without Aspergillosis	With Aspergillosis
Without Aspergillosis	4	0
With Aspergillosis	1	5

The PLS-DA model yielded 100% sensitivity and specificity for the calibration set, but the prediction accuracy was 90% since one sample without aspergillosis from the validation set was identified as a sick sample (false positive). The score plot of LV1 × LV2 was constructed, including samples from the validation set (Fig. 5). Some data

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points from the group without aspergillosis are closer to those with aspergillosis in this plot. In the ELISA test, a limit value is used (commonly optical density ≅ 0.5) and a sample with value below or above the limit is considered negative or positive for aspergillosis, respectively. Therefore, these data points may reflect the false-positives commonly observed in new bone marrow transplant patients. However, the presence of these samples does not compromise the robustness of the model on the calibration set or its prediction capability. This highlights that the model is efficient in predicting the presence of aspergillosis in unknown samples.

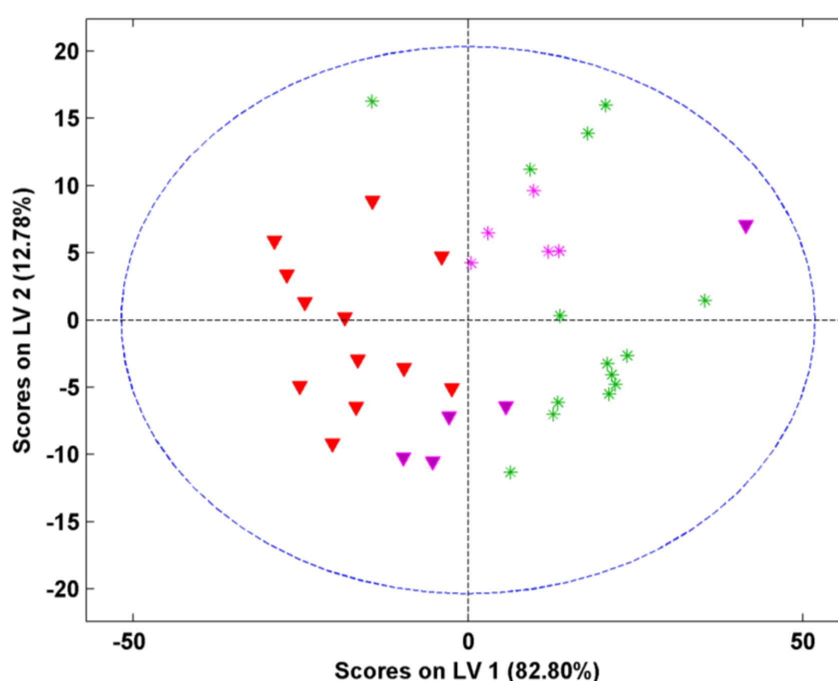

Fig. 5 PLS-DA scores plot obtained from LV1 x LV2. (▼) samples without aspergillosis from calibration set; (▼) samples without aspergillosis from validation set; (*) samples with aspergillosis from calibration set; (*) samples with aspergillosis from validation set.

CONCLUSIONS

Effective IA therapy is only possible following rapid diagnosis. Therefore, the use of reliable methods for detecting IA is important. In this study, DIMS combined with

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3 chemometric analysis was able to distinguish groups of immunocompromised patients
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5 with and without aspergillosis. Major advantages of this method are its speed and
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8 selectivity. This method uses a simple sample clean-up step and is sensitive in
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10 differentiating the disease group with high selectivity and repeatability. Here we propose
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12 an efficient model for predicting IA in unknown samples quickly. Therefore, using DIMS
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15 with chemometric analysis may provide a simple, low-cost, and robust alternative for
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17 rapid detection of aspergillosis for routine use.
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24 **CONFLICT OF INTEREST**
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27 *All authors declare that they have no conflict of interest.*
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34 **ACKNOWLEDGEMENTS**
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