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# Diagnosis of liver cancer using HPLC-based metabonomics avoiding false-positive result from hepatitis and hepatocirrhosis diseases

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**Abstract**

Metabonomics, the study of metabolites and their roles in various disease states, is a novel methodology arising from the post-genomics era. This methodology has been applied in many fields. Current metabonomics practice has relied on mass spectrometry (MS), gas chromatography–mass spectrometry (GC–MS), liquid chromatography–mass spectrometry (LC–MS) and nuclear magnetic resonance (NMR) to analyze metabolites. In this study, a novel approach of using high-performance liquid chromatography (HPLC) in conjunction with developed software was employed. Using the principal components analysis method (PCA), all (113) peaks of urinary metabolites with a *cis*-diol structure from patients with hepatitis and hepatocirrhosis were compared to those from liver cancer patients. The results showed that the metabonomics-PCA method might be useful to differentiate between patients with hepatocirrhosis and hepatitis from patients with liver cancer while lowering false-positive rate. These findings also suggest that a subset of the urinary nucleosides identified with metabonomics correlate better with cancer diagnosis than the traditional single tumor marker alpha-fetoprotein (AFP).


*Keywords:* Metabonomics; HPLC; Nucleosides; Methodological studies; Molecular diagnosis and prognosis

## 1. Introduction

Metabonomics is the method of studying, profiling and fingerprinting metabolites in various physiologic states [1]. This method has recently demonstrated enormous potentials in many fields such as plant genotype discrimination [2,3], toxicological mechanisms, disease processes and drug discovery [4–10]. One such recent application of this method included the rapid and non-invasive diagnosis of coronary heart disease [11–14]. In these methods, metabolite profiling is mainly used for the analysis of a class of metabolites. Metabonomics aims to include all classes of compounds and utilizes metabolic fingerprinting to maintain a rapid classification of samples according to their origin and biological relevance. In order to optimize and utilize metabonomics, a stable metabolite fingerprint must be achievable. While in traditional metabolites target analysis, only several predefined metabolites were selected to analyze [1]. In previous attempts, metabonomics data were generated by nuclear magnetic resonance (NMR), gas chromatography–mass spectrometry (GC–MS) and liquid chromatography–mass spectrometry (LC–MS) [15–17]. While these methods were adequate, work by Tuan et al. illustrated that high-performance liquid chromatography (HPLC) could be used in metabonomics. This method offered the advantage of controlling critical elements of experimental design while allowing for optimization of data acquisition and analysis [18].

One important area where the analysis of metabolites appears crucial is the study of nucleosides. Nucleosides are an important class of metabolites and have the potential roles of serving as tumor markers [19–22]. The study

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of nucleosides, however, has been hindered by the false-positive results of some benign diseases, especially inflammatory diseases. There are at least 93 modified nucleoside metabolites [23] that occur mainly from tRNA. This complicates the analysis of nucleosides as metabolite markers for cancer. These concerns become significant when designing rapid detection methods for discerning between liver cancer and an inflammatory disease such as acute or chronic hepatitis.

In this study, we developed an HPLC-based metabonomics method to distinguish between patients with hepatocirrhosis and hepatitis from those with liver cancer based on all peaks of urinary metabolites with *cis*-diol structure, including nucleosides. Using the self-developing software, all the peaks in the chromatograms of these urinary metabolites with a *cis*-diol structure from patients were matched to those of a predefined reference chromatogram. From the following pattern recognition results, lower false-positive result (7.40%) was obtained and potential markers were found.

## 2. Experimental

### 2.1. Collection of urine samples and extraction of nucleosides

Spontaneous urine samples were collected from 50 healthy adults, 77 patients with liver infectious diseases (27 hepatocirrhosis patients, 30 acute hepatitis patients, 20 chronic hepatitis patients) and 48 liver cancer patients. Their ages were $50.6 \pm 16.2$ (the age range was 20–85). All patients were from the First and Second Affiliated Hospitals of Dalian Medical University of China. All the diagnoses of these patients were confirmed by histopathology. Urine samples were collected and kept at $-20\,^{\circ}C$ until analysis. And there were no diet or other restrictions in the sample collection.

For the analysis of urinary *cis*-diol metabolites, the samples were thawed at room temperature. The spontaneous urine added with internal standard 8-bromoguanosine (Br8G) was extracted on a phenylboronic acid column as described elsewhere [23–25]. After the extraction, the class of *cis*-diol was the major composition of the samples for the phenylboronic acid column's specific affinity for *cis*-hydroxyl group. The eluent from the phenyl boronate column was evaporated to dryness in a vacuum system at 39–40 °C and dissolved in potassium dihydrogen phosphate ($KH_2PO_4$) buffer. And then a RP–HPLC process was employed to analyze the samples [21–25]. The HPLC system consisted of two Shimadzu HPLC-10ATVP pumps (Kyoto, Japan), an autoinjector model SIL 10ADVP, an SPD-10AVP UV–vis detector, set at 254 nm and a SCL 10AVP interface, a Hypersil ODS 5 μm C18 HPLC column (250 mm × 4.6 mm) (Elite, Dalian, China). And the creatinine concentrations of these samples were determined by capillary electrophoresis [21–25]. And a typical sample chromatogram is given in Fig. 1.

### 2.2. Data acquisition and processing

A data set of urinary nucleosides containing all the patients and healthy volunteers was obtained for the metabolite target analysis. Each pattern was described by 15 feature variables, which were the creatinine concentrations of 15 urinary nucleosides.

The other data set was collected for the metabolite profiling and metabonomics. All of the HPLC peaks information was recorded. The process of this data set analysis begins with the selection of a reference chromatogram (Fig. 1) that is typical of the whole set of *cis*-diol compound analyses and has the most peaks in all sample chromatograms. The second step of the data processing is to find all peaks in the reference chromatogram by using a peak detecting program. This step

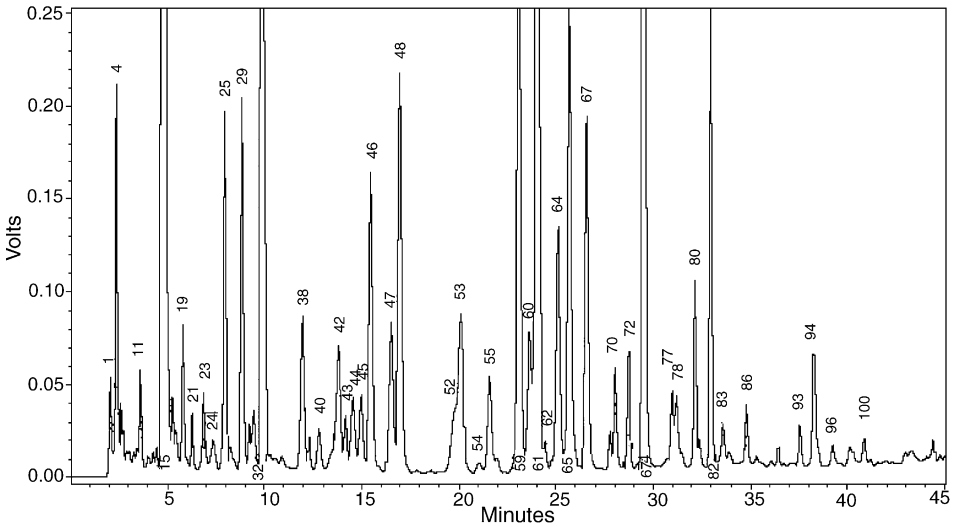


Fig. 1. A typical sample chromatogram, which was then selected as reference chromatogram.

uses a script for identifying peaks that both exceed a signal-to-noise ratio (S/N) of 1000 and that have peak widths of at least 12 s. All other chromatograms were then matched against this list of *cis*-diol targets. Six reference peaks were firstly identified and used to match the individual peaks between the reference chromatogram and other chromatograms. This was done to avoid retention time fluctuation between HPLC samples. The chromatogram was then separated into seven zones, each zone bordering a reference peak. On the basis of these reference peaks, the peak matching was carried out based on retention factor (e.g. retention time, capacity factor $k'$, or $\lg k'$) comparison between a sample chromatogram and the reference chromatogram.

After peak matching, the area of each peak was normalized to the internal standard 8-bromoguanosine and corrected by the concentration of creatinine because the ratios of nucleosides to creatinine in random samples were the same as those in 24-h samples [19–22], and the results from the random are as valid as those from urine collected over a 24-h period when nucleoside levels are expressed relative to creatinine [25,27–30]. A number of peaks that were detected in the reference chromatogram were apparently absent in the sample chromatograms. This primarily occurred because the comparison peaks had a S/N lower than 1000. Therefore, the true peak areas for these peaks were somewhere between zero and the detection limit. The value of $1 \times 10^{-6}$ was given to such peaks.

It should also be noted that use of a reference chromatogram has the advantage of producing examples all with the same number of attributes. The data for analysis is therefore a simple 2D matrix, enabling the data analyst to choose from many statistical and machine learning data analysis methods.

### 2.3. Peak matching algorithm

The peak matching was carried out based on the adjusted retention factor (arf) that was calculated according to the following formula:

$$\mathrm{arf}_i = \frac{\mathrm{rf}_i - \mathrm{RF}_j}{\mathrm{RF}_{j+1} - \mathrm{RF}_j}(\mathrm{ARF}_{j+1} - \mathrm{ARF}_j) + \mathrm{ARF}_j, \quad T_j < t_i \leq T_{j+1} \qquad (1)$$

where $\mathrm{arf}_i$ ($i = 1, \ldots, 113$) is the adjusted retention factor of the peak $i$; $\mathrm{rf}_i$ is the retention factor; $t_i$ is the retention time of the peak; $j$ ($j = 0, \ldots, C$) is the number of reference peaks. In this study, $C$ is equal to six. $\mathrm{ARF}_j$, $\mathrm{RF}_j$ and $T_j$ are the reference peak's adjusted retention factor, retention factor and retention time, respectively. $\mathrm{ARF}_0$, $\mathrm{RF}_0$ and $T_0$ were all equal to zero.

### 2.4. Pattern recognition method

Principal components analysis (PCA) is a mathematical way of determining the linear transformation of a sample of points in $N$-dimensional space, which exhibits the properties of the sample most clearly along the coordinate axes. The principal components (PCs) are displayed as a set of 'scores' ($t$), which highlight clustering or outliers, and a set of 'loadings' ($p$), which highlight the influence of input variables on $t$ [26]. The PCA algorithm used for the above analysis was modified from the Statistics Toolbox of Matlab.

## 3. Results and discussion

### 3.1. Metabolites target analysis

Metabolite target analysis pays great attention to several known bio-markers. In order to analyze the data an internal standard (Br8G) was used to quantify the concentrations of 15 urinary nucleosides: pseudouridine (Pseu), cytidine (C), uridine (U), 1-methyladenosine (m1A), inosine (I), 5-methyluridine (m5U), guanosine (G), xanthosine (X), 1-methylinosine (m1I), 1-methylguanosine (m1G), $N_4$-acetylcytidine (ac4c), 2-methylguanosine (m2G), adenosine (A), 2,2-dimethylguanosine (m22G) and 6-methyladenosine (m6A). Their concentrations were further calibrated by their respective creatinine concentrations [20–22,24,25,27]. Fifteen nucleosides were selected based on their bio-importance as tumor markers and whether their standard could be available. Significant differences were found in the mean concentrations of all 15 nucleosides (C, $P < 0.05$; other nucleosides, $P < 0.01$) between cancer and control patterns (healthy people). Metabolite target analysis was carried out by using principal components analysis method [20] on the HPLC–UV data of 15 urinary nucleosides. The positive ratio of cancer from these samples was 83.0% (Table 1). The level of the traditional biomarker, such as AFP, was also measured. The positive ratio of cancer for AFP was 73%, suggesting that the metabolites target analysis of the 15 urine nucleosides was superior to that of a traditionally accepted tumor marker.

Clinical diagnosis of cancer requires a low false-positive result when distinguishing it from other diseases. To investigate the false-positive rate, the urinary nucleoside concentrations in patients with acute hepatitis, chronic hepatitis and hepatocirrhosis were measured. An increase in the mean concentrations of seven nucleosides (Pseu, C, U, X, m1G, ac4c and m2G) (Fig. 2a) could be observed with patients with more severe liver diseases. The lowest mean concentrations coming from patients with acute hepatitis, and increasing in patients with chronic hepatitis, and highest in patients with hepatocirrhosis. While promising, we wanted to further understand

Table 1
Diagnostic positive ratio of liver cancer patients based on traditional biomarkers and PCA based on 15 urinary nucleosides

| PCA based on 15 urinary nucleosides | AFP (ng/mL) | | CEA | CA199 | CA125 |
|---|---|---|---|---|---|
| | >20 | >200 | | | |
| 39/47 | 34/46 | 27/46 | 2/33 | 10/26 | 13/25 |
| 83.0% | 73.9% | 58.7% | 6.1% | 38.5% | 52.0% |

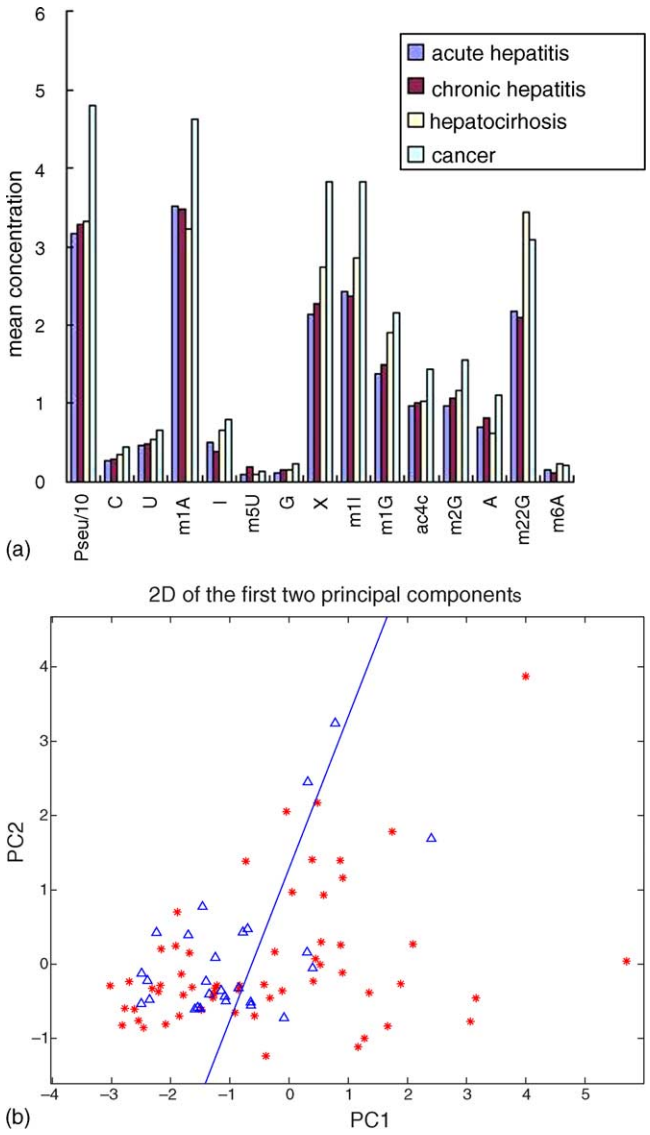


Fig. 2. (a) Mean concentrations of the 15 urinary nucleosides from liver disease patients. (b) PCA scores plot of hepatocirrhosis patients and cancer patients based on 15 nucleosides. (△) Hepatocirrhosis patients; (✱) cancer patients.

this trend in individual patients. Therefore, metabolite analysis of 15 nucleosides was analyzed with the PCA method. As all the constructed principal components are orthogonal, the object scores may be plotted against one another to represent the distribution of the objects in a space. Fig. 2b illustrates that the liver cancer patients and hepatocirrhosis patients were not distinguishable and suggests that the information of 15 nucleosides is not sufficient to distinguish liver cancer from other liver diseases.

### 3.2. Metabonomics of cis-diol metabolites

To avoid false-positive results, we developed a novel metabonomics method to maximize the information used from the original chromatograms. Unlike the NMR method, the retention factor of the HPLC method is not stable enough to provide the data set for a metabonomics investigation. While HPLC chromatograms are potentially information-rich entities and need to be refined to extract useful information from the raw data. Our data acquisition and pre-processing procedures were designed to extract the maximum reliable information from the chromatograms. The aim was to identify as many metabolites as possible and not just a predefined nucleoside set based on background knowledge of their biological importance as has been conventionally used in studies of metabolites. A peak matching software was, therefore, developed to recognize and match all the peaks appearing in a chromatogram.

Based on the peak detecting software, 113 peaks were found in the typical reference chromatogram (Fig. 1). For 125 patient samples and 50 healthy volunteer samples, this resulted in a processed data set of $113 \times 125$ (from patients) and $113 \times 50$ (from healthy volunteers) using real numbers after peak matching. No pre-processing or data reduction was carried out on these samples.

Based on the data from all peaks of metabolites with *cis*-diol structure, the corresponding PCA score plots (Fig. 3a) showed that patients with hepatitis and patients with hepatocirrhosis clustered in one region, while liver cancer patients were located in a different cluster region. While the cluster regions primarily adhere to these subsets of patients, some cancer patients appeared in the hepatocirrhosis and hepatitis area, while a few hepatocirrhosis patients appeared in the cancer pattern area. This may be related to cancer patients expressing some of the same symptoms as those with hepatocirrhosis and hepatitis, while the hepatocirrhosis and hepatitis have fewer of the symptoms expressed in cancer with a high RNA turnover. As liver disease occurs as a range of presentations, hepatocirrhosis patients deteriorating along the spectrum to liver cancer could explain the slight overlap in these cluster regions.

The concentration of AFP considered to be indicative of liver cancer is generally accepted to be 20 ng/mL, while a higher value may be in the range of 200 ng/mL. Based on the AFP criteria (>20 ng/mL), 50% (13/26) of hepatocirrhosis and 52.2% (12/23) of chronic hepatitis would be classified as liver cancer. If the higher concentration of AFP is used (>200 ng/mL), 11.5% (3/26) of hepatocirrhosis and 17.4% (4/23) of chronic hepatitis would be classified as liver cancer. Fig. 3a illustrates that only 7.40% of the hepatocirrhosis patients and none of the hepatitis patients are classified as cancer. These findings illustrate that the metabonomics-PCA method may be useful to differentiate between patients with hepatocirrhosis and hepatitis from patients with liver cancer while lowering false-positive rates. These findings also suggest that a subset of the urinary nucleosides identified with metabonomics correlate better with cancer diagnosis than the traditional single marker AFP.

In order to further distinguish between patients with hepatocirrhosis and those with liver cancer, another trial was done. The hepatitis and liver cancer patterns were analyzed by the

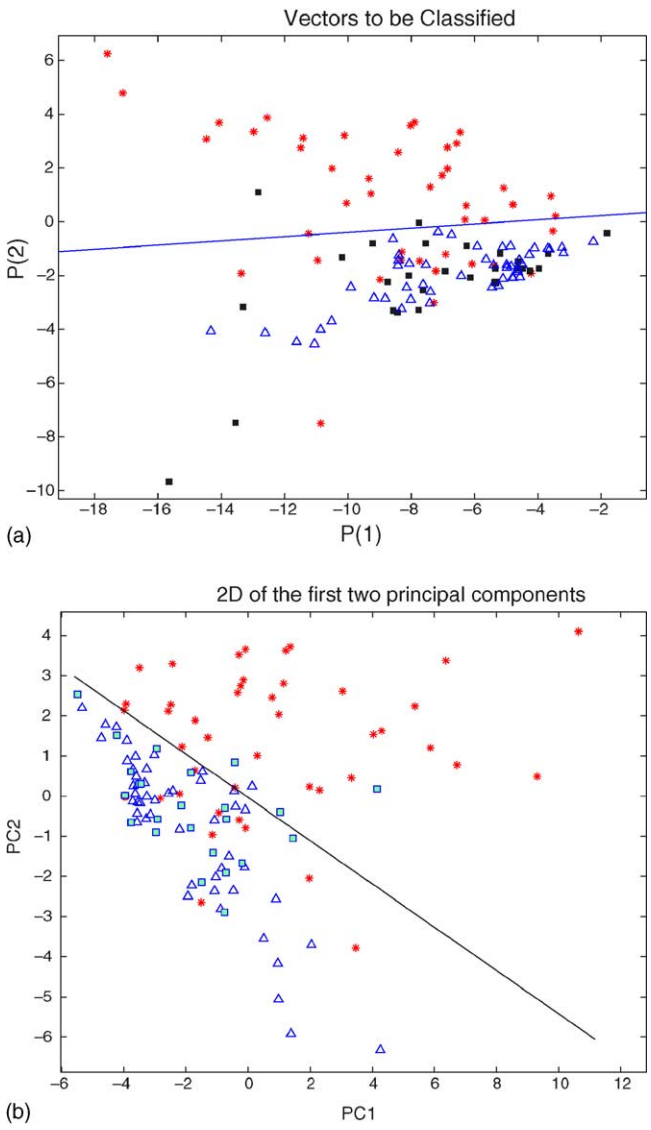


Fig. 3. (a) PCA scores plot of the metabonomics data (lg $k'$ was used as the retention factor in peak matching method). (b) Prediction of the positions of the hepatocirrhosis patients via regression analysis. (✱) Liver cancer; (△) hepatitis; (■) hepatocirrhosis.

metabonomics-PCA method mentioned above, and the hepatocirrhosis patterns were then fed to a principal components regression. Here, they were projected to the space produced by the first two principal components of the hepatitis and liver cancer patterns. The process was similar to the position prediction of the hepatocirrhosis patients. Fig. 3b reveals that 88.9% (24/27) of the hepatocirrhosis patterns appeared in the area of the hepatitis patterns using this regression model.

### 3.3. Reference peaks selection

As mentioned above, we have demonstrated that our HPLC-based metabonomics method is helpful in clinical diagnosis of various liver diseases. To get optimized peak matching performance, reference peaks should be carefully considered. The selected reference peaks in the peak matching method should satisfy the following requirements: (1) easily recognized in the reference and sample chromatograms, a peak with a significant height should be firstly considered; (2) a stable retention time; (3) smaller disturbance from neighbor peaks.

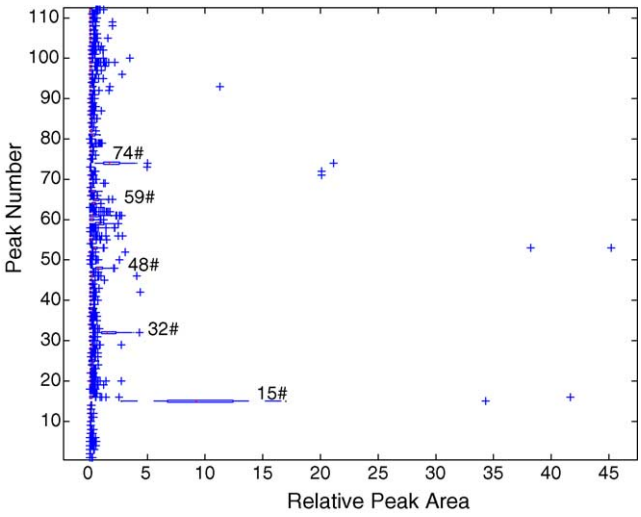


Fig. 4. Box plot of the metabolites. The box has lines at the lower quartile, median and upper quartile values. The whiskers are lines extending from each end of the box to show the extent of the rest of the data. Outliers (+) are data with values beyond the ends of the whiskers. If there is no data outside the whisker, a dot is placed at the bottom whisker.

The box plots (Fig. 4) for all the *cis*-diol metabolites give a quick impression of the original data. There was substantially more variability in peaks: 15#, 32#, 48#, 59# and 74# when compared to other peaks. These peaks were also higher than the others. Because of the unstable retention time, peak 59# was omitted and peak 53# was selected as one of reference peaks for its stable retention time. The internal standard Br8G fulfilled the first three requirements and was then added to the reference peaks set. In this study, the reference peaks included 15#, 32#, 48#, 53#, 74# and 80# (Br8G). The pre-defined arf values were then assigned to these reference peaks based on the distribution density of the peaks in the chromatograms. The region crowded with more target peaks was assigned to a larger arf scale. After calibration of the adjacent reference peaks, the adjusted retention factors of all peaks became stable. This allowed for the peaks to be correctly identified.

### 3.4. Elucidation of the contribution of the PCs

Pioneering studies of Borek and co-workers [28–30] suggest that excretion of elevated amounts of modified nucleosides in tumor bearing animals results from incremented tRNA turnover rather than cell death or the destruction of the tissue. Although the molecular mechanisms of this excretion are unclear, efforts have been made to use modified nucleosides as biochemical markers for neoplastic diseases [19–37]. Several modified nucleosides occurring at compar-

Table 2
First 10 peaks appearing in the first and second principal components

| PC1 | | | PC2 | | |
|---|---|---|---|---|---|
| Peak no.[a] | Constituent | Weight | Peak no.[a] | Constituent | Weight |
| 32 | m1A | 0.206 | 36 | Unknown | 0.241 |
| 48 | Unknown | 0.203 | 39 | Unknown | −0.232 |
| 23 | Unknown | 0.200 | 28 | Unknown | 0.220 |
| 15 | Pseu | 0.188 | 33 | Unknown | 0.204 |
| 14 | Unknown | 0.184 | 25 | Unknown | −0.200 |
| 13 | Unknown | 0.175 | 81 | Unknown | −0.199 |
| 44 | Unknown | 0.170 | 17 | Unknown | 0.188 |
| 59 | m1I | 0.165 | 27 | Unknown | 0.183 |
| 40 | Unknown | 0.164 | 67 | Unknown | −0.174 |
| 38 | Unknown | 0.158 | 77 | Unknown | −0.164 |

[a] Peak no. is the same as in Fig. 1.

atively high levels such as Pseu, dihydrouridine (Dhu), m1I, m2G, m1G have frequently been used to examine the differences between urinary nucleoside levels of cancer patients and normal subjects.

The contributions of the predominant peaks in the first and the second PC and the corresponding constituents are given in Table 2. It is clear that they are very different from those given in Fig. 2a and other nucleoside markers traditionally used [19–37]. Here, many unknown components are presented, especially in the second PC. These may represent previously overlooked regions of data that may be of great significance for the metabonomics analysis. Perhaps these peaks affect the diagnosis in a combination model and not in a single component mode. The result in Table 2 suggests that further work using HPLC–MS is necessary to identify the unknown components. These peaks may be potential components of novel markers that could aid the correct and reliable diagnosis of the liver cancer.

## 4. Conclusions

We conclude that the HPLC-based metabonomics is able to distinguish between cancer patients and healthy volunteers, and is further able to distinguish between patients with hepatocirrhosis patients and hepatitis from patients with liver cancer. These results suggest that our metabonomics method may play an important role in the post-genomics era, particularly in identifying new markers for clinical diagnosis of cancer and other diseases. Complementary to NMR, the HPLC-based metabonomics approach presents many advantages, such as simpler and cost efficiency.

## Acknowledgements

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