Characterization of *Fen-Daqu* Through Multivariate Statistical Analysis of ^{1}H NMR Spectroscopic Data

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ABSTRACT

J. Inst. Brew. 117(4), 516–522, 2011

Fen liquor is typical of Chinese light-flavour liquor (alcoholic spirit), which is fermented from sorghum with *Fen-Daqu* powder. *Fen-Daqu* is a saccharifying agent and fermentation starter in this fermentation process and in *Fen* traditional vinegar. To investigate the changes of biochemical components in *Fen-Daqu* during the incubation, samples at seven incubation stages were analyzed by ^{1}H nuclear magnetic resonance (NMR) spectrometry and principal component analysis (PCA). This revealed clear separation of the samples obtained from different incubation stages in the principal component plots by combining PC1 and PC2, which cumulatively accounted for 93.27% of the variance. The major compounds that contributed to discrimination were acetate/alanine, arginine, ascorbate, betaine, choline, ethanol, fructose, galactose, glucose, glucitol, glycerate, lactate, maltose, mannitol, phenylalanine, proline, propylene glycol, threonine and tryptophan. These compounds were regarded as the representative metabolites or biomarkers characteristic for each incubation stage and were related with microbiological changes of importance for quality control in *Fen-Daqu* production.

Key words: *Fen-Daqu,* fermentation starter, ^{1}H nuclear magnetic resonance, principal component analysis.

INTRODUCTION

Fen liquor is light-flavoured, and is one of the famous Chinese liquors (alcoholic spirit) with a history of over 1,500 years. *Fen* liquor is fermented and distilled from sorghum with *Fen-Daqu* powder made from barley and pea. *Daqu* is a saccharifying agent and fermentation starter for the production of Chinese liquor, and contains a range of microorganisms, various enzymes, metabolites and degradation products, and important flavour compounds that will contribute to the aroma of the final distillate[3,8,14]. According to the approximate temperature and level of cooling during the incubation step, *Fen-Daqu* can be classified into three types as follows: (i) *Fen-Houhuo,* high-temperature and medium cooling (ii) *Fen-Hongxin,* long high-temperature and low cooling; (iii) *Fen-Qingcha,* medium temperature and high cooling, and their proportion in *Fen* liquor production is 40%:30%:30%. *Fen-Daqu* is prepared from barley and peas by five steps: (1) ingredient formulation; (2) grinding and mixing; (3) shaping; (4) incubation (about 1 month) and (5) maturation (about 6 months). The incubation step is divided into seven stages: *Woqu, Shangmei, Liangmei, Chaohuo, Dahuo, Houhuo* and *Yangqu*. *Woqu* is the first day of incubation and the *Daqu* is still quite soft and thus may only be stacked to a maximum of three layers, to avoid deformation and allow good ventilation. *Shangmei* means "growth of filamentous fungal mycelium". Fungi occur as a natural inoculum on raw materials, water, rush mats, bran coats and in the environment. The temperature increases gradually, attaining 30–40°C in 2–3 days. *Liangmei* means a cooling down to prevent damage from overheating and a hardening of the *Daqu,* the temperature is around 25–35°C for 2–3 days. The key incubation period for microbial succession is "*Chaohuo*", the temperature increases to about 43–47°C and this stage takes about 5–6 days. The *Dahuo* stage takes about 4–6 days and the temperature reaches a maximum of around 52°C. The *Houhuo* stage takes about 5–6 days and the temperature decreases gradually to approximately 30–35°C. *Yangqu* (pre-maturation) takes about 5–7 days and the aim of this stage is to allow the equilibration of moisture, acidity and enzyme activity[18].

There have been a few studies on the end product of sauce-flavour, light-flavour and strong-flavour *Daqu*. The results showed that many biochemical compounds in *Daqu* served as flavour compounds or flavour precursors in the liquor obtained by this type of *Daqu*[3,14,17]. We hypothesize that the profile of the biochemical characteristics in *Daqu* during the incubation period can reflect the specific fermentation events and biochemical reactions associated with the microbial succession and metabolism taking place during its stages. However, up to now, no biochemical characteristics of *Fen-Daqu* during the various stages of incubation have been reported.

Nuclear magnetic resonance (NMR) is one of the important non-targeted techniques. It can produce rapid, nondestructive, stable and highly reproducible results[5]. It has the advantage that all kinds of small metabolite molecules can be measured simultaneously[1,9]. Principal component analysis (PCA) is a multivariate statistical analysis

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Publication no. G-2011-1128-1147

technique that is used for data reduction. Metabolomic techniques combining NMR and PCA have been applied to the metabolic profiling of various kinds of *Daqu* products, fermented soybean, wine, beer and cheese[2,4,6,11,13,14,16].

In this study, we used ^{1}H NMR spectroscopy followed by PCA for the analysis of the biochemical compounds from extracts of *Fen-Daqu* samples obtained during the successive stages of the incubation steps. The objective of the study was to reveal the major components of *Fen-Daqu*, and also to assess whether these components could be used to discriminate *Fen-Daqu* intermediate products obtained during the successive stages of incubation.

MATERIALS AND METHODS

Sampling

Fen-Daqu samples were obtained from the Xinghuacun Fenjiu Group, Shanxi province, China. *Daqu* was fermented and matured in stacked layers. Samples were collected at the end of *Woqu, Shangmei, Liangmei, Chaohuo, Dahuo, Houhuo* and *Yangqu* stages of *Fen-Houhuo, Fen-Hongxin* and *Fen-Qingcha*. Each sample was obtained by randomly selecting from each upper, middle and lower stacked layer and mixed together as an experimental sample. Samples were stored at –80°C until used.

Extraction of polar compounds from *Fen-Daqu*

One hundred milligrams of ground *Daqu* sample was transferred into a centrifuge tube and then 1.5 mL of cold Milli-Q water (~0°C, ice bath) was added into the tube and vortexed at 2,500 oscillations/min for 60 sec using a Biospec Beadbeater (Mini-Beadbeater-8, Biospec, Bartlesville, USA) without added beads. The tube was then kept on ice for 10 min and then centrifuged for 10 min at 16,060 × *g* at 4°C. Finally, one millilitre of supernatant was transferred to a new tube and stored at –80°C until analysis, as described previously[14]. Each experiment was performed in triplicate.

NMR measurements

For the NMR measurements, each extract was mixed with an equal volume of cold loading buffer (0.1 M of sodium phosphate, pH 7.0, containing 10% D_2O (v/v), 1 mM TSP (3-trimethylsilylpropionate) as an internal standard, 100 mM imidazole and 0.2% w/v sodium azide) and centrifuged at 16,060 × *g* at 4°C. A 500 µL aliquot of the mixture was transferred into a 5 mm NMR tube for determination. All ^{1}H NMR spectra were measured at 300 K using an Avance III NMR spectrometer (Bruker, Germany, proton frequency 600.13 MHz, 14.1 T). For each sample, 128 scans were recorded with the following parameters: pulse sequence, noesygppr1d; relaxation delay, 4 sec; mixing time (for noesy), 1s; acquisition time, 2.28 s; number of steady states transients (dummy scans), 4; gradient pulse times, 1 ms; solvent suppression, presaturation with spoil gradient; spectral width, 7,184 Hz; time domain size, 32 k.

Data analysis

The spectral region from 0.60 ppm to 9.00 ppm was segmented into regions of 0.04 ppm width giving a total of 210 integrated regions per NMR spectrum. The region from 4.40 to 5.30 was excluded from the analysis because of the residual signal of water in the aqueous extracts, whereas those from 7.35 to 7.50 and 8.40 to 8.60 were excluded from the analysis because the residual signal of imidazole. All spectral data were scaled to total intensity

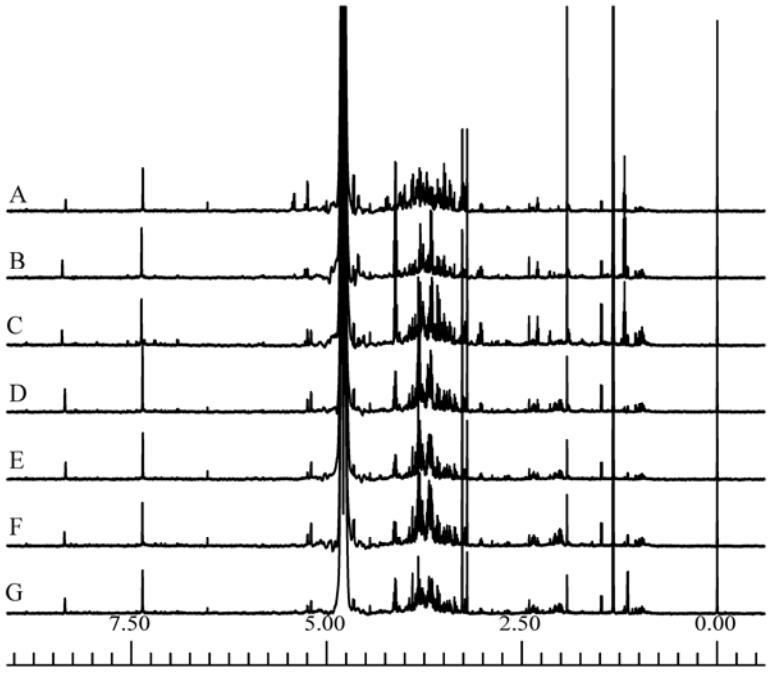

Fig. 1. Representative ^{1}H NMR spectra of aqueous extracts of *Fen-Daqu,* obtained from (A) *Woqu,* (B) *Shangmei,* (C) *Liangmei,* (D) *Chaohuo,* (E) *Dahuo,* (F) *Houhuo,* and (G) *Yangqu.*

Table I. Presumptive biochemical compounds and their concentrations in *Fen-Daqu.* Values are in micromoles per gram of *Daqu* dry matter by means of Chenomx.

Metabolite	*Woqu*	*Shangmei*	*Liangmei*	*Chaohuo*	*Dahuo*	*Houhuo*	*Yangqu*
1,6-Anhydro-β-D-glucose	2.20	1.28	1.92	1.21	1.50	1.60	0.86
2-Aminobutyrate	0.87	0.27	0.63	1.49	0.61	1.43	0.54
2-Hydroxyglutarate	0.98	nd[a]	nd	1.01	2.04	0.66	1.06
4-Aminobutyrate	4.59	3.69	6.60	4.85	2.85	3.58	0.55
4-Carboxyglutamate	nd	0.85	1.37	1.18	2.40	1.85	1.71
Acetate	7.02	51.10	32.85	9.04	13.27	6.47	5.78
Acetoacetate	1.31	0.69	0.85	0.73	0.68	1.42	0.58
Adenosine	0.71	nd	0.38	0.46	0.26	0.47	0.71
Alanine	4.69	5.28	12.15	9.74	8.55	7.57	5.12
Alloisoleucine	0.38	0.35	0.73	0.58	0.42	0.80	0.25
Anserine	0.83	0.82	0.70	1.08	1.17	1.20	0.43
Arginine	3.01	1.74	2.79	3.01	0.75	3.23	1.51
Ascorbate	3.36	1.66	3.54	1.85	2.75	2.15	2.22
Asparagine	1.64	1.08	0.79	1.29	0.81	1.86	0.38
Aspartate	2.74	1.06	3.15	2.72	1.46	1.35	0.50
Betaine	4.33	2.57	5.00	5.97	6.09	5.42	8.39
Caffeine	0.20	0.09	0.43	0.26	0.27	0.19	0.18
Carnitine	0.28	0.14	0.69	0.56	0.63	0.49	0.31
Choline	6.32	3.67	4.78	4.79	4.68	4.16	3.55
Creatinine	0.64	0.17	0.24	0.27	0.33	0.14	0.15
Cystine	1.48	nd	nd	1.44	1.05	1.28	0.95
Dimethylamine	nd	nd	0.07	0.15	0.21	0.22	0.16
Ethanol	11.31	43.89	22.97	6.20	3.41	1.78	4.46
Fructose	27.44	5.74	3.72	4.30	3.25	3.35	2.96
Fucose	0.48	1.00	0.78	0.49	0.29	0.41	0.50
Fumarate	2.82	0.08	0.26	0.97	2.35	1.25	1.16
Galactitol	2.26	0.74	2.04	1.82	1.99	2.33	1.83
Galactonate	2.75	0.75	1.36	1.68	1.97	1.72	2.40
Galactose	10.50	12.24	11.44	10.22	4.42	6.71	5.26
Glucitol	4.75	2.46	3.35	5.11	3.83	4.54	3.84
Gluconate	5.28	2.19	4.09	3.20	2.32	2.84	2.18
Glucose	60.72	14.26	23.04	19.91	19.61	19.96	8.63
Glutamate	2.14	2.51	5.82	5.33	4.74	5.26	4.00
Glutamine	1.31	1.16	1.82	1.67	1.38	1.52	0.84
Glutaric acid monomethyl ester	0.23	0.39	0.28	0.56	0.54	0.36	0.35
Glycerate	4.67	2.46	5.05	3.53	3.90	4.14	2.73
Glycerol	5.91	9.88	18.90	11.81	11.34	9.12	10.08
Glycine	3.21	3.30	5.10	4.36	3.77	3.35	2.19

(continued on next page)

[a] Not detected.

of the corresponding spectrum, and then analyzed by principal component analysis (PCA) with Pareto scaling. PCA was performed with AMIX software (version 3.7.10; Bruker BioSpin, Rheinstetten, Germany). The output from the PCA analysis consisted of score plots giving an indication of the differentiation of the classes in terms of metabolome similarity and loading plots giving an indication as to which NMR spectral regions were important with respect to the classification obtained in the score plots. Compounds were identified and quantified with Chenomx software (Version 6.0; Chenomx, Edmonton, Canada).

RESULTS AND DISCUSSION

Visual inspection of ^{1}H NMR spectra and assignment of compounds

Representative ^{1}H NMR spectra of aqueous extracts of *Fen-Daqu* during its production are presented in Fig. 1. A wide range of biochemical compounds could be assigned in each spectral matrix that provided complementary information on overall changes of the biochemical compounds during the production of *Fen-Daqu*. About 80 biochemical compounds were presumptively identified by Chenomx software 6.0 as shown in Table I.

In sample preparation, the extracts of *Fen-Daqu* contained added imidazole and sodium azide, these compounds were not added in the previous study[14]. Imidazole was used as pH indicator and the pH values of samples were in the range 6.68–7.05. Sodium azide was used to inhibit the growth of bacteria.

Table I showed that the amounts of sugars such as glucose, fructose, maltose, sucrose, ribose and lactose gradually decreased during incubation. Obviously these decreases reflect the assimilation of carbon as a source of energy by the evolving microbiota. Such microbiological developments in *Fen-Daqu* were reported previously[7]. In addition, it was reported that fungi had a unique distribution in the starter of *Fen* liquor. They possessed high α-amylase and glucoamylase activities, which enabled the utilization of raw starch as a carbon source[12].

Table I also shows that the concentration of proline, homoserine, tyrosine and threonine were lower during the early stages than during the later stage of production. During the middle stage, especially the *Dahuo* stage, the temperature in the *Daqu* blocks was increased to 52°C. It had been stated that the goal of the *Dahuo* stage is to enhanced proteolysis and the accumulation of amino acids[18]. During the middle stage, the concentrations of acetate,

Table I. (continued from previous page).

Metabolite	*Woqu*	*Shangmei*	*Liangmei*	*Chaohuo*	*Dahuo*	*Houhuo*	*Yangqu*
Glycylproline	2.93	2.12	2.47	3.12	1.94	3.71	2.59
Guanidoacetate	5.51	4.15	3.62	7.25	6.92	5.52	2.02
Histamine	1.29	0.69	1.30	0.53	0.87	0.79	0.67
Histidine	0.95	0.63	1.01	0.69	1.90	0.63	1.08
Homocysteine	2.31	nd	0.85	0.41	0.53	1.10	nd
Homoserine	1.50	2.21	2.82	2.32	3.54	3.49	2.62
Isoleucine	0.55	0.62	1.86	1.46	1.21	1.69	0.72
Isopropanol	1.31	0.99	1.01	nd	0.47	1.22	1.55
Lactate	1.41	115.83	139.58	103.50	66.39	55.45	28.76
Lactose	4.22	2.39	3.81	2.98	2.38	1.73	1.28
Leucine	0.93	1.58	2.97	2.73	2.56	2.70	0.93
Lysine	0.82	2.28	3.28	2.94	2.86	1.77	0.42
Malate	5.14	0.80	0.73	3.04	2.08	3.75	2.72
Malonate	0.56	nd	nd	0.43	0.47	0.34	0.40
Maltose	9.48	2.79	1.74	2.03	2.08	2.49	1.39
Mannitol	3.57	7.79	10.54	6.27	10.07	6.38	2.78
Mannose	3.09	2.06	5.70	4.82	5.46	4.00	4.21
Methanol	3.50	3.77	3.82	2.69	3.64	5.27	2.42
Methionine	0.28	0.51	1.27	0.81	0.60	0.66	0.38
myo-Inositol	6.72	2.46	3.35	2.36	1.72	2.35	1.29
O-Phosphocholine	1.10	0.39	1.33	1.08	0.63	0.21	0.24
Ornithine	nd	1.71	1.72	0.93	0.45	nd	0.53
Phenylalanine	0.30	0.41	2.35	0.18	2.49	1.21	0.40
Proline	1.70	2.32	6.11	17.78	16.20	16.53	13.16
Propylene glycol	0.25	1.35	2.50	0.94	0.78	1.47	2.53
Pyruvate	0.67	nd	0.76	0.34	0.60	1.18	0.55
Ribose	15.19	4.67	6.80	7.00	7.64	3.99	3.13
Saccharopine	nd	0.31	1.16	1.58	nd	0.86	nd
Succinate	0.60	2.41	2.80	1.69	1.59	1.38	0.97
Sucrose	3.19	1.16	1.25	0.82	0.28	1.10	1.42
Taurine	2.46	1.20	2.06	0.94	3.11	3.97	1.98
Threonine	0.99	2.81	3.09	3.98	2.11	2.26	3.69
Trigonelline	2.15	nd	2.39	nd	1.31	2.21	0.86
Trimethylamine N-oxide	0.91	0.20	0.60	0.65	0.62	1.63	0.34
Tryptophan	0.65	1.52	1.96	2.04	0.80	1.06	0.63
Tyramine	nd	0.17	0.96	0.51	0.08	0.27	0.28
Tyrosine	0.23	1.09	1.34	0.78	1.65	1.51	0.33
Uracil	0.74	1.64	2.01	1.45	0.71	1.05	0.64
Valine	1.48	1.77	3.56	2.58	2.36	1.70	0.95
Xylose	1.96	nd	5.67	3.64	1.51	nd	2.50
π-Methylhistidine	1.29	nd	0.55	0.97	1.68	0.84	1.30

alanine, lactate, leucine, glycine, glycerol, mannitol and valine were relatively higher than during the early and later stages, as shown in Table I. Lactate is produced by lactic acid bacteria[10]. The changes in lactate concentration showed a positive correlation with the changes in the lactic acid bacteria concentration during these stages. The experimental results showed that the lactic acid bacteria counts were highest at during the *Shangmei, Liangmei* and *Chaohuo* stages, and were in the range of 5.7–6.4 Log CFU g^{-1}. However, lactate was not detected in the end product of light-flavour *Daqu*[14], so it could be that the lactate was reduced during the maturation step.

According to the flavour characteristics of the liquor obtained, several types of *Daqu* can be distinguished, such as light-flavoured, strong-flavoured, and sauce-flavoured *Daqu*. It has been reported that glycerol, acetate, carnitine and malate were identified as biomarkers of light-flavoured *Daqu*[14]. Table I shows that the concentration of glycerol, acetate and malate were relatively higher during the incubation step. Acetate was also reported as the representative aroma compound in light-flavoured liquor, and mainly as ethyl acetate[15]. Ethyl acetate and 3-methyl-1-butanol acetate were found at relatively high concentrations in the *Shangmei* and *Liangmei* stages (data not shown).

Principal component analysis

PCA was performed encompassing all of the samples and enabled a discrimination among samples. The PCA scores plot (Fig. 2) showed that, samples collected at each stage of the production of *Fen-Daqu* could be discriminated clearly. The first two principal components (PC1 and PC2) cumulatively accounted for 93.27% of the total variation. Separation of the different samples of *Fen-Daqu* in the score plot was achieved by combining PC1 and PC2. In the score plot, samples obtained from the *Woqu, Shangmei* and *Liangmei* stages were separated clearly from each other. The score plots for the samples obtained from *Liangmei, Chaohuo* and *Dahuo* stages were relatively close to each other, but were nevertheless separated (Fig. 4c and 4d). Similarly, score plots for the samples obtained from the *Dahuo, Houhuo* and *Yangqu* stages were separated clearly from each other. The distances between the score plots of the incubation stages indicate that the metabolite profiles undergo the biggest changes

from A (*Woqu*) to B (*Shangmei*) stage and from the E (*Dahuo*) to F (*Houhuo*) stage.

The biochemical compounds in the aqueous extracts of *Fen-Daqu* could be distinguished using PCA, and is shown in the loading plot for PC1 and PC2 (Fig. 3). The major compounds that contributed to discrimination were acetate/alanine, arginine, ascorbate, betaine, choline, ethanol, fructose, galactose, glucose, glucitol, glycerate, homoserine, lactate, maltose, mannitol, phenylalanine, propylene glycol, threonine and tryptophan. Of these compounds, acetate/arginine, fructose, galactose, glucose, glucitol, lactate, maltose, mannitol, phenylalanine, threonine and tryptophan contributed mainly to discrimination by PC1, while alanine/ascorbate, betaine, choline, ethanol, glucose, glycerate, homoserine, lactate, maltose, propylene glycol, threonine and tryptophan contributed mainly to discrimination by PC2.

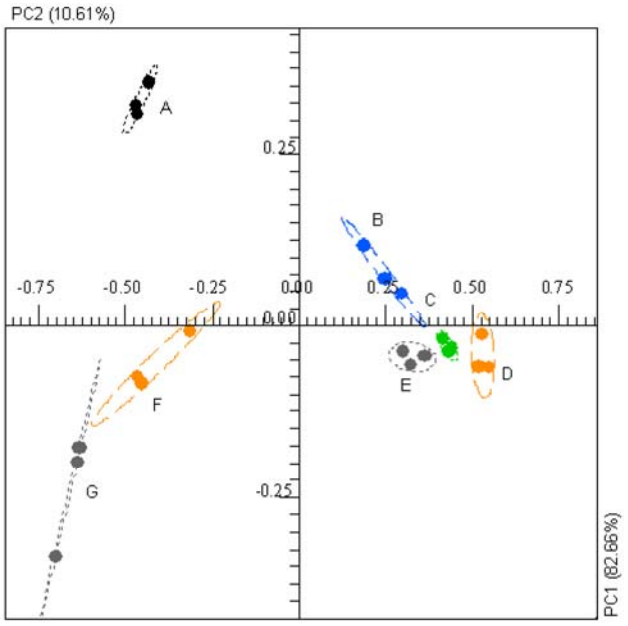

Fig. 2. PCA score plot of *Fen-Daqu* during the incubation (A) *Woqu,* (B) *Shangmei,* (C) *Liangmei,* (D) *Chaohuo,* (E) *Dahuo,* (F) *Houhuo,* and (G) *Yangqu.*

The biomarkers of each incubation stage were identified by pairwise comparison of peak intensities among the seven stages. Figure 4 shows the pairwise PCA comparisons of each incubation stage of *Fen-Daqu.* All score plots displayed a significant separation between the respective stages of the production process. The loading plots indicate the biochemical compounds that were responsible for the separation of clusters.

As shown in Fig. 4a, the score plot shows a separation between the *Woqu* and *Shangmei* stages. Loadings plot shows that the biochemical compounds responsible for the separation were increased in the concentrations of acetate, lactate, glycerol, ethanol, mannitol, homoserine, phenylalanine, propylene glycol and decreased in the concentrations of choline, betaine, glucose, fructose, aspartate, maltose, sucrose and taurine. Figure 4b shows the separations of the samples obtained from the *Shangmei* and *Liangmei*

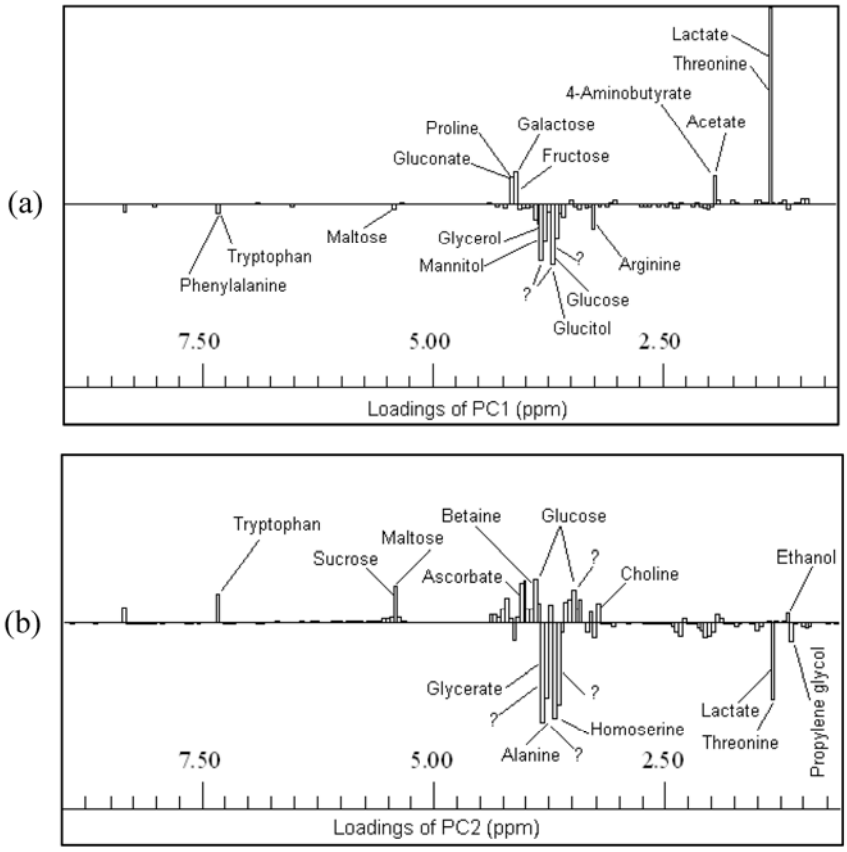

Fig. 3. PCA loading plot for PC1 (a) and PC2 (b) of *Fen-Daqu* during the incubation.

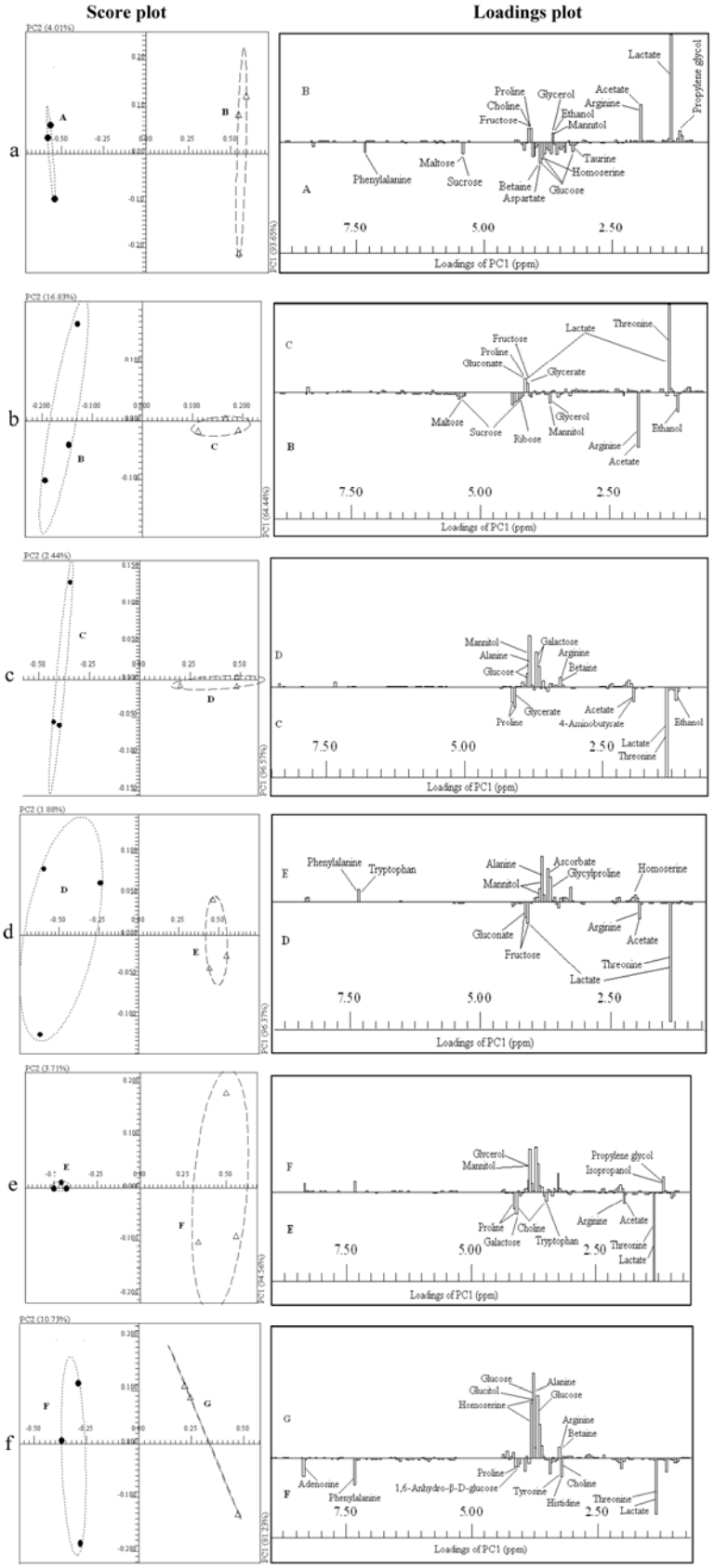

Fig. 4. PCA of *Fen-Daqu* extracts of incubation stages: (a) A: *Woqu* versus B: *Shangmei,* (b) B: *Shangmei* versus C: *Liangmei*, (c) C: *Liangmei* versus D: *Chaohuo,* (d) D: *Chaohuo* versus E: *Dahuo,* (e) E: *Dahuo* versus F: *Houhuo,* and (f) F: *Houhuo* versus G: *Yangqu*.

stages; these were caused by increases in the concentration of lactate, arginine, gluconate, glycerate, glycerol, proline, mannitol, ribose, sucrose, threonine and decreases in the concentration of acetate, ethanol, maltose, and fructose. Figure 4c shows the separations of the samples obtained from the *Liangmei* and *Chaohuo* stages that were caused by increases in the concentrations of lactate, arginine, proline, threonine, and decreases in the concentrations of 4-aminobutyrate, galactose, glycerate, glucose, acetate, betaine, alanine, ethanol and mannitol. Figure 4d shows the separations of the samples obtained from the *Chaohuo* and *Dahuo* stages that were caused by increases in the concentrations of acetate, ascorbate, homoserine, phenylalanine, and mannitol and decreases in the concentrations of alanine, arginine, lactate, gluconate, glycylproline, threonine, and tryptophan. Figure 4e shows the separations of the samples obtained from the *Dahuo* and *Houhuo* stages; these were caused by increases in the concentrations of arginine, galactose, isopropanol, proline, propylene glycol, threonine, tryptophan and decreases in the concentrations of acetate, choline, glycerol, lactate, and mannitol. Similarly, Fig. 4f shows a clear separation between the *Houhuo* and *Yangqu* stage. Loadings plots show that the biochemical compounds responsible for the separation were increased concentrations of betaine, histidine, adenosine, threonine, and decreased concentrations of alanine, arginine, choline, homoserine, glucitol, lactate, phenylalanine, proline, tyrosine, and 1,6-anhydro-β-D-glucose. The absolute concentrations of all biochemical compounds are presented in Table I.

To this day, the manufacturing process of *Fen* liquor still relies on workers' experience. The production of *Fen-Daqu* is still the constitution of the traditional fermentation technology without added microorganisms. The quality of *Daqu* cannot be kept stable, even for the same batch of products. This study is the first joint application of ^{1}H NMR to investigate the changes in the biochemical components in *Fen-Daqu* during the incubation step. Having gained this important insight into the changes of biochemical components of *Fen-Daqu,* we potentially have the knowledge to make substantial improvements to the liquor fermentation process, such as improving the beneficial strains, and so further study of microbial ecology during the incubation step of *Fen-Daqu* will be necessary.

In conclusion, the changes in the biochemical compounds in *Fen-Daqu* during the incubation step were revealed in this study. This could help *Daqu* producers monitor the progress of the *Daqu* manufacturing process, by measuring specific biomarkers for each stage, and to verify the authenticity of commercially produced *Fen-Daqu*.

ACKNOWLEDGEMENTS

We thank Mr. Hu Gao-Fei for NMR support and the Shanxi Xinghuacun Fenjiu Group Company for *Daqu* sampling assistance and advice. This study was funded by the National Natural Science Foundation of China (No. 31071592) and the KNAW-China Joint Research Project (No. 07CDP015) from the Royal Netherlands Academy of Arts and Sciences.

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(Manuscript accepted for publication November 2011)