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Effects of nitrogen catabolite repression-related amino acids on the flavour of rice wine

Xinrui Zhao,[1,2] Huijun Zou,[3] Guocheng Du,[1,2] Jian Chen[1,2] and Jingwen Zhou[1,2]*

The quality of rice wine is highly dependent on the content of the flavour compounds produced by the budding yeast *Saccharomyces cerevisiae*. In this study, the effects of three amino acids (arginine, glutamate and glutamine) related to nitrogen catabolite repression on the formation of flavour compounds were investigated. Each of these amino acids could promote the growth of *S. cerevisiae*, and a total of 83 flavour compounds were found in a model system of rice wine production. The effects of arginine, glutamate and glutamine on the content of the higher alcohols, amino acids and esters were significant, whereas the effects on the aldehydes and organic acids were slight. The results of this study could facilitate the development of new strategies to control the flavour pattern and improve the quality of rice wine.

Keywords: amino acids; flavour compounds; *Saccharomyces cerevisiae*; principal component analysis

Introduction

Rice wine is one of the traditional alcoholic beverages brewed from cereals *(1)*. Normally, it is produced from rice with yeast and a special saccharifying agent (*Qu*, which is made from a natural culture of moulds, bacteria and yeasts on wheat) *(2,3)*. The production of rice wine is complex and strictly controlled (summarized in Fig. 1) *(4)*. Dozens of steps are required to produce high-quality rice wine with the desired odour and flavour, and these are highly dependent on the content of the organic aromatic compounds produced during fermentation *(5)*.

At least five types of flavour compounds, including alcohols, esters, aldehydes, organic acids and amino acids *(6)*, contribute to the mellow and soft flavour of high-quality rice wine. Production of the desired flavour compounds depends mainly on the quality of the raw materials, the fermentation properties of the producing strains (*Saccharomyces cerevisiae*) and fermentation conditions *(7)*. Manufacturers of rice wine have focused on finding suitable strains of *S. cerevisiae* because many flavour substances are produced directly or indirectly by the yeast *(7)*.

Nitrogen is an important nutrient for the growth of *S. cerevisiae* *(8)*. However, the utilization of different nitrogen sources is in a specific order *(9)*. When preferred nitrogen sources are present in the medium, the use of non-preferred nitrogen sources is decreased significantly by nitrogen catabolite repression (NCR) *(10)*. An earlier study found that arginine (Arg), glutamate (Glu) and glutamine (Gln) were the three important nitrogen sources involved in NCR *(11)*. These amino acids in the medium, which can repress the use of non-preferred nitrogen sources, are used preferentially during fermentation *(11)*. Furthermore, amino acids have been used as additional sources of assimilable nitrogen to enhance the generation of aromatic compounds *(12,13)*. The changes in the flavour patterns induced by adding essential nitrogen sources during rice wine production have received little attention. It is therefore important to investigate the relationship between the formation of flavour compounds and Arg, Glu and Gln supplements during the production of rice wine.

Yeast strains in a medium containing Arg as the sole nitrogen source had the best fermentation characteristics in earlier experiments *(14)*. Moreover, it was found that the addition of Arg improved the growth of the yeast significantly *(11)*. Glu, which is a major bridge between carbon and nitrogen metabolism *(15)*, can be converted into α-ketoglutarate during the tricarboxylic acid cycle or can be used to synthesize proteins *(15)*. Gln represents 15% of the total cellular nitrogen and is a preferred nitrogen source for yeast *(16)*.

To our knowledge, there has been no study on the effect of individual amino acids on the growth of *S. cerevisiae* or the formation of flavour compounds during the production of rice wine. The effects of Arg, Glu and Gln on the growth of *S. cerevisiae*, fermentation characteristics and the formation of flavour compounds were identified in this study. In addition, several individual flavour compounds were reported in the model rice wine system. Further, flavour compounds affected significantly by the addition of Arg, Glu or Gln were identified. The results of this work could provide guidance for the production of high-quality rice wine

* Correspondence to: Jingwen Zhou, Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China. E-mail: zhoujw1982@jiangnan.edu.cn

[1] Key Laboratory of Industrial Biotechnology, Ministry of Education, School of Biotechnology, Jiangnan University, 1800 Lihu Road, Wuxi, Jiangsu 214122, China

[2] Synergetic Innovation Centre of Food Safety and Nutrition, 1800 Lihu Road, Wuxi, Jiangsu 214122, China

[3] Zhejiang Guyuelongshan Shaoxing Wine Company, 13 Yangjiang Road, Shaoxing, Zhengjiang 312000, China

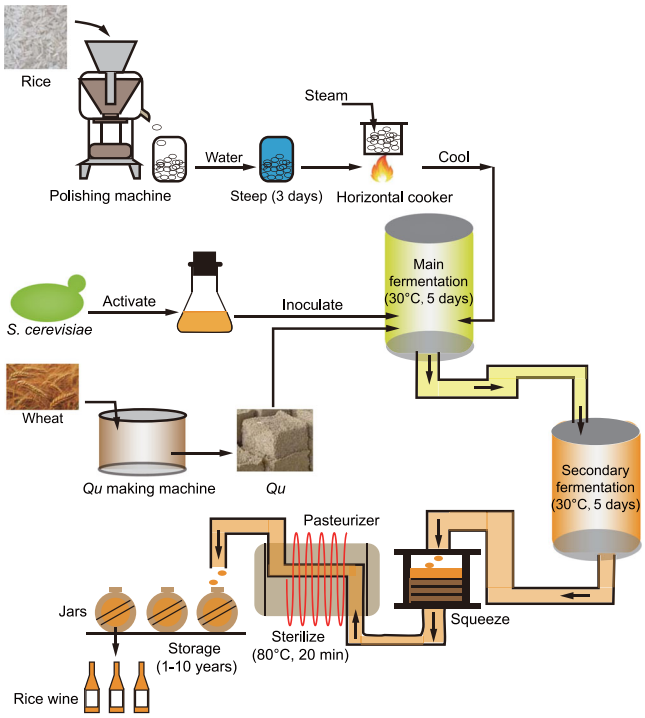

Figure 1. The process of rice wine production in China.

and facilitate the development of new strategies to control the flavour of rice wine.

Materials and methods

Yeast strain

The *S. cerevisiae* diploid strain N85 was provided by the Guyuelongshan Shaoxing Wine Co. Ltd (China) *(11)*.

Cultural conditions for fermentation

S. cerevisiae strain N85 was cultivated in YPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) at 30°C for 20 h with shaking (200 rpm). YNB medium (1.6 g/L yeast nitrogen base, 20 g/L glucose) with added Arg, Glu or Gln (each 10 mM) was inoculated with *S. cerevisiae* N85 and incubated in flasks at 30°C for 36 h with shaking (200 rpm). Samples were withdrawn every 6 h to measure the concentration of glucose, ethanol and urea and to examine the value of OD_{600} in the fermentation broth. The same analyses were performed with three biological replicates and mean values were used for further calculations.

Model rice wine production system

A model system for rice wine production was designed according to the manufacturing process of Shaoxing rice wine in China. The process of fermentation has been described in detail *(17)*. After fermentation for 25 days, samples were taken to measure the concentrations of the flavour compounds in the model system. All experiments were performed with three biological replicates and mean values were used for further calculations.

Assay of glucose, ethanol and urea

Urea was measured by an HPLC system (Agilent 1200; Palo Alto, CA, USA) equipped with a fluorescence detector using automated derivatization with xanthydrol *(18)*. Glucose and ethanol were measured by the same system equipped with a refractive index detector *(19)*.

Assay of flavour compounds

All standard flavour compounds were obtained from Sigma-Aldrich (St Louis, MO, USA). Amino acids were measured by an HPLC system (Agilent 1200; Palo Alto, CA, USA) equipped with a column (Zorbax Eclipse AAA; 4.6 × 150 mm) as described *(20)*. The analysis of volatile flavour compounds was performed with a TurboMatrix HS-16 Headspace Sampler (Perkin Elmer, Norwalk, CT, USA) and a GC-2010 gas chromatograph equipped with a mass spectrometer (GCMS-QP2010 plus; Shimadzu, Kyoto, Japan). A polar phase chromatography capillary column (30 m × 0.25 mm i.d., film thickness 0.25 μm; Rtx-WAX, Restek Corp., Bellafonte, PA, USA) was used as described *(21)*. The parameters set for the headspace sampler were: sample temperature, 60°C; needle temperature, 105°C; transfer temperature, 110°C; timing programme, 30 min thermostatting, 1 min pressurization, 20 min purge. The parameters set for gas chromatography were as follows: the injector, splitless model at 250°C and oven temperature programme at 50°C for 4 min, followed 5°C/min to 120°C (1 min) and 8°C/min to 220°C (2 min). The carrier gas was helium and the flow rate was 1 mL/min *(6)*. The mass spectrometer was set at electron impact mode at 70 eV and qualitative analysis for flavour compounds was performed using the full scan with a range of 50–500 amu *(6)*.

Statistical analysis

SPSS 18.0 software (SPSS Inc., Chicago, IL, USA) was used for principal component analysis of the volatile flavour compounds. The analyses of variations in flavour compounds and data presentation were performed with TIGR Multiexperiment Viewer (TMeV; http://www.tm4.org/) *(22)*.

Results and discussion

Effects of three amino acids on fermentation characteristics

After fermentation for 36 h, the concentrations of glucose, ethanol and urea and the values of OD_{600} in the different media were measured (Fig. 2). Each amino acid added to the medium promoted growth of the yeast. In medium supplemented with Arg, glucose (20 g/L) was metabolized within 18 h and the final OD_{600} at 36 h was increased by 30.9% compared with the control. The effect of Glu or Gln on growth was less than that of Arg. Glucose was metabolized within 24 h following the addition of Glu or Gln and the final OD_{600} at 36 h was increased by 13.9 and 24.1%, respectively. As the biosynthesis of flavour compounds in rice wine is related to the growth of yeast *(23)*, the enhanced cell growth might affect the formation of flavour compounds qualitatively or quantitatively.

As ethanol is the most important flavour component of rice wine, the effects of Arg, Glu and Gln on the formation of ethanol, the most important flavour component of rice wine *(6)*, were examined. The results showed that these amino acids could enhance the production of ethanol by 6.0, 21.5 and 15.3%, respectively. A

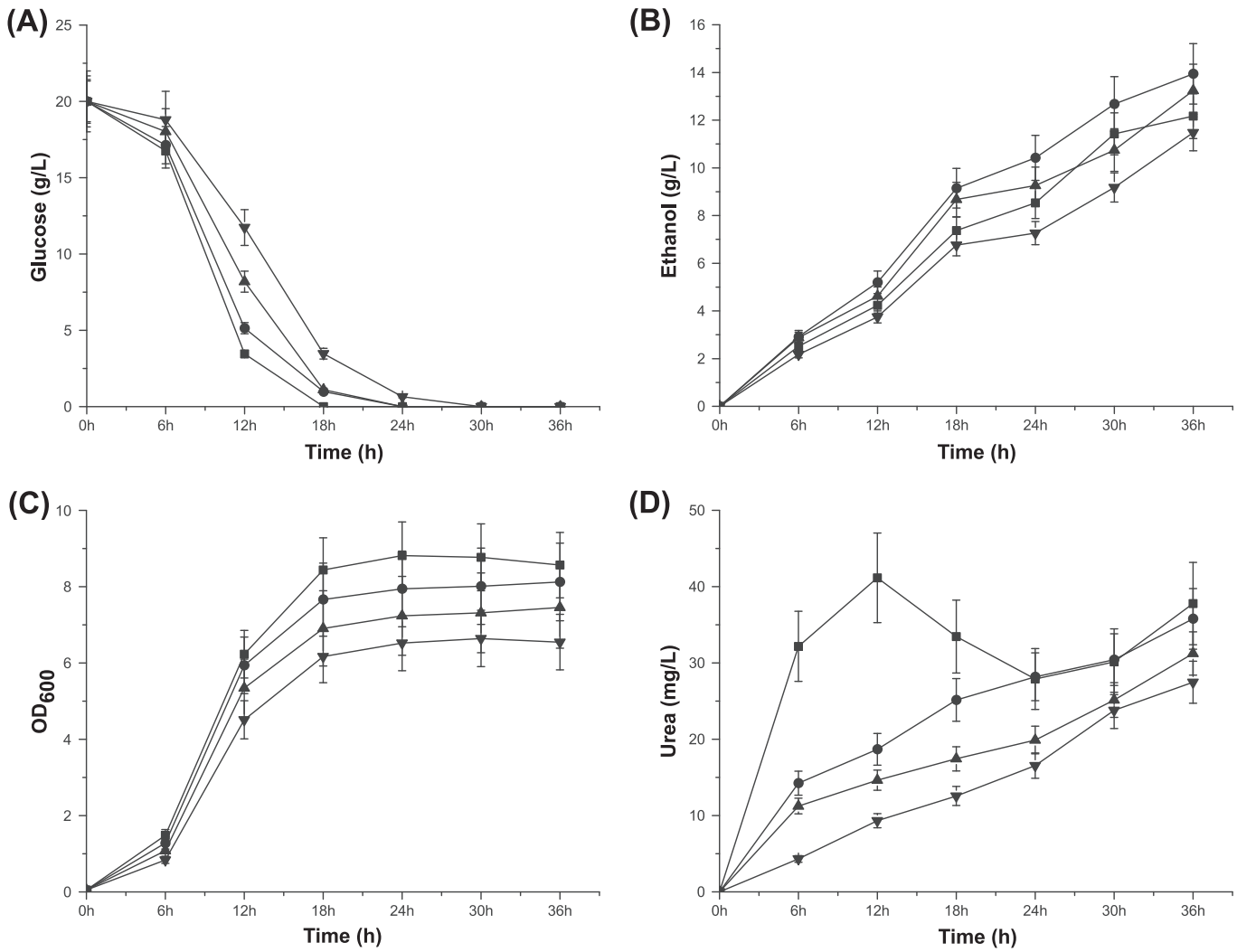

Figure 2. The effect of three amino acids on the fermentation characteristics of yeast. The YNB medium with addition of (10 mmol/L) arginine (■), glutamate (●) or glutamine (▲). The YNB medium without any amino acids was used as the control (▼). The cells were harvested and the concentrations of urea and the nitrogen source were measured every 6 h during the 36 h of fermentation. (A–D) The concentrations of glucose (A), ethanol (B) and urea (D), and the value of OD_{600} (C).

similar effect was obtained by over-expressing the *GLT1* gene (encodes glutamate synthase) to increase the concentration of Glu and Gln in *S. cerevisiae (24)*. The concentration of urea, which is one of the precursors of ethyl carbamate (a potential carcinogen) and one of the intermediate metabolites of Arg, in different media was measured. It was found that urea accumulated rapidly in parallel with the usage of Arg in the initial stage of fermentation. Furthermore, the existence of Gln resulted in an increase in urea concentration compared to the control. This phenomenon is controlled by the regulation of NCR through the phosphorylation of activators (Gln3p and Gat1p) for the transcription of urea metabolic enzymes *(11)*.

Effect of Arg, Glu and Gln on the formation of alcohols and esters

After 25 days of simulated fermentation of rice wine in the model, five types of flavour compounds (83 in total), including alcohols, aldehydes, amino acids, esters and organic acids, were identified (Table 1). The composition of the flavour compounds in the fermentation broth was altered greatly compared with the control. The concentrations of most aldehydes and organic acids were changed only slightly under each set of growth conditions, whereas those of a good many higher alcohols, amino acids and esters were changed significantly (Fig. 3).

Alcohols are the most abundant aromatic compounds in rice wine *(4)*. A total of 13 higher alcohols were identified in rice wine and isoamyl alcohol, phenethyl alcohol, isobutyl alcohol and 1-propanol were the most abundant (Table 1). When Arg, Glu and Gln were added into the medium, the concentration of isoamyl alcohol, 1-propanol, isobutyl alcohol, 1-butanol and 2-butanol increased significantly, but the concentration of the other higher alcohols changed only slightly (Fig. 3). In general, the addition of Glu and Gln promoted the formation of greater amounts of higher alcohols compared with Arg. As shown earlier for grape wine, appropriate higher alcohols (<300 mg/L) can have a desirable influence on the taste *(25)*. As the concentration of total higher alcohols increased from 169.59 to 268.85 mg/L (Table 1), controlling the content of the three amino acids could be an efficient tool for regulating the quality of rice wine.

Table 1. Flavour compounds in different model systems of rice wine (mg/L)

Flavour compound	Control	Arginine	Glutamate	Glutamine
1. Alcohols				
Isoamyl alcohol	66.43	107.31	125.92	117.80
Phenethyl alcohol	54.14	71.13	62.15	68.34
Isobutyl alcohol	24.58	44.72	41.12	39.73
1-Propanol	16.89	24.17	30.18	29.19
1-Butanol[a]	2.16	3.76	4.19	3.97
3-Methyl-2-hexanol	1.56	1.48	1.27	1.17
3-Ethoxy-1-propanol[a]	1.28	1.23	1.37	1.27
2,3-Butanediol	1.34	1.36	1.16	1.26
1-Hexanol	0.63	0.92	0.85	1.04
Benzyl alcohol	0.27	0.28	0.25	0.24
2-Butanol	0.26	0.41	0.36	0.39
1-Octanol	0.03	0.03	0.02	0.02
Dodecanol[a]	0.02	0.02	0.01	0.01
Total	169.59	256.81	268.85	264.42
2. Aldehydes				
Acetaldehyde[a]	4.23	4.13	3.32	3.06
Benzaldehyde	2.13	1.98	1.73	1.45
Furfural[a]	0.48	0.46	0.33	0.35
Caprylaldehyde	0.13	0.15	0.11	0.10
Nonaldehyde	0.08	0.08	0.09	0.07
Capraldehyde	0.07	0.07	0.06	0.06
Total	7.12	6.87	5.64	5.09
3. Amino acids				
Alanine	549.20	564.90	523.60	558.10
Glutamate	372.40	367.40	382.10	392.60
Arginine	347.90	513.00	447.80	423.90
Proline	333.80	318.40	356.10	342.30
Aspartate	286.90	316.90	304.70	293.60
Leucine	269.10	391.30	417.70	439.20
Phenylalanine	218.80	221.70	231.80	210.20
Glycine	218.60	209.50	204.20	199.10
Serine	174.10	187.10	203.40	189.80
Tyrosine	153.20	172.90	155.30	163.80
Valine	149.10	196.40	222.20	218.10
Lysine	137.90	151.10	136.00	143.60
Isoleucine	130.30	182.50	212.60	197.40
Histidine	81.70	94.30	91.30	98.50
Glutamine	79.20	105.30	87.60	93.80
Threonine	76.70	74.50	83.70	88.10
Asparagine	42.10	63.50	50.70	57.10
Tryptophan	34.50	37.10	28.70	32.80
Cystine	23.20	28.40	25.30	24.40
Methionine	9.90	16.60	12.50	13.40
Total	3688.60	4212.80	4177.30	4179.80
4. Esters				
Ethyl lactate	69.26	83.19	98.42	93.14
Ethyl acetate	22.16	24.13	30.74	27.56
Diethyl succinate	10.17	12.57	14.43	15.28
Ethyl butyrate[a]	0.45	0.57	0.62	0.64
γ-Nonanoic lactone	0.25	0.29	0.33	0.35
Ethyl valerate	0.18	0.22	0.26	0.25
Ethyl oleate[a]	0.18	0.20	0.26	0.19
Methyl dodecanoate[a]	0.16	0.14	0.13	0.18
Ethyl octylate	0.16	0.15	0.17	0.14
Phenylethyl acetate	0.13	0.16	0.20	0.18
Ethyl palmitate	0.13	0.11	0.14	0.16
Isoamyl acetate	0.07	0.09	0.12	0.10
Methyl oleate	0.07	0.05	0.08	0.10
Ethyl caproate	0.04	0.05	0.06	0.05
Ethyl isovalerate	0.03	0.03	0.05	0.04
Ethyl dodecanoate[a]	0.02	0.03	0.02	0.02
Ethyl phenylacetate[a]	0.02	0.01	0.02	0.02
Ethyl propionate	0.02	0.02	0.03	0.02
Phenethyl isobutyrate[a]	0.02	0.02	0.01	0.02
Ethyl benzoate[a]	0.01	0.02	0.01	0.01
Methyl proline	1.23	1.11	1.34	1.30
Ethyl alanine	0.74	0.68	0.76	0.75
Ethyl leucine	0.50	0.60	0.62	0.66
Ethyl phenylalanine	0.22	0.23	0.20	0.21
Ethyl threonine	0.17	0.18	0.15	0.18
Ethyl isoleucine	0.15	0.21	0.19	0.22
Ethyl serine	0.14	0.13	0.12	0.13
Ethyl valine	0.12	0.16	0.15	0.16
Total	106.80	125.35	149.63	142.06
5. Organic acids				
Acetic acid	2.86	2.95	2.81	2.72
Lactic acid[a]	1.88	1.96	2.07	2.18
Caproic acid	1.69	1.42	1.53	1.47
Citric acid	1.38	1.48	1.54	1.44
Palmitic acid	0.76	0.86	0.61	0.55
Tartaric acid	0.75	0.73	0.80	0.83
Stearic acid	0.73	0.83	0.63	0.52
Malic acid	0.25	0.26	0.25	0.27
Lauric acid[a]	0.24	0.22	0.23	0.20
Succinic acid	0.18	0.19	0.16	0.17
Oxalic acid	0.14	0.11	0.12	0.13
Pyruvic acid	0.12	0.13	0.14	0.15
Myristic acid	0.11	0.09	0.08	0.12
Fumaric acid[a]	0.10	0.12	0.09	0.11
Caprylic acid	0.07	0.05	0.07	0.06
Heptanoic acid	0.04	0.04	0.03	0.03
Total	11.30	11.44	11.16	10.95

[a]The characteristic flavour compounds in Guyuelongshan rice wine.

Esters comprise the most important group of compounds affecting flavour components in fermented beverages and the vast majority in rice wine are secondary metabolites produced by *S. cerevisiae (7)*. These are formed mainly through the reaction between lipids and acetyl-CoA *(26)*. In total, 28 esters (including eight amino acid esters) were identified, among which ethyl lactate, ethyl acetate and diethyl succinate were the most abundant (Table 1). The concentration of most esters was increased by

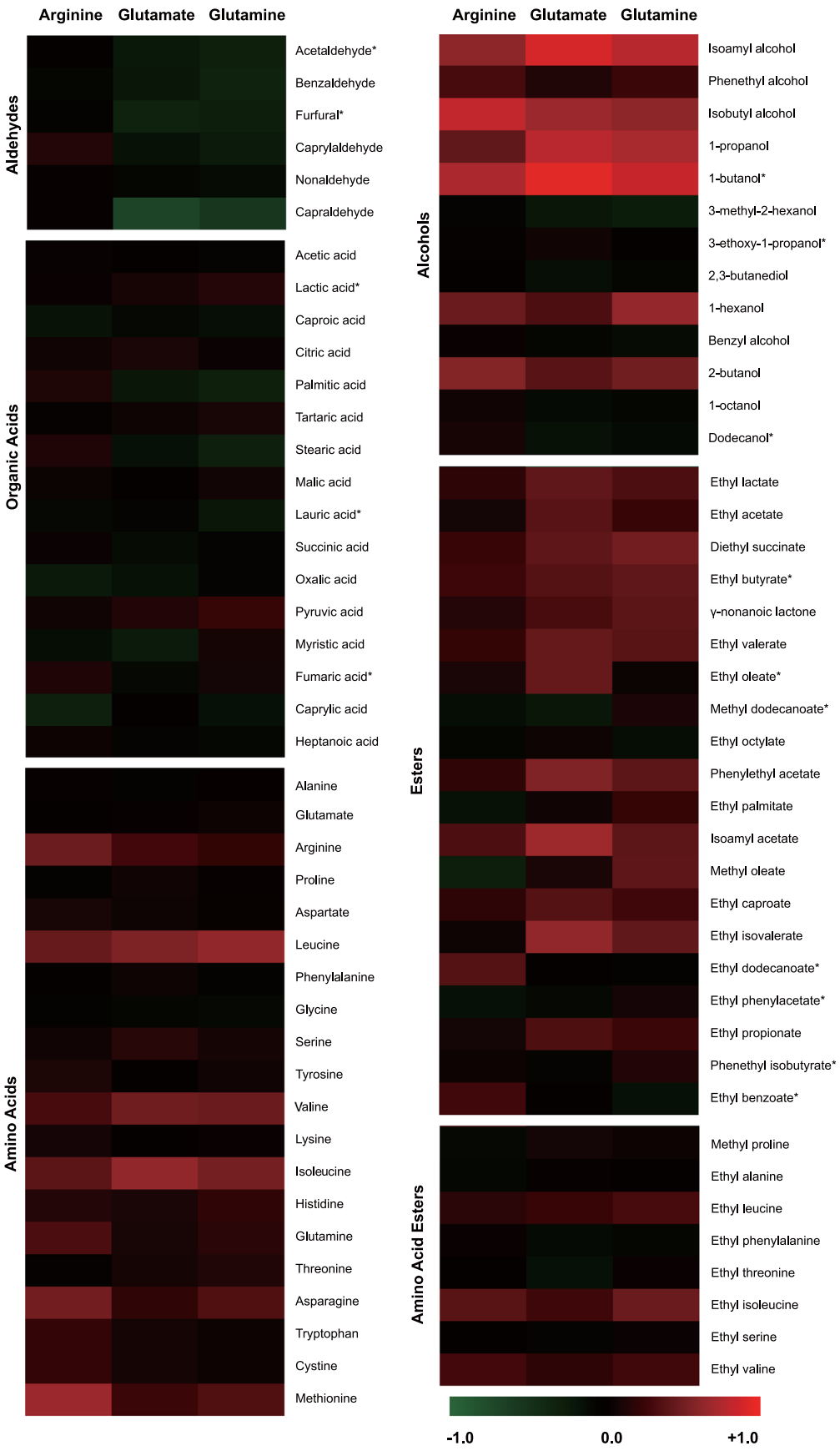

Figure 3. Global view of the effects of the three amino acids on the flavour compounds in rice wine. Black, no significant difference in the amounts of flavour compounds among the samples with the added amino acids; red and green, compounds that were more (red) or less (green) abundant in the samples compared with the control. The intensity of the colours is proportional to the fold increase or decrease, with maximal intensity corresponding to 1.0-fold as represented on the colour scale (bottom).This figure is available in colour online at wileyonlinelibrary.com/journal/brewing.

adding Arg, Glu or Gln into the medium and the effect of Gln was greatest (Fig. 3). Esters are direct contributors to the flowery and fruity aroma of rice wine, and increasing their concentrations by the addition of these amino acids improved the sensory value of rice wine significantly *(7)*. Amino acid esters are found in grape wine but this is the first report of their presence in rice wine *(27)*. The eight amino acid esters identified were methyl proline, ethyl alanine, ethyl leucine, ethyl phenylalanine, ethyl threonine, ethyl isoleucine, ethyl serine and ethyl valine (Table 1). Some amino acid esters are of sensory importance in grape wine and similar compounds in rice wine might contribute to the taste *(28)*.

Effects of Arg, Glu and Gln on the formation of amino acids, aldehydes and organic acids

Amino acids are essential for the growth of yeast and they are the precursors of many important flavour compounds, including isoleucine, leucine and valine *(29)*. Thus, amino acids are important to the final flavour of rice wine *(25)*. Therefore, it was important to examine the profile of the amino acids in order to evaluate the quality of the rice wine *(30)*. All 20 common amino acids were found in the rice wine and Ala, Arg and Leu were the most abundant among them (Table 1). The addition of Arg, Glu or Gln increased the concentration of the branched-chain amino acids (isoleucine, leucine and valine) significantly, by 40.4, 55.4 and 55.8%, respectively (Fig. 3). As these amino acids are involved in the synthesis of branched-chain higher alcohols (such as isoamyl alcohol, isobutyl alcohol, propanol and butanol) through the Ehrlich pathway, these results help to explain the reason for the increased concentrations of the related higher alcohols during fermentation *(31)*.

Compared with the other three types of flavour compounds, although six aldehydes and 16 organic acids were quantified, all changes in the content of these flavour compounds induced by the addition of Arg, Glu and Gln were $<$20% (Fig. 3). The most abundant aldehydes found in the rice wine were acetaldehyde and benzaldehyde (Table 1), which contribute to the apple and citrus scents of rice wine *(32)*. However, some studies have reported that these two aldehydes are also important for the bitter almond aroma in Chinese rice wine *(5)*. In addition, acetic acid was the most abundant organic acid in the rice wine (Table 1). The content of organic acids in rice wine must be appropriate as otherwise there is a notable influence on the taste. There was no obvious influence on the content of aldehydes and on organic acids, which was beneficial for maintaining the quality of the rice wine.

Principal component analysis to identify the main flavour compounds affected by three important amino acids

Many flavour compounds were found to be influenced by Arg, Glu and Gln; thus, it was important to identify the compounds most affected. All changes in the content of flavour compounds were analysed by principal component analysis *(6)*. After data reduction using SPSS 18.0 software, all variables could be explained by three principal components (PC1, PC2 and PC3). All extracted eigenvalues of these three principal components were $>$1.0 (Table 2), which could account for 100% of the differences among the original data *(6)*. Furthermore, evaluation values of PC1, PC2 and PC3 were obtained for medium with added Arg, Glu and Gln, respectively (Table 3). On the basis of the evaluation values of the two most important principal components (PC1 and PC2; Fig. 4), the effects of Arg, Glu and Gln could be divided into two groups.

Table 2. Eigenvalue, percentage of variance and cumulative percentage explained by three principal components in different model systems of rice wine

Principal component	Eigenvalue	Variance (%)	Cumulative (%)
1	40.98	49.37	49.37
2	24.83	29.92	79.29
3	17.19	20.71	100.00

Table 3. The evaluation values of different model systems of rice wine in the three principal components

Sample	PC1	PC2	PC3
Control	−0.26	−1.47	0.19
Arginine	−1.27	0.76	0.22
Glutamate	0.49	0.25	−1.39
Glutamine	1.03	0.46	0.99

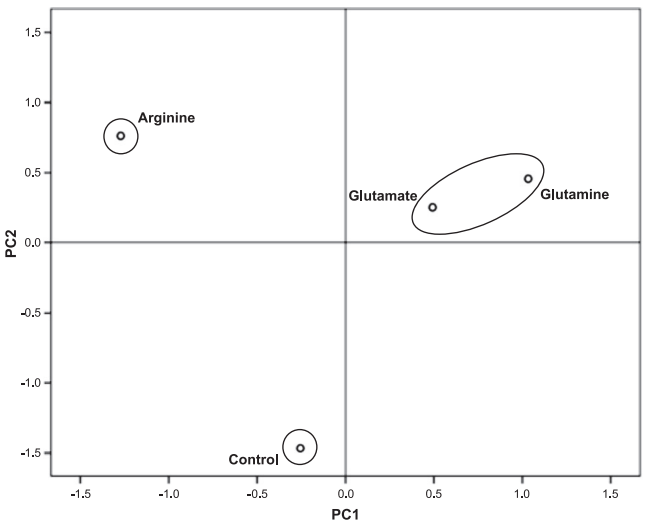

Figure 4. Score plot obtained from the principal component analysis on the basis of the flavour compounds in the different media.

The addition of Glu or Gln had similar effects on the formation of flavour compounds, whereas arginine exhibited a different role.

The principal components with positive evaluation values were selected to examine the effects of Arg, Glu and Gln on flavour compounds *(33)*. PC2 (0.76) and PC3 (0.22) were selected for Arg; PC1 (0.49) and PC2 (0.25) were selected for Glu; PC1 (1.03), PC2 (0.46) and PC3 (0.99) were selected for Gln. Several flavour compounds were identified according to the positive loadings of PC1, PC2 and PC3 (Table 4). The amounts of acetic acid, arginine, aspartate, caprylaldehyde, citric acid, cystine, ethyl benzoate, ethyl dodecanoate and stearic acid were affected significantly by the addition of Arg. The amounts of even more flavour compounds, including 1-butanol, 1-hexanol, 2-butanol, citric acid, ethyl butyrate, ethyl isoleucine, ethyl leucine, ethyl valine, histidine, isoamyl alcohol, isobutyl alcohol, isoleucine, leucine, phenethyl alcohol and valine, were increased by the addition of Glu. Furthermore, the formation of 1-butanol, arginine, citric acid, ethyl caproate, isoamyl acetate, isoamyl alcohol, isobutyl alcohol and isoleucine were associated mainly with the addition of Gln.

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Table 4. The loadings of the flavour compounds in principal components

Sample	PC1	PC2	PC3
1. Arginine (the loadings of PC2 and PC3 > 0.3)			
Acetic acid		0.44	0.50
Arginine		0.89	0.38
Aspartate		0.82	0.57
Caprylaldehyde		0.49	0.31
Citric acid		0.32	0.71
Cystine		0.91	0.41
Ethyl benzoate[a]		0.67	0.51
Ethyl dodecanoate[a]		0.85	0.31
Stearic acid		0.39	0.44
2. Glutamate (the loadings of PC1 and PC2 > 0.3)			
1-Butanol[a]	0.83	0.50	
1-Hexanol	0.69	0.66	
2-Butanol	0.53	0.85	
Citric acid	0.63	0.32	
Ethyl butyrate[a]	0.92	0.40	
Ethyl isoleucine	0.65	0.73	
Ethyl leucine	0.89	0.43	
Ethyl valine	0.59	0.81	
Histidine	0.70	0.67	
Isoamyl alcohol	0.88	0.41	
Isobutyl alcohol	0.56	0.78	
Isoleucine	0.89	0.36	
Leucine	0.87	0.50	
Phenethyl alcohol	0.39	0.91	
Valine	0.92	0.36	
3. Glutamine (the loadings of PC1, PC2 and PC3 > 0.2)			
1-Butanol[a]	0.83	0.50	0.24
Arginine	0.25	0.89	0.38
Citric acid	0.63	0.32	0.71
Ethyl caproate	0.91	0.25	0.34
Isoamyl acetate	0.86	0.21	0.47
Isoamyl alcohol	0.88	0.41	0.24
Isobutyl alcohol	0.56	0.78	0.27
Isoleucine	0.89	0.36	0.28

[a]The characteristic flavour compounds in Guyuelongshan rice wine.

A list of flavour compounds produced in response to the addition of Arg, Glu or Gln to the growth medium is reported in rice wine for the first time. Although the effects of Arg and Glu were investigated earlier *(34)*, their roles in the formation of flavour compounds were not investigated at that time. In this study, it was found that the roles of Arg were extensive and complex *(35)*. The flavour compounds produced in response to the addition of Arg into the growth medium included amino acids, organic acids and esters. During fermentation, Arg was degraded into ornithine and urea and these were converted into Glu, ammonium and carbon dioxide *(36)*. Therefore, the roles of Arg with regard to flavour compounds were dependent primarily on Glu and ammonium. In addition, it was found that Glu and Gln affected mainly the formation of higher alcohols, which is related to the branched-chain amino acids isoleucine, leucine and valine in *S. cerevisiae (37)*. Some researchers have attempted to increase the production of higher alcohols by enhancing the activity of the branched-chain amino acid transaminase in yeast *(38)*. The results obtained in this study showed that the addition of Glu or Gln could achieve the same effect. This procedure was simple and effective and it could reduce the amount of metabolic engineering required on the yeast strains used for the production of rice wine.

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