DOI: 10.26599/FSHW.2023.9250033

Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

Application of SPE-GC/MS in resolution of metabolism pattern of higher alcohols in rat plasma

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ABSTRACT: Higher alcohols are key factors affecting sensory quality and post-drinking comfort of alcoholic beverages. A strategy combining solid-phase extraction and gas chromatography-mass spectrometry (SPE-GC/MS) was established to analyze the metabolism pattern of higher alcohols in rat plasma after gavage of four common alcoholic beverages including huangjiu, baijiu, wine and brandy. Seven milliliter of dichloromethane was determined as the optimal extraction condition, and eight higher alcohols were precisely quantified with detection limits of 1.82-11.65 μg/L, recoveries of 89.07%-110.89% and fine repeatability. The fastest absorption and elimination rates of plasma total higher alcohols were observed in baijiu and huangjiu group, respectively, and the highest peak concentration was found in brandy group. Additionally, the metabolic rate of plasma isoamyl alcohol in huangjiu group was faster than that in wine group at the same intragastric administration dosage. This study may provide potential insight for evaluation of alcoholic beverage quality.

Keywords: Higher alcohols; alcoholic beverages; SPE-GC/MS; pharmacokinetic parameters.

1. Introduction

Higher alcohols, also known as fusel alcohols and fusel oil, are the collective name for high-boiling monohydric alcohols with more than two carbon atoms, mainly including 1-propanol, 1-butanol, isobutanol, isoamyl alcohol, 1-hexanol, and phenethyl alcohol [1]. These alcohols are ubiquitous in different categories of alcoholic beverages such as fermented wine (huangjiu, wine, etc.) and distilled liquors (baijiu, brandy, etc.).

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Received 25 January 2023
Received in revised from 6 March 2023
Accepted 27 March 2023

Huangjiu, namely traditional Chinese rice wine, is popular in southeast China due to its unique aroma, subtle flavor and low alcohol content. In traditional Chinese huangjiu, higher alcohols are mainly derived through the bilateral fermentation of yeast, and its production is facilitated in the case of sluggish yeast growth under a high concentration of fermented mash [2]. In wine, baijiu, and brandy, it is also derived through yeasts metabolism in two ways, including the glucose-substrated anabolic pathway and amino acid-substrated catabolic pathway [3].

It is crucial for a harmonious taste of alcoholic beverages in the ratio between higher alcohols and other constituents such as organic acids and esters [4]. At the proper concentration, higher alcohols are one of the key aroma sources in alcoholic beverages, providing a fine flavor along with a full-bodied taste. However, its disproportion or excess may exert negative effects on the sense of wine body and even be harmful to human health [5]. With stronger toxic and anesthetic effects than ethanol, higher alcohols can cause congestion of nervous system, leading to severe headache and hangover after binge alcoholic beverage consumption [6]. And the toxicity of higher alcohols becomes severer with the increase in molecular weight because of the relative low oxidation rate and long residence time in the body. Xie et al. studied the effects of different contents and ratios of higher alcohols on the degree of intoxication in mice, and found the key role of the isobutanol/isoamyl alcohol ratio therein [7]. Sun et al. proved that higher alcohols, especially isoamyl alcohol and phenethyl alcohol, were key factors in alcoholic beverage-induced ataxia of *Cryprinus carpio* and also one of the major causes for headache and deep drunkenness through compound exposure and omission experiments [8]. Hedlund and Kiessling confirmed the ability of higher alcohols to inhibit ethanol metabolism *in vivo* and their triggering effect of headache and deep drunkenness by rat experiments [9]. Moreover, the excessive higher alcohols may increase the burden on the liver and cause liver damage [10]. Therefore, it is necessary to develop an appropriate detection method to monitor the metabolism of higher alcohols *in vivo*.

Despite extensive attention paid by scientific researchers to post-drinking discomforts caused by a high content or disproportion of higher alcohols, current research concerned about intoxication and hangover mainly focused on the metabolism of ethanol. The degree of intoxication is obvious positively correlated with the blood alcohol concentration (BAC), and different stages of intoxication is divided into three phases according to the change of BAC: excitation phase, ataxia phase and coma phase. Additionally, ethanol metabolism may cause stimulatory effects on the nervous system, liver and other organs [11]. There have been multiple reports on the detection of BAC using headspace-gas chromatography (HS-GC) or headspace-solid phase micro extraction-gas chromatography/mass spectrometry (HS-SPME-GC/MS) [12]. However, the detection methods of higher alcohols are mainly concentrated in samples with relatively simple matrices such as wine and vinegar. The main methods for qualitative and quantitative analysis of higher alcohols in alcoholic beverages were GC-MS combined with pretreatments like SPE, liquid-liquid extraction (LLE), and headspace solid-phase micro-extraction (HS-SPME) [13-15]. Nevertheless, there were few studies on the detection of higher alcohols in plasma, which may be attributed to the low content of higher alcohols in blood and the fact that direct detection in plasma sample could be influenced by the complex blood matrix. As reported, SPE has

been widely used in the separation and extraction of volatile compounds in various samples. It separates target compounds from the sample matrix based on the forces between porous media and target compounds, followed by elution and enrichment with an extraction solvent and nitrogen sweeping. Among the main categories of SPE, ion-exchange SPE is widely used in pretreatment of biological samples and environmental monitoring, and the polymer resins like polystyrene divinylbenzene (PS-DVB) are commonly used fillers [16]. Considering the low content of plasma higher alcohols and the complicated composition of plasma, SPE pretreatment can detach impurities in the matrix that interfere with separation and analysis, enrich low-concentration higher alcohols, and reduce the usage of organic solvents.

In this study, a detection strategy for plasma higher alcohols was established by optimizing extraction condition and method validation, and it was applied to investigate the metabolism pattern (concentration-time curves and pharmacokinetic parameters) of higher alcohols in rat plasma after gavage with four kinds of common alcoholic beverages (including two fermented wine, huangjiu and wine; two distilled spirits, baijiu and brandy). The findings offered a basis for assessing the metabolic differences of higher alcohols *in vivo* and a new perspective for evaluating the quality of alcoholic beverages.

2. Materials and methods

2.1 Reagents and samples

Standards of higher alcohols including 1-propanol, isobutanol, 1-butanol, isoamyl alcohol, 1-pentanol, 1-hexanol, 1-heptanol, phenethyl alcohol were obtained from Sigma Aldrich (Shanghai, China). Dichloromethane, methanol and other solvents of chromatographic grade were purchased from Aladdin Bio-Chem Technology (Shanghai, China). The Envi-Chrom P PS-DVB (polystyrene-divinylbenzene, 250 mg) cartridge were purchased from Sigma Aldrich (Shanghai, China).

A total of four alcoholic beverages were purchased from local market in Wuxi, China. Two fermented wine samples (huangjiu and wine, 14% vol ethanol content) and two distilled spirits (baijiu and brandy, 52% vol ethanol content) were used in the experiment to investigate metabolism pattern of higher alcohols *in vivo*. The fermented wine samples used in the method establishment experiment were the same as those in the metabolism pattern experiment. The type and content of higher alcohols in the four alcoholic beverages was analyzed by HS-SPME combined with GC-MS according to the reports of Tao and Tang et al [15, 17], and the results were shown in Figure 2.

2.2 Animals experiments

Specific pathogen free (SPF) wild-type Wistar rats (male, aged 7-8 week and weighed 280-300 g) were obtained from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The rats (n = 348) were housed in a controlled environment at (22 ± 2) ℃ with (40-60)% humidity and a 12-h light/dark cycle. Rats had access to water and fodder *ad libitum*, and their body weight (bw) and general health were closely monitored. The experimental protocol complied with the license of Animal Management and Use Committee of Jiangnan University (license ID: JN. No 20211015W1021031).

After a one-week acclimatization period, rats were randomly divided into six treatment groups for two experiments including the method establishment and the metabolism pattern study. Before the treatment began, all the rats were orally administrated with 10 mL/kg bw of saline solution daily for two days to adapt to the gavage operation. For the method establishment experiment, forty-eight rats were divided into the control group (n = 36) and the fermented wine group (n = 12). After a six-hour fasting period, the two groups of rats were respectively administered with saline solution and huangjiu at a single dose of 10 mL/kg bw. Then, the rats were sacrificed at 60 min after gavage and blood sample were collected. With regard to the metabolism pattern study, the rest rats were equally divided into four groups, including huangjiu group, wine group, baijiu group and brandy group, with 75 rats per group. After the same fasting period of six hours, all experimental groups were given alcoholic beverage solutions of 10 mL/kg bw by gavage. Five rats in each group were sacrificed at corresponding time point (15, 30, 45, 60, 75, 90, 105, 120, 150, 180, 240, 300, 360, 480 and 600 min) and rat blood were collected through abdominal aorta after isoflurane anesthesia (2 vol%) in an inhalation anesthetic apparatus (Figure 1), then the rats were sacrificed through cervical dislocation. The blood sample was injected into a blood collection vessel pre-added with 1.92 mg Na_2-Ethylene diamine tetraacetic acid (Na_2EDTA), and then centrifuged at 3000 g for 10 min to obtain the upper plasma. The plasma sample were stored at -80 ℃ for analysis. The dose of each higher alcohol of four alcoholic beverages were calculated according to the data in Figure 2 and the body weight of rats and results were shown in Table S1.

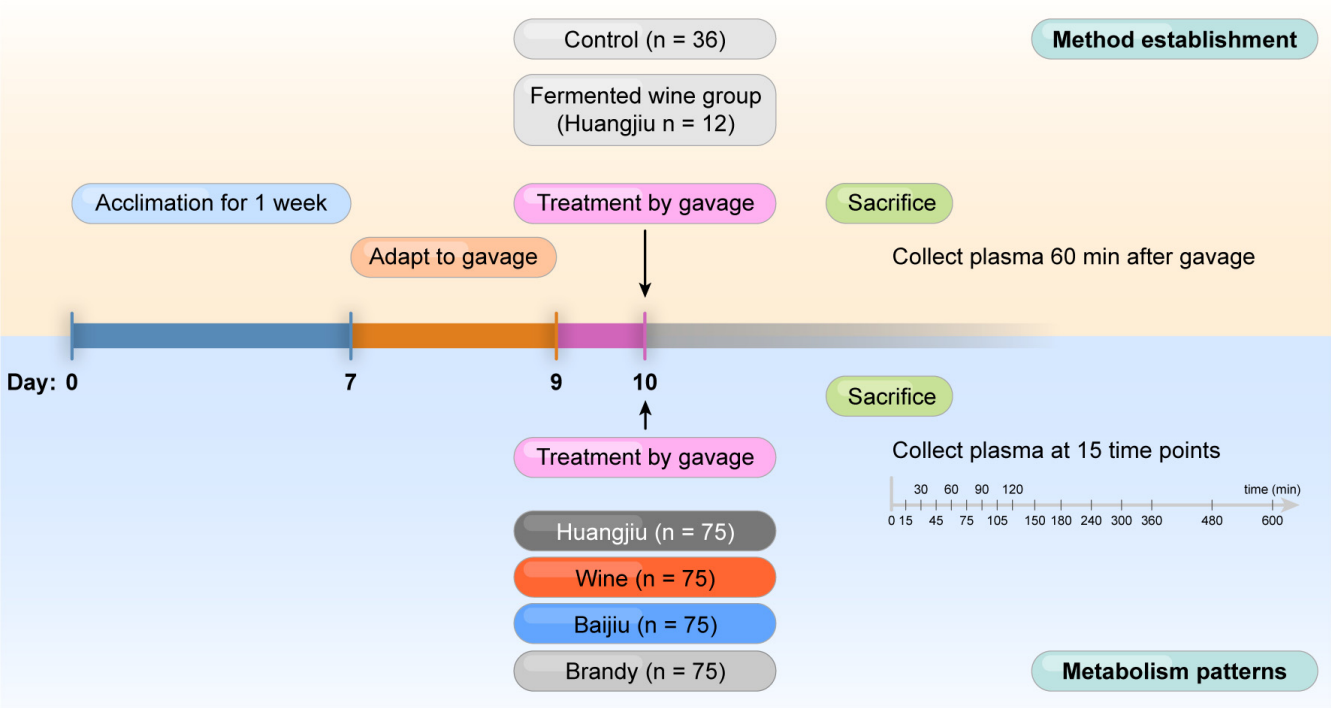

Fig. 1 The grouping and treatment of rats.

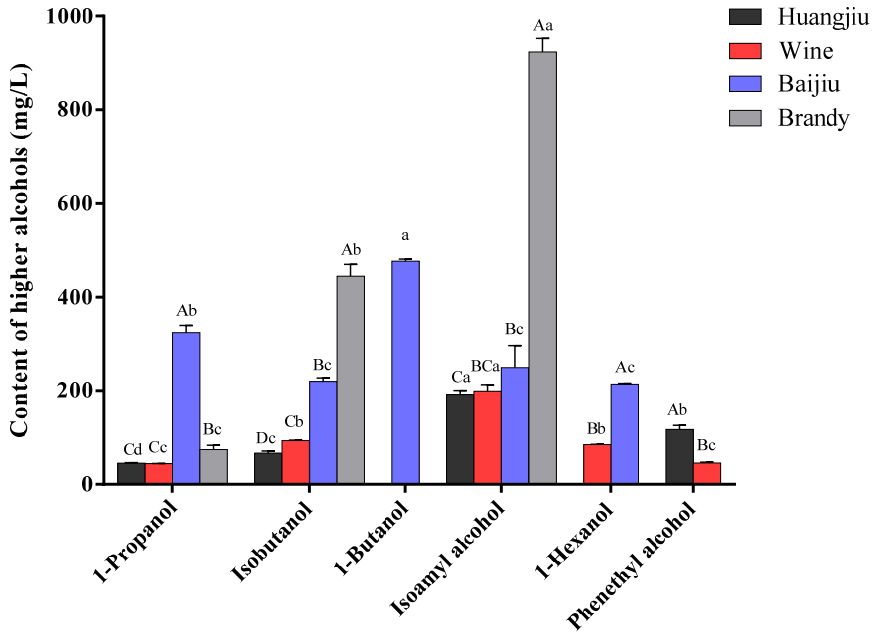

Fig. 2 Composition and content of higher alcohols in different alcoholic beverages. Different uppercase letters (A, B, C, D) represent significant ($P < 0.05$) differences between different alcoholic beverages (for the same higher alcohol), and different lowercase letters (a, b, c) represent significant ($P < 0.05$) differences between different higher alcohols (in the same alcoholic beverage).

2.3 Optimization of SPE procedure

The SPE procedure was slightly modified according to previous report [18]. Plasma sample was filtered through 0.80 μm aqueous membrane, then 3 mL of treated plasma sample was added to the SPE cartridge (pre-activated with 6 mL methanol and 6 mL ultrapure water in turn), followed by washing with 6 mL of ultrapure water and 7 mL of dichloromethane to elute the higher alcohols. The eluate was dried with anhydrous Na_2SO_4 then concentrated to 0.2 mL through nitrogen sweeping, and finally the sample (1 μL) was injected into GC-MS for analysis.

The SPE procedure was optimized by two parameters, namely the type and volume of elution solvents. Four kinds of elution solvents were selected, containing dichloromethane, 1-pentane, ethyl ether and trichloromethane. Four different volumes of elution solvents were adopted, including 3, 5, 7 and 9 mL. The plasma sample of twenty-four rats in control group were used as matrix, and the concentration was set to 250 μg/L (the preparation was described in 2.4.3) with 1-propanol, isobutanol, isoamyl alcohol, phenylethanol, etc., respectively. Three replications for each elution solvent and volume of dichloromethane were conducted and the recoveries under each parameter were calculated through standard curve.

2.4 Identification and quantitation of higher alcohols

2.4.1 GC-MS analysis

GC-MS analysis was carried out using a Thermo Trace1300 gas chromatograph coupled to a Thermo ISQ7000 mass detector with DB-wax chromatographic column (30 m × 0.25 mm, 0.25 μm, Thermo Fisher

Scientific, Massachusetts, USA). The inlet temperature was maintained at 250 ℃, and splitless injection was conducted. The column temperature was held at 40 ℃ for 2 min, then increased to 230 ℃ at 7 ℃/min and held for a further 10 min. The carrier gas was helium (>99.999%) at a flow rate of 1.0 mL/min. In addition, the solvent delay time was set at 4.2 min and the injection volume was 1 μL.

The MS conditions were as follows: Ionization mode was EI and ionization energy was 70 EV. The transfer line to the mass spectrometer was heated to 240 ℃ and ion source temperature was 240 ℃. Ion scanning module was selected for qualitative and quantitative analysis.

2.4.2 Identification and quantitation of target compounds

Peak identification was based on comparison of retention times (RTs) and mass spectra fragments to standard compounds. Selected ion monitoring (SIM) mode was applied for quantitative analysis with target masses of m/z 59 and 60 for 1-propanol, m/z 41, 43 and 74 for isobutanol, m/z 41, 43 and 56 for 1-butanol, m/z 42, 55 and 75 for isoamyl alcohol, m/z 39, 42 and 55 for 1-pentanol, m/z 43, 56 and 69 for 1-hexanol, m/z 43, 70 and 83 for 1-heptanol, and m/z 65, 91 and 122 for phenethyl alcohol. All target masses for each compound were used to establish calibration for method validation and quantitation analysis of plasma sample.

Stock solutions of higher alcohols were prepared by dissolving 8 standard references in anhydrous ethanol at a concentration of 10 mg/mL and stored at -20 ℃ in seal. The stock solution was diluted to 200, 50, 25, 10, 5, 2 and 1 mg/L. Seven calibration solutions were prepared by adding 30 μL stock solution with different concentrations to the plasma sample obtained from control group, and the concentrations of working solution were 2000, 500, 250, 100, 50, 20 and 10 μg/L. The working solutions were extracted and analyzed using the same parameters as rat plasma sample.

2.4.3 Method validation

During the repeatability test, higher alcohols (250 μg/L) were chosen as the test concentration (30 μL of 25 mg/L higher alcohol working solution was spiked to 3 mL of blank rat plasma). The test was divided into intra-day and inter-day repeatability, and results were expressed in relative standard deviation (RSD). The determination of limit of detection (LOD) and limit of quantification (LOQ) referred to the reports of Zhou et al. [19], and quantitative analysis was performed using the above standard curve.

After obtaining the plasma sample of rats in fermented wine group, corresponding higher alcohol working solutions (30 μL) with concentrations of 5, 10 and 20 mg/L were spiked to 3 mL plasma sample to increase the concentration by 50, 100 and 200 μg/L. Recoveries were determined at these three different concentrations by comparing the detection result of spiked plasma sample with added concentration, and three replications were implemented. The retention time, linearity after optimization and relevant parameters of method validation were shown in Table 1.

Table 1 The retention time, linearity and method validation parameters of of the SPE-GC/MS method on detecting higher alcohols in plasma. Different lowercase letters (a, b, c) represent significant ($P < 0.05$) differences between different higher alcohols with the same addition.

Higher alcohols	Retention time (min)	Calibration curves		R^2	Repeatability (%)		LOD (μg/L)	LOQ (μg/L)	Recoveries (%)		
		Slope	Intercept		Intra-day RSD	Inter-day RSD			50 μg/L	100 μg/L	200 μg/L
1-Propanol	4.71	13346	$5.888*10^6$	0.9942	5.65	5.22	5.20	17.32	101.05±12.38a	107.13±2.98ab	109.51±8.34a
Isobutanol	5.60	3247	-15591	0.9917	5.58	6.69	3.43	11.44	89.07±18.16a	102.27±6.23b	105.77±6.04ab
1-Butanol	6.62	36739	$1.161*10^6$	0.9956	3.15	3.58	2.23	7.45	100.10±5.72a	110.89±8.21a	99.85±1.61b
Isoamyl alcohol	7.85	210107	$9.069*10^7$	0.9909	4.26	6.54	4.26	14.20	95.46±12.99a	101.90±6.14b	95.87±6.75c
1-Pentanol	8.70	85096	$4.669*10^7$	0.9927	2.20	1.54	8.61	28.70	104.21±15.49a	99.29±1.42b	105.80±8.62ab
1-Hexanol	10.71	115940	$2.202*10^6$	0.9914	6.81	8.77	4.92	16.40	104.93±0.27a	99.90±0.82b	101.23±0.07ab
1-Heptanol	12.63	66180	$5.398*10^7$	0.9952	5.43	9.64	11.65	38.82	108.05±6.98a	103.40±0.01ab	104.51±0.75ab
Phenethyl alcohol	20.25	286573	$4.426*10^6$	0.9905	3.97	3.67	1.82	6.08	96.05±5.25a	102.14±3.11b	99.76±2.06b

2.5 Determination of ethanol and higher alcohols in rat plasma after gavage with different alcoholic beverages

The concentration of rat plasma ethanol of huangjiu, baijiu, wine and brandy group were analyzed through a HS-GC/MS strategy referring to the report of Cordell et al. [12]. The types and content of plasma higher alcohols of the four alcoholic beverage groups were determined by the optimized SPE-GC/MS strategy described in 2.3 and 2.4, and the pharmacokinetic parameters of plasma ethanol and higher alcohols were analyzed by DAS 3.0.

2.6 Statistical analysis

The results were reported as mean ± standard deviation (SD). An analysis of variation (ANOVA) was performed to determine the significance of differences using IBM SPSS Statistics Version 19.0 (IBM Corp., Armonk, NY) with a significance level of 0.05. The pharmacokinetic parameters were analyzed by DAS 3.0 software.

3. Results

3.1 Composition and content of higher alcohols in different alcoholic beverages

The composition and content of higher alcohols in four different alcoholic beverages (huangjiu, wine, baijiu and brandy) were determined using HS-SPME-GC/MS, as shown in Figure 2. A total of six higher alcohols (five aliphatic alcohols and one aromatic alcohol) were detected with concentrations ranging from 44.34 to 923.46 mg/L, and 1-propanol, isobutanol and isoamyl alcohol were observed in all four alcoholic beverages. In huangjiu and wine, the total content of higher alcohols was similar (421.70 and 468.36 mg/L, respectively) and both were dominated by isoamyl alcohol (accounting for about 45% of the total higher alcohols). Four common higher alcohols, namely, 1-propanol, isobutanol, isoamyl alcohol, and phenethyl alcohol, were found in both fermented alcoholic beverages, but 1-hexanol was only present in wine. As for baijiu and brandy, the varieties of higher alcohols were more abundant in baijiu than those in brandy (additionally, 1-butanol and 1-hexanol in baijiu), but the total amount of higher alcohols was close in the two distilled spirits (about 1450 mg/ L), which was three times more than that of the fermented wines. Furthermore, 1-butanol and isoamyl alcohol were the largest portion of higher alcohols in baijiu and brandy, respectively, accounting for 32% and 64% each.

3.2 Optimization of SPE procedure

Regarding the SPE process for the recovery of higher alcohols in plasma, in order to find the optimum conditions, the type and volume of elution solvents were optimized. A total of four kinds of elution solvents (dichloromethane, 1-pentane, ethyl ether and trichloromethane) and four gradient concentration of elution solvent (3, 5, 7, and 9 mL) were investigated, and the recoveries of eight higher alcohols in plasma were measured under different elution conditions (Figure 3).

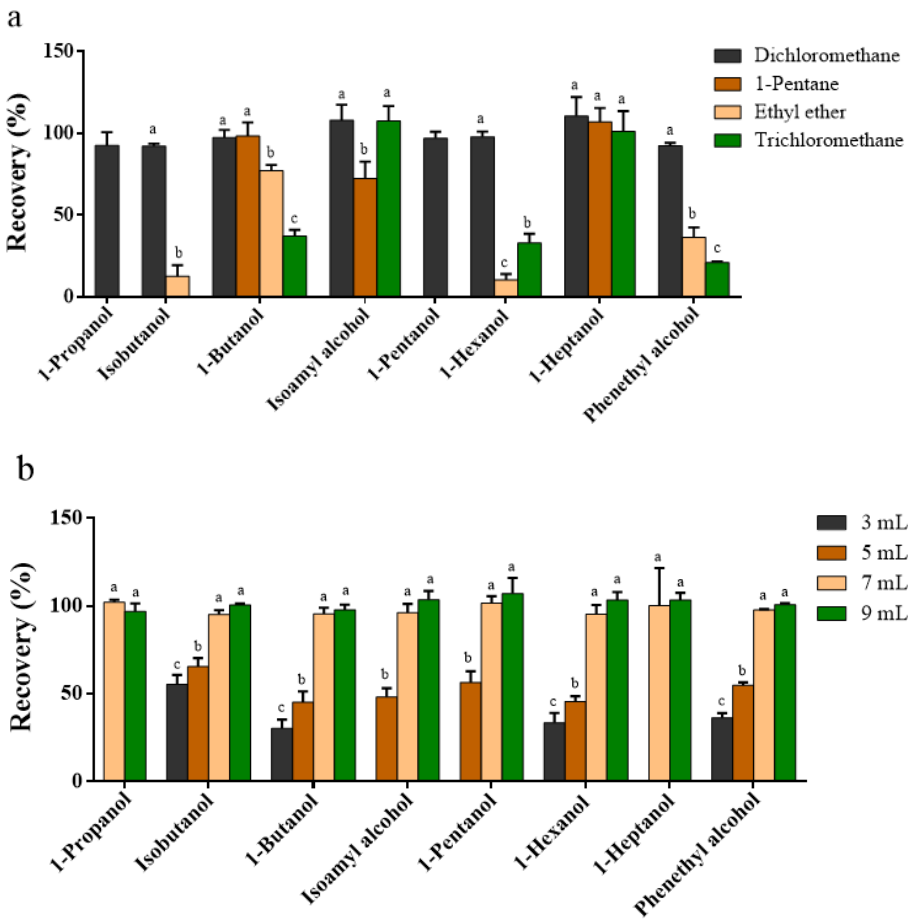

Fig. 3 Effects of different elution solvents (a) and volume of dichloromethane (b) on the extraction recoveries (%). Different lowercase letters (a, b, c) represent significant ($P < 0.05$) differences between different elution solvents or volumes of dichloromethane (for the same higher alcohol).

All the eight higher alcohols were extracted by dichloromethane, and the recoveries ranged from 92.59% to 110.63%. Only three out of eight higher alcohols (1-butanol, isoamyl alcohol and 1-heptanol) were eluted by 1-pentane, and the recovery rate of isoamyl alcohol was significantly ($P < 0.05$) lower than that eluted by dichloromethane (72.58% versus 108.24%). The 1-propanol, isoamyl alcohol, 1-pentanol and phenethyl alcohol could not be eluted by ethyl ether, and recoveries of isobutanol, 1-butanol, isoamyl alcohol and 1-hexanol in ethyl ether was all lower than those in dichloromethane. Isoamyl alcohol and 1-heptanol were extracted by trichloromethane from the rat plasma, although the extraction efficiencies of trichloromethane on 1-butanol, 1-hexanol and phenethyl alcohol were relatively low (< 37.35%). The results above indicated that dichloromethane was the proper elution solvent.

As for the optimization of elution solvent volume, four gradients of dichloromethane were adopted, and the recoveries of higher alcohols were improved by the increase of the volume of elution solvent. When the solvent volume was three milliliters, 1-propanol, isoamyl alcohol, 1-pentanol, and 1-heptanol were not detected, and the recoveries of four higher alcohols detected were lower than 55.34%. When using five milliliters as the solvent volume, two higher alcohols (1-propanol and 1-heptanol) were not observed and the recoveries of other six higher alcohols were lower than 65.38%. when the solvent volume was increased to

seven milliliters, all eight higher alcohols were detected with recoveries of 91.74%-102.10%. With the further increase in solvent volume, the elution of eight higher alcohols achieved equilibrium, and their recoveries did not vary. Therefore, seven milliliters of dichloromethane were determined as the optimum extraction condition.

3.3 Method validation

3.3.1 Linearity, repeatability and sensitivity

The developed SPE-GC/MS method for the analysis of higher alcohols in rat plasma was validated using International Conference of Harmonization (ICH) guidelines [20]. The retention time, linearity (slopes, intercepts and correlation coefficients R^2 of calibration curves), repeatability (intra-day RSD% and inter-day RSD%), accuracy and precision (LOD and LOQ) of the detection method were calculated as shown in Table 1 and the chromatogram were displayed in Figure S1.

The detection of eight higher alcohols in rat plasma was completed within 25 min, and the correlation coefficients (R^2) were found to be > 0.99 for all compounds calculated by seven-point calibration curves using external standard, indicating that the method can achieve rapid analysis with high degree of correlation and good linearity. The LOD and LOQ of higher alcohols analyzed by the SPE-GC/MS strategy were in the ranges of (1.82-11.65) μg/L and (6.08-38.82) μg/L, respectively, suggesting that this detection method was suitable for qualitative and quantitative analysis at low levels. The intra-day and inter-day precision RSD% of all higher alcohols in rat plasma were in the ranges of 2.20%-6.81% and 1.54%-9.64%, respectively, which were all within the permissible limits and reflected the precision of the method.

3.3.2 Recoveries

The recoveries of the developed method were assessed by the complete analysis in triplicate of eight higher alcohol standards spiked at various concentration levels (50, 100 and 200 μg/L) in rat plasma sample prior to the SPE procedure for higher alcohols. The recoveries for each higher alcohol in plasma were shown in Table 1 and quantitative analysis data were provided in appendix Table S2.

Four higher alcohols, 1-propanol, 1-butanol, 1-pentanol and phenethyl alcohol, were detected in rat plasma after gavage for 60 min with concentrations ranged from 121.64 to 220.99 μg/L. The other four higher alcohols were also found after spiking with higher alcohols standards. The recoveries of higher alcohols were all good throughout the range of concentrations tested, which all exceeded 89%. All the above results indicated that the developed method provided adequate linearity, repeatability, sensitivity, and recoveries.

3.4 Concentration-time curves of plasma ethanol and higher alcohol

The developed SPE-GC/MS strategy was used to monitor the composition and content of higher alcohols in rat plasma after gavage of four alcoholic beverages (huangjiu, baijiu, wine, and brandy), and the fate of higher alcohols in blood was depicted by a nonlinear fitting graph plotting their concentration on the y-axis and the time of plasma sampling on the x-axis. In addition, the concentration-time (C-T) profiles of plasma ethanol and total higher alcohols were provided (Figure 4).

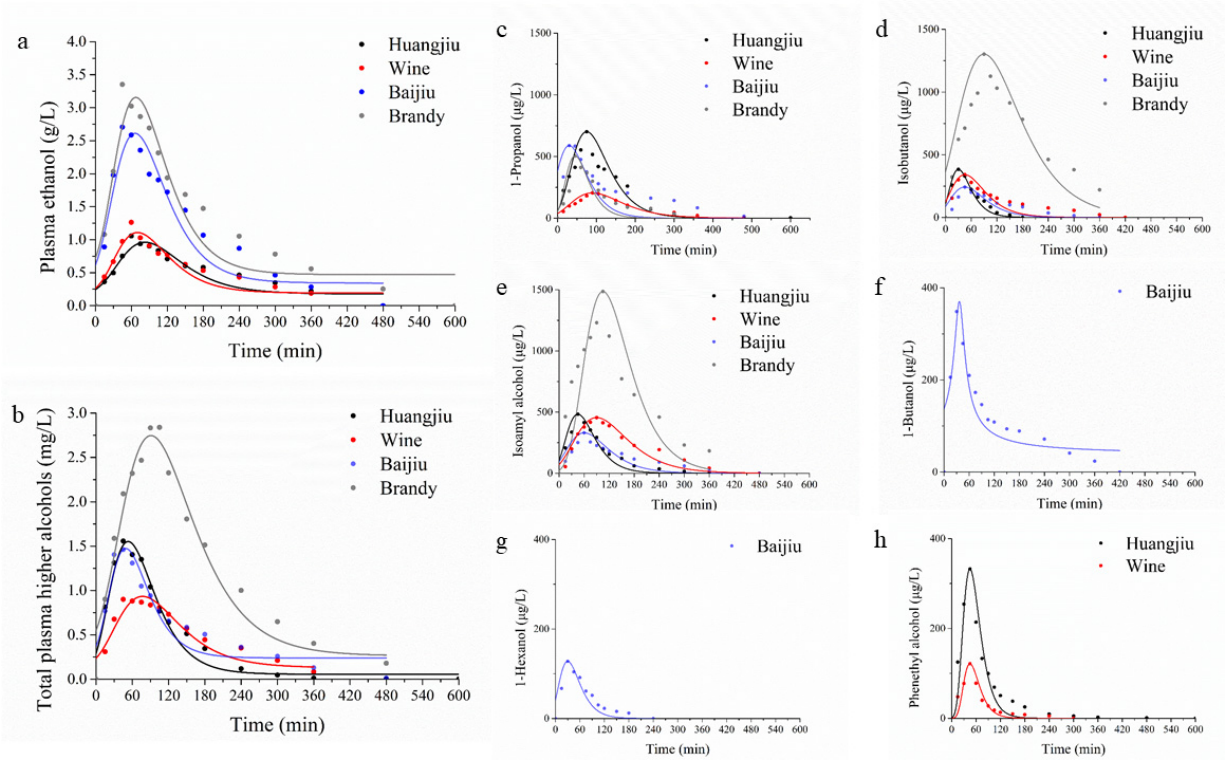

Fig. 4 Concentration-time profiles of ethanol, total higher alcohols and each higher alcohol in rat plasma after gavage with different alcoholic beverages. (a) plasma ethanol, (b) plasma total higher alcohols, (c) 1-propanol, (d) isobutanol, (e) isoamyl alcohol, (f) 1-butanol, (g) 1-hexanol, (h) phenethyl alcohol.

Plasma ethanol in the two fermented wine groups reached the peak at about 60 min and the peak concentration were approximately 1 g/L. For the two distilled spirit groups, the peak time of plasma ethanol were slightly earlier than that of the fermented wine groups at about 45 min, and the maximum concentration were around 3 g/L. The total higher alcohols also reached the highest levels at (45-60) min although the total concentration was only about one-thousandth of that of ethanol in rat plasma (0.9-2.8) mg/L. It was worth noting that the concentration of total higher alcohols in the plasma of rats after oral administration of brandy was remarkably high, while that of wine was the lowest, and that of baijiu and huangjiu was similar. Besides, the elimination of plasma ethanol and total higher alcohols may last for several hours and no obvious difference was found.

In the four alcoholic beverage groups, the variation pattern of each higher alcohol over time in rat plasma were similar to that of ethanol and total higher alcohols, and all the C-T profiles consisted of two phases, that is, a rapid rise to peak (absorption phase) followed by a steady decline until complete clearance (elimination phase). A total of six higher alcohols were identified in the rat plasma after gavage with huangjiu, baijiu, wine and brandy, and 1-propanol, isobutanol, and isoamyl alcohol were found in all the four alcoholic beverage groups with peak concentrations ranging from 200 to 1500 μg/L. The peak time of plasma 1-propanol in distilled spirit groups were faster than that in fermented wine groups, and the highest peak concentration was found in huangjiu group, while the lowest was observed in the wine group together with the longest time to peak. Large amounts of isobutanol and isoamyl alcohol were detected in the plasma of the brandy-gavage rats,

and their peak time (over 90 min) and elimination time (over six hours) were relatively late, which distinctly distinguished it from the other alcoholic beverage groups. In addition, phenethyl alcohol was detected in the plasma of both huangjiu and wine groups, both peaked at about 45 min (332.27 and 121.46 μg/L), but completely diminished at about 240 and 180 min, respectively. Only a small quantity of 1-butanol and 1-hexanol were detected in rat plasma of baijiu group, and the peak concentrations were less than 350 μg/L.

3.5 Pharmacokinetics of plasma ethanol and higher alcohols

The C-T data of plasma ethanol and higher alcohols were estimated using DAS 3.0 for the following pharmacokinetic parameters, including peak concentration (C_{max}), time to peak concentration (T_{max}), elimination rate constant (K), absorption rate constant (Ka), area under the C-T curves (AUC) and mean residence time (MRT), as shown in Table 2. In terms of plasma ethanol, the T_{max} ranged from 49 to 75 min in the four groups of rats, with an increasing order of baijiu < wine < brandy < huangjiu, and the C_{max} was 2 - 3 fold higher after consumption of distilled spirits than that of fermented wines. Regarding total higher alcohols in plasma, huangjiu group showed the shortest T_{max}, followed by baijiu and then by wine and brandy, while the largest and the smallest C_{max} were observed in brandy group and wine group, respectively, and the baijiu group and huangjiu group were in the middle. In addition, the fastest absorption and elimination rates of plasma total higher alcohols were found in baijiu and huangjiu group, respectively. After consumption of four alcoholic beverages, both T_{max} and C_{max} of total higher alcohols were lower than those of ethanol in rat plasma. In addition, relative higher Ka and K values along with shorter MRT were found in plasma higher alcohols than those in plasma ethanol.

With regard to pharmacokinetic parameters of individual higher alcohols, there were remarkable difference between the same higher alcohol in different alcoholic beverage groups or different higher alcohols in the same alcoholic beverage group. Specifically, under no significant ($P > 0.05$) differences in the initial gavage dosage, a shorter T_{max}, a greater C_{max}, and a three-times higher AUC of plasma 1-propanol were detected in the huangjiu group compared with those of the wine group. Likewise, there was no significant differences ($P > 0.05$) in the initial gavage dosage of isoamyl alcohol between the huangjiu and wine groups, but the former demonstrated a shorter T_{max}, higher K and Ka values, a smaller AUC and a shorter MRT in plasma. Additionally, the C_{max} and K of isobutanol were higher and the MRT was shorter in the huangjiu group than those in the wine group, while there were no significant differences ($P > 0.05$) in T_{max}, Ka, K, and MRT of phenethyl alcohol between the two groups. As for the two distilled spirit groups, baijiu administration resulted in a higher Ka and AUC, a lower K, and a longer MRT of plasma 1-propanol compared with that in brandy. Notably, the AUC of plasma isobutanol in baijiu group was only about one tenth of that in brandy group, followed by higher K and Ka values as well as a shorter MRT. Moreover, the Ka of plasma isoamyl alcohol in baijiu group was significantly ($P < 0.05$) higher than that in brandy group, but the AUC was smaller and MRT was shorter.

Table 2 Pharmacokinetic parameters of plasma ethanol, total higher alcohols, and individual higher alcohol C-T profiles of each group. Different lowercase letters (a, b, c) represent significant ($P < 0.05$) differences among target compounds in each column.

Target compound	Alcoholic beverages	T_{max} (min)	C_{max} (μg/L)	Ka (10^{-2} min^{-1})	K (10^{-2} min^{-1})	AUC (mg/L*min)	MRT (min)
Ethanol*	Huangjiu	74.56±9.79bc	0.84±0.11b	2.80±0.52cd	0.51±0.04f	238.95±13.45c	232.73±20.86a
	Wine	55.42±5.17d	1.06±0.03b	4.10±0.65cd	0.59±0.02f	254.88±13.72c	194.24±4.49b
	Baijiu	48.92±2.34de	2.34±0.02a	4.50±0.44c	0.72±0.05ef	463.44±16.02b	161.92±8.27cd
	Brandy	62.48±4.09cd	2.58±0.30a	3.40±0.49cd	0.59±0.05f	637.48±95.48a	200.07±10.42b
Total higher alcohols	Huangjiu	32.49±3.08ef	1292.54±37.14e	6.10±0.80bc	1.30±0.06c	151.50±4.97h	93.49±5.21f
	Wine	54.29±1.26d	823.42±13.02f	3.50±0.19cd	0.80±0.02e	158.95±3.11h	153.33±1.66cd
	Baijiu	32.50±5.13ef	1267.17±78.35e	7.60±2.00a	0.91±0.08de	187.66±13.53g	123.86±5.11e
	Brandy	59.34±9.11cd	2613.19±165.03c	3.40±0.84cd	0.72±0.01ef	556.04±14.41d	169.83±6.85c
1-Propanol	Huangjiu	65.28±7.26cd	701.36±24.46fg	2.40±0.51d	0.93±0.13de	95.37±15.08i	151.84±14.85cd
	Wine	87.76±12.93ab	205.13±3.17k	1.60±0.35d	0.82±0.09e	32.94±3.64lm	188.49±23.67bc
	Baijiu	38.84±2.59e	588.39±20.89h	6.50±1.10b	0.68±0.09ef	95.76±10.69i	163.42±15.74c
	Brandy	56.27±3.76d	504.53±18.35i	2.80±0.27cd	1.00±0.07de	53.23±1.28k	134.04±7.67de
Isobutanol	Huangjiu	25.58±0.52f	383.59±17.24j	5.60±0.29bc	2.60±0.17a	16.83±0.50n	56.67±2.02g
	Wine	31.54±7.91ef	341.38±7.7j	9.10±3.80a	0.77±0.06ef	49.21±2.47kl	143.18±6.21d
	Baijiu	48.52±2.38de	242.86±3.45k	3.60±0.21cd	1.05±0.13d	25.62±3.45mn	124.19±12.26de
	Brandy	78.56±0.64b	1302.60±57.92e	2.10±0.08d	0.67±0.02ef	238.28±8.57f	194.55±3.78b
Isoamyl alcohol	Huangjiu	39.84±2.43e	482.47±13.8i	4.20±0.39cd	1.40±0.09c	40.84±2.37l	97.15±5.24f
	Wine	66.13±2.95c	455.1±14.35ij	2.60±0.19d	0.78±0.02e	65.15±3.72j	167.18±4.76c
	Baijiu	53.90±3.89d	330.75±15.61j	3.60±0.36cd	0.78±0.07e	48.47±1.32kl	156.36±11.32cd
	Brandy	89.38±12.79a	1485.37±36.15d	1.70±0.37d	0.73±0.00ef	275.71±5.00e	199.16±17.11b
Phenethyl alcohol	Huangjiu	37.96±2.43e	332.27±5.90j	4.50±0.39c	1.40±0.13c	28.42±2.31m	96.16±7.11f
	Wine	38.71±9.17e	121.46±9.76l	4.20±1.40cd	1.60±0.24b	8.90±0.76n	88.59±16.61fg
1-Butanol	Baijiu	38.59±3.64e	348.32±17.71j	6.70±0.81b	0.68±0.10ef	47.10±3.97kl	164.12±19.42c
1-Hexanol	Baijiu	26.18±3.24f	127.80±8.45l	7.20±1.70b	1.70±0.34b	7.84±0.16n	71.93±10.28g

* The concentration units of C_{max} and AUC for plasma ethanol were g/L and g/L*min.

4. Discussion

4.1 Higher alcohols in alcoholic beverages

Higher alcohols are by-products of yeast fermentation in alcoholic beverages, approximately 65% of which are produced by the Ehrlich pathway for amino acid catabolism and the other 35% by the Harris pathway for sugar metabolism [21]. In four representative alcoholic beverages, huangjiu, wine, baijiu and brandy, a total of three straight-chain aliphatic alcohols (1-propanol, 1-butanol and 1-hexanol), two branched-chain aliphatic alcohols (isobutanol and isoamyl alcohol) and an aromatic alcohol (phenethyl alcohol) were accurately detected using HS-SPME-GC/MS. Higher alcohols were present in total concentrations ranging between 0.2 and 1.2 g/L in fermented wines (huangjiu and wine) and between 0.5 and 1.5 g/L in distilled spirits (baijiu and brandy), which was consistent with literature reports [21, 22]. Previous studies showed that isobutanol and isoamyl alcohol, as the main branched-chain alcohols, were synthesized from the corresponding α-keto acid intermediates in the branched-chain amino acid metabolic pathway for the degradation of valine and leucine, thus amino acid composition was one of the key factors affecting the levels of higher alcohols during fermentation of alcoholic beverages [23, 24]. Besides, aliphatic alcohols such as propanol, 1-butanol, and isobutanol were considered to be important contributors to baijiu flavor, and phenethyl alcohol was considered to be one of the most important aromatic alcohols affecting wine flavor [25]. The production of higher alcohols during fermentation mainly depended on the yeast strain and inoculum concentration. Different strains of *saccharomyces cerevisiae* determined the formation and metabolism of higher alcohols, and increasing the yeast inoculum reduced the levels of higher alcohols [26, 27]. Furthermore, the content and types of higher alcohols in alcoholic beverages were also affected by other factors such as raw materials, fermentation temperature, pH of mash [28]. The unique processes of individual alcoholic beverage also changed the composition of higher alcohols, such as the concentration of saccharifying agents during huangjiu fermentation, clarification during wine brewing, and the flavor style and pit ages of baijiu [29, 30]. The above were the main reasons for the difference in the composition of higher alcohols in the four alcoholic beverages.

4.2 Detection of plasma higher alcohols using SPE as pretreatment

Compared with traditional separation method such as LLE, SPE possesses advantages including low organic solvent consumption, simplicity, rapidity and improved sample clean-up and is widely used in different biological/analytical fields such as environmental, clinical, pharmaceutical and biomedical research [31]. HS-SPME combined with GC-MS is a common strategy for quantifying higher alcohols in alcoholic beverages due to its fast, efficient, and solvent-free advantages. However, it was rarely used as a preparation method for biological samples, and the application of HS-SPME in quantification of plasma higher alcohols did not succeed in this study (data not shown). Moreover, detection sensitivity of the proposed SPE strategy was obviously higher in comparison with HS-SPME (LOD of propanol: 5.20 μg/L versus 1.61 mg/L), as reported by previous research [32]. This may be attributed to the minimal extractive phase of HS-SPME

compared to SPE, as expected for non-exhaustive versus exhaustive methods, along with the interference of complicated matrix such as the binding effects of plasma proteins and other macromolecules [31, 33]. Typical SPE sorbents include unmodified sorption materials such as pure silica, magnesium silicate, alumina and diatomaceous earth, as well as polymeric resins such as PS-DVB, divinylbenzene-co-N-vinylpyrrolidone (DB-NVP) [34]. PS-DVB polymeric sorbents, which overcome the limitations of bonded silica, are the most widely used in the separation of many volatile compounds [34, 35]. Our study showed that PS-DVB resin could separate higher alcohols well from plasma, which may be because the PS-DVB material itself contained a relatively large number of active aromatic sites on the hydrophobic surface and thus had greater analyte retention (mainly polar compounds, like alcohols) [34]. In addition, the extraction efficiency of higher alcohols depended not only on the polarity of the liquid solvent, but also on its solubility in the system [36, 37]. Overall, extraction techniques and clean-up methods are essential prerequisites for the development of accurate analytical assays, and the SPE strategy employing PS-DVB as sorption material and dichloromethane as elution solvent achieved precise separation of higher alcohols from plasma sample.

4.3 Metabolism pattern of higher alcohols in plasma

Our results show clearly that the metabolism of both ethanol and higher alcohols in rat plasma was divided into an absorption-distribution phase and an elimination-excretion phase. The C-T profiles ascended sharply in an almost linear way during absorption, whereas it declined gently during elimination. That is, the metabolism pattern of higher alcohols and ethanol shared certain characteristics in rat plasma after gavage, and similar results were obtained by Mitchell et al. and Jones [38, 39]. These findings indicated that, higher alcohols, as evidenced by a similar absorption-elimination process, may be absorbed, distributed, metabolized, and excreted through enzyme systems similar to those of ethanol *in vivo*. The enzyme systems involved in the ethanol metabolism have been studied extensively, and the major metabolic pathways include the alcohol dehydrogenase (ADH)-catalyzed ethanol oxidizing system and the microsomal ethanol oxidizing system (MEOS) in hepatocytes [40]. Most ethanol is catalyzed by the two enzyme systems for dehydrogenation, followed by acetaldehyde, which is subsequently dehydrogenized to ethanoic acid. It was reported that higher alcohols probably were also metabolized through this enzyme system and oxidized to corresponding aldehydes, and the accumulated metabolites may lead to headache and had an impact on ethanol metabolism during this period [9]. Therefore, the enzyme system is a key determinant for the metabolism of higher alcohols. Furthermore, the effects of different dietary composition, such as ethanol concentration, protein or lipid, which may exert influence on the activity of enzyme system, on the metabolism of higher alcohols should also be taken into account.

Consuming similar amounts of ethanol with different alcoholic beverages resulted in considerably different pharmacokinetic parameters of plasma higher alcohols, such as C_{max}, T_{max}, metabolic rate, etc., which may be due to the different contents and types of higher alcohols in the beverage samples [17]. Interestingly, it was found that the metabolism of some higher alcohol still varied greatly even at the same initial gavage dose. For example, higher MRT and AUC of plasma isoamyl alcohol were observed in wine

group than those in huangjiu group, which represented a longer time of presence of isoamyl alcohol molecule in the body and a decreased metabolism. This may be attributed to other distinct components of these alcoholic beverages, such as amino acids, polypeptides, polysaccharides, and organic acids. For instance, some peptides inhibited the increase of plasma alcohols by stabilizing production of coenzyme (NAD^+) and then maintained tricarboxylic acid cycle due to its high concentration of alanine and leucine [41]. Besides, some polysaccharides may influence the alcohol metabolism by increasing the activity of hepatic ADH and ALDH [42]. Therefore, the above was the main reasons for resulting in different metabolism of plasma higher alcohols after consumption of the four alcoholic beverages. Overall, consuming distilled spirits such as baijiu/brandy produced higher peak concentration of blood ethanol and higher alcohols than that of huangjiu or wine, indicating that the peak alcohol concentration and the aggregate exposure to alcohol of organs other than the liver and gut may vary remarkably depending on the type of alcoholic beverage and the rate at which it was consumed.

Metabolism of individual plasma higher alcohol also varied widely within the same alcoholic beverage in addition to the differences among the four groups. The metabolic differences of higher alcohol individuals may depend on their initial concentrations in the same group, and may also be related to the molecular weight (carbon chain length) and configuration of higher alcohols themselves. Distinct protein structure and electrophilicity of ADH involving in the metabolism of alcohols could result in the different substrate specificity on the alcohols, and the alcohols with branching in the side chain may diminish the activity of the enzyme and lower its efficiency [10, 43, 44], which needs further experiments to verify.

According to previous researches, higher alcohols may be an important cause of intoxication and hangover after heavy drinking. Furthermore, drunkenness and headache were severer after consumption of alcoholic beverages with more complex ingredients such as whiskey [45]. Nevertheless, the assessment of discomfort was mainly through subjective descriptions such as headache, thirst and lack of proof based on rigorous controlled trials. In this study, the metabolism pattern of plasma higher alcohols *in vivo* was explored preliminarily and the results showed obvious differences among different alcoholic beverage groups. These findings may be helpful for assessment of the role of higher alcohols in intoxication and hangover in further study.

5. Conclusion

An appropriate detection method for higher alcohols in rat plasma using SPE-GC/MS strategy was developed. Optimal extraction condition was obtained by eluting 3 mL plasma with 7 mL of dichloromethane in a PS-DVB cartridge, and the method showed fine linearity, detection limits, repeatability and recoveries. The optimized method was applied to plasma of rats administered with different alcoholic beverages, and a higher metabolic rate of plasma total higher alcohols in huangjiu group than that of wine group and a generally higher absorption rate in baijiu group than that of brandy group were observed. These findings not only provided data support for a new perspective of evaluation of alcoholic beverage quality, but also contributed a

theoretical basis for better understanding of the specific impact of higher alcohols on intoxication and hangover after binge drink, which need more extensive investigation combined with multiple factors such as dietary pattern.

Ethics statement

The protocol was approved by the Experimental Animal Ethics Committee of Jiangnan University (Wuxi, Jiangsu, China).

Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (22138004 and 32001828).

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