Quality evaluation of different varieties of dry red wine based on nuclear magnetic resonance metabolomics

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Abstract
The metabolites that provide the aroma and flavor to wine are the products of several influences, such as grape cultivar, geographic location and associated environmental features, viticultural practices, and vinification techniques, which are central to production protocols, quality evaluation and development of wine regions. Accordingly, we initiated the requisite studies to investigate the differences in the dry red wine metabolites of different grape varieties. The proton-nuclear magnetic resonance technique (1H-NMR) combined with multivariate statistical analysis was used to investigate the changes of metabolite levels in Cabernet Sauvignon, Merlot and Cabernet Gernischt dry red wines vinified in Changli, Hebei province, China, in 2017. The results showed that the types of metabolites in different varieties of dry red wines were similar, but the content was significantly different. The main contributors to the differences in Cabernet Sauvignon, Merlot and Cabernet Gernischt dry red wines were ethyl acetate, lactic acid, alanine, succinic acid, proline, malic acid, and gallic acid, indicating 1H-NMR method combined with multivariate statistical analysis can distinguish these three types of dry red wines from each other. It provides a benchmark for further comparative study on wine quality and the verification of wine authenticity.

Keywords: Proton nuclear magnetic resonance technology, Metabolites, Multivariate statistical analysis, Wine varieties

Introduction
Dry red wine is a kind of natural alcoholic beverage with health care function. Long-term moderate drinking can delay aging, prevent and inhibit cardiovascular disease and cancer. As dry red wine contains colored substances in the peel or pulp, it is mainly in red color, such as deep ruby red, ruby red, magenta, crimson, brown red and so on. Changli is one of the important wine grape producing areas in China, with superior geographical location, unique geomorphological features, good soil and meteorological ecological conditions. Changli is located at 39°24′–40°37′ north latitude, which is the same latitude as Bordeaux, France. The unique geographical characteristics of bordering the Bohai Sea in the East and Yanshan Mountain in the North have created Changli production area. The annual sunshine duration is 2600–2800 h, the mean temperature difference between day and night is 12 degrees, and the annual rainfall is 400–600 mm. Therefore, it is necessary to study the metabolites of dry red wine in Changli region. Meanwhile, with the continuous development of the wine market, red wine counterfeiting and adulteration incidents have also occurred. Considering consumers’ pursuit of wine quality and safety, it is significant to find a convenient and fast way to identify different kinds of dry red wines, which provides a certain reference for consumers to choose dry red wine.

Nuclear magnetic resonance (NMR) technology has become an important detection method to obtain structural information of metabolites because of its fast analysis speed, simple sample preparation, good repeatability...
and good stability [1]. The combination of $^1$H NMR and pattern recognition technology has been widely used not only in food industry, but also in wine and some liquid beverages such as oil, juice, green tea, beer, etc. [2–5]. Ali et al. [6] used NMR technology combined with pattern recognition analysis to study the inhibitory effect of wine metabolites on tumor necrosis factor. The results showed that the inhibitory effect of wine on tumor necrosis factor was affected by the influence of the vintage and type of wine on the metabolites. Mazzei et al. [7] used NMR techniques to analyze the wines from vineyards with different climatic and soil characteristics in Campania, Italy. The results showed that the differences between wines are associated with climate, the content of carbonate and organic matter in soil and other factors. As a commonly used data analysis method, multivariate statistical method is often combined with NMR technology, and is widely used in the determination and classification of food sources [8–11]. Lee et al. [12] used NMR techniques and multivariate statistical analysis method to distinguish wines from different vintages. The results showed that the main compounds causing differences in wines in different years were 2,3-butanediol, lactic acid, alanine, proline, γ-aminobutyric acid, choline, and polyphenols. Zhu et al. [13] used $^1$H-NMR combined with pattern recognition technology and multivariate statistical analysis method to make a distinction between Cabernet Sauvignon and Shiraz red wine brewed in Shanxi in 2016, and found key contributors to differences were proline, tartaric acid, glycerol, lactic acid, choline, succinic acid and gallic acid. All these results showed that $^1$H-NMR combined with multivariate analysis was a good tool for identifying the different wines.

This study investigated the differences of metabolites in different varieties of dry red wine based on $^1$H-NMR metabolomics and multivariate statistical analysis methods, provided an effective and feasible method for the identification of dry red wine varieties, and offered reasonable advice for consumers to choose suitable dry red wines.

Materials and methods

Wine sample

The wines used in this experiment were provided by Hebei Changli Grape Wine Co., Ltd., and all the single varieties of wine in 2017 were made by the standard process, using the following techniques: De-stemmed and crushed the grapes, and then added the yeast to ferment at 25 °C for 8–10 days, followed by pressing the pomace gently. The wine was separated and tank-switched, and then sampled for pretreatment. The physical and chemical indicators of all wine samples were in line with the requirements of the Chinese National Standard (GB15037-2006), and the samples were stored at −4 °C for further use.

NMR spectroscopic analysis

NMR sample pretreatment

Ten milliliters of wine were taken, and then centrifuged at the speed of 3000 rpm at −4 °C for 20 min. Three milliliters of supernatant fluid were pre-frozen at −80 °C for 12 h, and then lyophilized for 48 h. The lyophilized product was dissolved in 400 μL of oxalate buffer (pH = 4; Shanghai Suyi Chemical Reagent Co., Ltd., China), 140 μL D$_2$O (deuterated degree > 99.9%; Qingdao Tenglong Microwave Technology Co., Ltd., China) and 60 μL of 0.5% DSS (4,4-dimethyl-4-silapentanesulfonate), the mixture were centrifuged at the speed of 13,000 rpm for 20 min. At last, 500 μL of the supernatant was loaded into a 5 mm nuclear tube for NMR analysis. Each sample was tested 4 times.

NMR spectrum acquisition

$^1$H-NMR spectra of wine samples were taken on an AVANCE 600 Nuclear magnetic resonance Spectrometer (Bruker Co., Ltd., Germany). The experiments were carried out at a constant temperature of 298 K. The $^1$H-NMR operating frequency was 600.23 MHz and the spectral width was 7183.9 Hz. The number of sampling points was 32k. The relaxation delay was set to 2 s and the sampling time was set to 2.3 s. The linewidth enhancement factor was 0.3 Hz. The NOESYGPPR1D sequence was used to suppress the water peak signal, and the number of scans was set at 256 times.

NMR spectral data processing

The chemical shift interval between 0 and 10.0 ppm in NMR spectrum was integrated at the section of 0.005 ppm by using Software AMIX. The DSS peaks of −0.5–0.5 ppm, 1.74–1.84 ppm, and 2.90–2.95 ppm, the residual ethanol peaks of 1.18–1.22 ppm and 3.57–3.72 ppm, and the residual water peak of 4.8–4.96 ppm were removed. The integral data obtained by nuclear magnetics was normalized and then imported into Software SIMCA-P 12.0 for multivariate statistical analysis. In order to establish a more reasonable regression model, partial least square discriminant analysis (PLS-DA) was used to strengthen the separation between the observation groups. In addition, PLS-DA also helps to understand which components carry category separation information, and the fitting degree of PLS-DA is verified by external model verification experiments.
Results

Identification of metabolites in wine

One-dimensional NMR spectrum can provide structural information of metabolites. The NMR spectra of Cabernet Sauvignon, Merlot and Cabernet Gernischt wines were shown in Fig. 1. As seen from the figure, most of the metabolites in the three wines were concentrated in the range of δH 9.00–δH 0.00. According to the related literature [14–17], the main characteristic peaks in the NMR spectrum of the corresponding wines were assigned, and the results were shown in Table 1.

It can be seen from the identified metabolites that these substances mainly included amino acids, organic acids, sugar, and phenolic, etc. These metabolites represented the overall metabolome of wine, indicating that $^1$H-NMR can analyze these metabolites synchronously with high throughput [18]. At the same time, it could be found that most of the small molecule metabolites in wine samples were the same, which meant that the composition of small molecule metabolites in wine was relatively stable. However, the contents of metabolites in different types of wine samples were different, and each type of wine had its own characteristic spectrum, indicating that metabolic spectrogram in different types of wine samples described their physiological and biochemical states. Therefore, it is necessary to process these spectral data to find out the markers. The characteristic peaks in the NMR spectrum correspond to different metabolites in the wine samples, and the peak intensity (such as peak area) represented the relative content of the corresponding metabolites [19]. Consequently, the NMR spectrum can effectively show the composition and content of metabolites in the wine samples, and can be studied as a metabolic fingerprint.

Differences in metabolites between different varieties of dry red wine

To find out the main metabolites that cause the differences in different types of dry red wine, NMR data of the dry red wine samples were imported into SIMCA P-12.0 software for Partial least squares discrimination analysis (PLS-DA). The PLS-DA model was established and the scores plot, cross-validation plot and loading plot of Merlot and Cabernet Sauvignon dry red wine was shown in Fig. 2. As seen from the scores plot, the two types of dry red wines could be clearly separated on the PC1 axis, and the cumulative contribution rate $R^2_X = 0.935$, $R^2_Y = 0.999$, $Q^2 = 0.996$, indicating that the model was
### Table 1: $^1$H NMR assignment of metabolites in wines

| Keys | Compound                      | $^1$H-NMR chemical shift                      | Group                                                                 |
|------|-------------------------------|-----------------------------------------------|----------------------------------------------------------------------|
| 1    | Valine                        | 0.88(d), 1.02(d)                              | C4H3, C5H3                                                            |
| 2    | 2,3-Butanediol                | 1.15(d)                                       | C1H3 + C4H3                                                          |
| 3    | Ethanol                       | 1.19(t), 3.56(q)                              | C2H3 + C3H2                                                          |
| 4    | Proline                       | 2.00(m), 2.07(m), 2.35(m), 3.33(m), 3.42(m), 4.16(m) | γ-CH3 β-CH2 β'-CH2γ-CH2δ-CH2δ-CHα-CH2                                    |
| 5    | Succinic acid                 | 2.65(s)                                       | C2H2 + C3H2                                                          |
| 6    | Ethyl acetate                 | 1.26(t), 4.16(q)                              | C4H3, C3H2                                                          |
| 7    | Tartaric acid                 | 4.51(s)                                       | C2H + C3H                                                          |
| 8    | β-Glucose                     | 4.61(d)                                       | βC1H                                                                |
| 9    | α-Glucose                     | 5.33(d)                                       | αC1H                                                                |
| 10   | Gallic acid                   | 7.14(s)                                       | C2H + C6H                                                            |
| 11   | Glycerol                      | 3.58(q), 3.67(m), 3.81(m)                     | C2H2, C3H2, C1H                                                       |
| 12   | Lactic acid                   | 1.39(d), 4.16(m)                              | C3H2, C2H                                                           |
| 13   | Choline                       | 3.20(s)                                       | N-CH3                                                                |
| 14   | α-α-Glucuronic acid           | 5.35(d)                                       | C1H                                                                |
| 15   | Malic acid                    | 2.74(dd), 2.87(dd), 4.46(q)                   | βCH3 β'CH2, CH                                                     |
| 16   | Citric acid                   | 2.82(d), 2.94(d)                              | C2H2 + C4H2, C2H4 + C4H6, C4H8                                        |
| 17   | Alanine                       | 1.51(d)                                       | βCH2                                                                |
| 18   | Tyrosine                      | 6.86(d), 7.19(d)                              | C2H(C3H)                                                            |
| 19   | D-Sucrose                     | 5.46(d), 3.55(dd), 3.72(dd), 3.90(dd), 4.215(d), 4.05(dd), 3.88(dd) | C1H, C2H, C3H, C4H, C1'H, C2'H, C3'H                                  |
| 20   | γ-Aminobutyric acid           | 2.50(t), 1.96(m), 3.05(t)                     | α-CH3 β-CH2 γ-CH2                                                    |

Letters in parentheses indicate the peak multiplicities: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) and dd (doublet of doubles).

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**Fig. 2** PLS-DA model derived from the $^1$H NMR spectra of Merlot and Cabernet Sauvignon dry red wine. PLS-DA scores plot (a), PLS-DA cross-validation plot (b), PLS-DA loading plot (c).
reliable. The permutation test was a widely used and effective method to verify the model quality. Results of cross-validation plot indicated that the values of $R^2$ and $Q^2$ did not exceed the quality parameters of the actual model, once again demonstrating this PLS-DA model was of good reliability and predictability. Major metabolites that contributed to the discrimination of the two types of dry wine could be obtained from the PLS-DA loading plot. In the loading plot, the higher peak indicated higher content of the corresponding metabolites, while the lower one indicated lower content of the corresponding metabolites [13]. As seen from the loading plot, compared with Cabernet Sauvignon dry red wine, Merlot dry red wine had higher levels of proline, ethyl acetate, valine, 2,3-butanediol, succinic acid, glycerol, but lower levels of lactic acid, β-glucose, malic acid, tartaric acid, α-D-glucuronic acid, choline, alanine and gallic acid.

The PLS-DA scores plot, cross-validation plot and loading plot of Merlot and Cabernet Gernischt dry red wine was shown in Fig. 3. As seen from the scores plot, the two types of dry red wine were clearly distinguished on the PC1 axis, and the cumulative contribution rate $R^2_X = 0.943$, $R^2_Y = 0.998$, $Q^2 = 0.996$, indicating that this model was of good quality. As seen from the cross-validation plot of the permutation test in PLS-DA model, the values of $R^2$ and $Q^2$ did not exceed the quality parameters of the actual model, further illustrated that the reliability and predictability of the model were excellent. It could be found from the loading plot that compared with Cabernet Gernischt dry red wine, Cabernet Sauvignon dry red wine contained higher levels of choline, valine, malic acid, alanine, tartaric acid, ethyl acetate, lactic acid, proline, but lower levels of 2,3-butanediol, succinic acid, α-D-glucuronic acid, glycerol and gallic acid.

The main metabolites of these three wines were quantitatively analyzed and the results were shown in Table 2. The content of these metabolites could be obtained by

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![Fig. 3](image-url)  
**Fig. 3** PLS-DA model derived from the $^1$H NMR spectra of Merlot and Cabernet Gernischt dry red wines. PLS-DA scores plot (a). PLS-DA cross-validation plot (b). PLS-DA loading plot (c)
calculating the ratio of the peak area generated by the proton on a specified group of the test substance to that of the added internal standard DSS in the one-dimensional $^1$H-NMR spectrum. As seen from the Table 2, the main metabolite content was significantly different, which was consistent with the PLS-DA model. The contents of succinic acid and glycerol in Merlot wine were the highest, gallic acid content in Cabernet Sauvignon wine was the highest, and the contents of lactic acid, alanine, proline and malic acid in Cabernet Gernischt wine were the highest. This finding could offer advice for the consumers to choose the suitable wine for their needs.

**Discussion**

Since the $^1$H-NMR measurement requires almost no sample pretreatment, the inherent properties of the sample are well retained. A large number of studies have demonstrated that metabolites detected by $^1$H-NMR can be used to non-destructive identification of wine varieties. Our results showed that there was little difference in the composition of metabolites in different varieties of dry red wines, while the content of these metabolites was quite different. The principal components responsible for the differences were ethyl acetate, lactic acid, alanine, succinic acid, proline, malic acid, glycerol, and gallic acid. These metabolites were significant, whose content were closely related to the flavor, taste and functional activity of wine.

Glycerol is the most abundant byproduct from yeasts alcoholic fermentation [20]. It has a slightly sweet taste and a viscous nature, which contributes to mouth-feel perception [21]. Several parameters, including pH, temperature, sulfite concentration and yeast strain, have been shown to influence the final glycerol levels in wine.

### Table 2 Content of the main metabolites in Merlot, Cabernet Sauvignon, and Cabernet Gernischt dry red wines (g/L)

| Metabolites    | Contents ($\bar{x} \pm SD, n = 4$) |
|----------------|-----------------------------------|
|                | Merlot                        | Cabernet Sauvignon | Cabernet Gernischt |
| Ethyl acetate  | $1.706 \pm 0.010^a$            | $1.519 \pm 0.028^b$ | $1.744 \pm 0.008^a$ |
| Lactic acid    | $0.459 \pm 0.015^c$            | $0.508 \pm 0.029^b$ | $0.622 \pm 0.030^a$ |
| Alanine        | $0.016 \pm 0.001^c$            | $0.037 \pm 0.001^b$ | $0.065 \pm 0.004^a$ |
| Succinic acid  | $1.440 \pm 0.027^a$            | $1.312 \pm 0.016^b$ | $1.122 \pm 0.054^c$ |
| Proline        | $3.891 \pm 0.157^b$            | $3.226 \pm 0.044^e$ | $4.311 \pm 0.081^a$ |
| Malic acid     | $4.076 \pm 0.240^b$            | $4.954 \pm 0.166^a$ | $5.022 \pm 0.027^a$ |
| Glycerol       | $14.776 \pm 0.197^a$           | $13.989 \pm 0.092^b$ | $13.245 \pm 0.275^c$ |
| Gallic acid    | $0.113 \pm 0.002^b$            | $0.135 \pm 0.003^a$ | $0.102 \pm 0.004^b$ |

All statistical analyses were performed using SPSS 17.0 software (SPSS Inc., Chicago, USA). Data are presented as the mean ± SEM. The results were analysed by one-way ANOVA followed by Tukey’s HSD post hoc test. A p-value < 0.05 was considered statistically significant.
amino acids to grape juice of the Merlot variety can
et al. [29] found that addition of alanine and other three
during the fermentation process. Hernández-Orte
its a low content due to a large amount of consumption
essential substance for the growth of yeast, and it exhib-
content of proline in wine depended on environmental
increase in the concentration of proline was beneficial
to the balance of the wine. Lee et al. [12] found that the
is rich in proline [14]. Chang et al. [28] found that the
protein, which has a strong affinity for polyphenols,
mouthfeel, which will improve the nutritional value of
the wine [26]. The proline content may be important
act with ethanol, organic acids, sugars to form a unique
sweetness and bitterness, amino acids can also inter-
for the wine’s “mouth” or “body”, because a salivary
protein, which has a strong affinity for polyphenols, is rich in proline [14]. Chang et al. [28] found that the increase in the concentration of proline was beneficial to the balance of the wine. Lee et al. [12] found that the content of proline in wine depended on environmental factors and the variety of wine grape fruit. Alanine is an essential substance for the growth of yeast, and it exhibits a low content due to a large amount of consumption during the fermentation process. Hernández-Orte et al. [29] found that addition of alanine and other three amino acids to grape juice of the Merlot variety can
significantly decrease sulphured notes. Both of proline and alanine can soften the taste of the wine, and their contents in Cabernet Gernischt wine are the highest, further illustrated that Cabernet Gernischt wine has the softest taste.
Gallic acid, the most abundant phenolic compound in wine [30], is mainly derived from grape seeds and grape stems, as well as in contact with oak during fermentation process [14]. In this experiment, the gallic acid content of Cabernet Sauvignon wine is significantly higher than that of Merlot and Cabernet Gernischt wines. Researches have shown that gallic acid has anti-tumor effects and can resist a variety of carcinogenic substances [31]. Therefore, the bioactivity and health function of Cabernet Sauvignon wine in this experiment may be the highest among the three types of wines. Malic acid is an important organic acid in dry red wine, which are inherent in grape berry [32]. A large number of studies have shown that malic acid has many important biological activities, for example, it can effectively improve the body’s exercise capacity, anti fatigue, accelerate the metabolism of carboxylate, protect the heart, and improve memory, etc. Malic acid content in Cabernet Sauvignon is higher, this result also supports that the bioactivity and health function of Cabernet Sauvignon wine in this experiment may be the highest among the three types of wines.
In this study, samples of Cabernet Sauvignon, Merlot and Cabernet Gernischt dry red wines vinified at Changli of Hebei province in 2017 were analyzed by 1H NMR and multivariate statistical analysis methods. The main contributors to the notable differences among these three types of wines were identified. It provides a new technical solution for the adulteration identification of wine varietals, and also offers advice for the consumers to choose these three wines according to their personal preferences: they can choose Merlot wine in pursuit of the richer flavor and taste, Cabernet Gernischt wine in pursuit of the softer taste, and Cabernet Sauvignon wine in pursuit of the better healthy function.

Abbreviations
1H-NMR: Proton-nuclear magnetic resonance technique; PLS-DA: Partial least squares discrimination analysis; NMR: Nuclear magnetic resonance.

Authors’ contributions
Formal analysis: ZX; Funding acquisition: FX; Methodology: ZJ; Validation: XS; Writing-original draft: GJ; Writing-review & editing: HB. All authors read and approved the final manuscript.

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Competing interests
The authors declare no Competing interests.

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