Food Research International 125 (2019) 108574

Contents lists available at ScienceDirect

Food Research International

journal homepage: www.elsevier.com/locate/foodres

High-resolution pure shift NMR spectroscopy offers better metabolite discrimination in food quality analysis

Yu Bo[a], Jianghua Feng[a], JingJing Xu[a], Yuqing Huang[a], Honghao Cai[b], Xiaohong Cui[a,*], Jiyang Dong[a,*], Shangwu Ding[c,d], Zhong Chen[a]

[a] Department of Electronic Science, Xiamen University, Xiamen, China
[b] School of Science, Jimei University, Xiamen, China
[c] Natl Sun Yat Sen Univ, Dept Chem, Taiwan
[d] Natl Sun Yat Sen Univ, Ctr Nanosci & Nanotechnol, Kaohsiung 80424, Taiwan

ARTICLE INFO

Keywords:
Pure shift spectroscopy
PSYCHE
High-resolution NMR
Homonuclear decoupling
Adulteration of honey
Originality discrimination of tea

ABSTRACT

PSYCHE homonuclear decoupling, a prominent pure shift NMR method, is successfully applied to adulteration discrimination of honey and geographical originality identification of tea. Effects of homonuclear couplings are efficiently suppressed, producing resolution-enhanced spectra. The pair wise honey and tea samples are well separated in OPLS-DA models with high predictability. Due to the well-resolved and accurate assignment of singlet resonances after decoupling, PSYCHE is advantageous in the identification of differential components and accurate quantification of compound concentrations presented by enhanced volcano and Beeswarm plots of honey samples, while the analysis of NOESY is easily interfered by overlapped resonances, which is further proved by the STOCSY analysis, displaying the spectral stability and reproducibility. Experimental results show that PSYCHE can improve the spectral resolution of natural complex products such as honey and tea and be combined with multivariate statistical analysis and serve as a supplementary technique to the standard methods, especially for samples systems composed by a few high-content compounds.

1. Introduction

As a non-invasive and powerful method, nuclear magnetic resonance (NMR) spectroscopy has been frequently used in structure determination of compounds and componential analyses of chemical mixtures. Among its numerous pulse sequences, proton-proton scalar coupling is very useful for structural identification by providing multiplicity and coupling constants (Grimes & O'Connell, 2011; Molinski, 2010; Zangger, 2015). However, extensive signal splitting often results in severe signal overlapping and makes it difficult to extract the spectral information in ^{1}H NMR spectra due to the limited chemical shift range (about 10–15 ppm) (Zangger & Sterk, 1997). Regarding this issue, on one hand, ^{13}C and two-, even higher-dimensional NMR spectroscopy such as COSY, HSQC have been developed to overcome the poor signal dispersion in proton spectra. On the other hand, some techniques called "pure shift" NMR have been presented based on the concept of homonuclear broadband decoupling (Aguilar, Faulkner, Nilsson, & Morris, 2010; Meyer & Zangger, 2013). Ernst and co-workers were the first to put forth this idea and later suggested an experimental route of homonuclear broadband decoupled proton spectra based on 2D *J*-resolved experiments in 1976 (Aue, Karhan, & Ernst, 1976; Ernst & Primas, 1963). After that, Bax, Mehlkopf, & Smidt, 1979, introduced a constant-time method consisting of a spin echo along with different times before and after the 180° pulse (Bax et al., 1979). Three years later, BIRD (bilinear rotational decoupling) method based on the inversion of ^{12}C-bound protons while keeping ^{13}C-bound protons unchanged was introduced by Garbow (Garbow, Weitekamp, & Pines, 1982). However, because of low sensitivity and usually complex processing schemes, those techniques have not been widely used.

Before long, Zangger and Sterk proposed a method which is more easily implemented with less processing complexity, called slice-selective decoupling or Zangger-Sterk (ZS) method (Zangger & Sterk, 1997). It is achieved by a combination of a soft and a hard 180° pulse along with a weak pulsed field gradient. To obtain high resolution decoupled spectra, it needs to record several individual pieces of an FID (free induction decay) in separate increments with varying evolution times. When joining the refocusing FID blocks of the obtained FIDs (named as data chunking technique), a pure shift spectrum can be obtained through Fourier transform of the new joined FID. Due to each signal being excited in a narrow region of the sample tube only, the sensitivity

* Corresponding authors.
E-mail addresses: cuixh@xmu.edu.cn (X. Cui), jydong@xmu.edu.cn (J. Dong).

https://doi.org/10.1016/j.foodres.2019.108574
Received 11 April 2019; Received in revised form 16 July 2019; Accepted 21 July 2019
Available online 22 July 2019

of slice selective decoupling experiments is significantly lower than that of regular 1D spectrum. Another very promising decoupling method called PSYCHE (pure shift yielded by chirp excitation) was reported by Morris in 2014 (Foroozandeh et al., 2014), in which two symmetric, low power, frequency-swept chirp pulses are applied during a weak pulsed field gradient in order to invert the passive spin to refocus scalar coupling while keeping the active ones unchanged (Foroozandeh, Adams, Kiraly, Nilsson, & Morris, 2015; Nilsson & Morris, 2007). The sensitivity is also reduced compared to regular spectra, but still far above most other pure shift methods, such as slice-selective or BIRD decoupling. Nevertheless, the ZS decoupling method can be easily implemented in the indirect dimension of multidimensional experiments and has been used in two-dimensional homonuclear TOCSY (Morris, Aguilar, Evans, Haiber, & Nilsson, 2010) and NOESY (Aguilar, Colbourne, Cassani, Nilsson, & Morris, 2012) with enhanced resolution. Several experiments employ PSYCHE to decouple the acquisition dimension of 2D spectra, such as 2D F1-PSYCHE-TOCSY (Foroozandeh, Adams, Nilsson, & Morris, 2014; Foroozandeh et al., 2016) and 2D *J*-ZQS-PSYCHE (Kiraly, Foroozandeh, Nilsson, & Morris, 2017).

Recently, PSYCHE homonuclear decoupling has been incorporated in inversion recovery and CPMG NMR experiments to significantly enhance spectral resolution, thereby enabling unambiguous measurement of relaxation times, T_1 and T_2, as showcased by a specific application in the study of drug-protein interactions (Dal Poggetto, Castanar, Adams, Morris, & Nilsson, 2017; Kakita, Shukla, Bopardikar, Bhattacharya, & Hosur, 2016). Besides, a new experiment PSYCHE-iDOSY was reported, which, by integrating diffusion weighting with PSYCHE method for pure shift NMR spectroscopy, allows DOSY spectra to be recorded with ultrahigh resolution and improved sensitivity (Foroozandeh et al., 2016).

Honey and tea are two kinds of popular foods throughout the world. Honey contains a large amount of fructose and glucose and some minor components such as organic acids, amino acids and polysaccharides. Pure honey possesses high nutrition and economic value, which, unfortunately, also drives its adulteration (Kortesniemi et al., 2016; Padovan, De Jong, Rodrigues, & Marchini, 2003). Similar to honey, tea is another popular food. Tea is rich in tea polyphenols, caffeine, theanine, vitamins and other phytochemicals (Dai et al., 2017; Pan et al., 2015). Besides, tea grown in different geographical origins generates different organoleptic characters due to the compositional variations. Recently, NMR spectroscopy has widely been used in qualitative analysis of food, especially when combined with multivariate statistical analysis, which is considered a very promising method in food quality analysis (Marcone et al., 2013; Meng et al., 2017; Shen et al., 2018.)

In this study, we apply PSYCHE homonuclear decoupling method to obtain the high-resolution NMR spectra of honey and tea. Two groups of honey samples consisting of pure ones and adulterated ones and two groups of tea samples from different geographical origins are characterized by PSYCHE homonuclear decoupling sequence. The componential differences of two pairwise groups based on PSYCHE spectra are then explored by multivariate statistical analysis including the principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA). Specifically, the PSYCHE spectra of honey samples were taken as an example to test the feasibility of the discriminate model. The stability and reproducibility of PSYCHE spectroscopy for quantitative analysis were also estimated using STOCSY (statistical total correlation spectroscopy) which displays the correlation between two different resonances. Meanwhile, NOESY as the conventional ^{1}H NMR spectroscopy is also employed to assist resonance assignment and make analytical comparison with that of the pure shift spectroscopy. Finally, the feasibility of PSYCHE spectroscopy to apply in complex natural products is further proved in the originality analysis of tea.

2. Materials and methods

2.1. Sample collection and preparation

2.1.1. Honey samples

Twenty-seven Chinese honey samples were provided by Animal, Plant, and Food Inspection Center of Jiangsu Entry-Exit Inspection and Quarantine Bureau, China. Fifteen of them were natural honey (unadulterated) and the other samples were adulterated honey. The adulterated samples were partly mixed with a certain amount of syrups including rice syrup and invert syrup. All honey samples were kept under the dark condition till NMR experiments with a temperature of 17–25 °C to avoid significant changes and crystallization (Venir, Spaziani, & Maltini, 2010).

Since the pH values of honey samples are between 3.4 and 5.2, honey samples were prepared by dissolving 150 mg of honey in 500 μL of 600 mM deuterated phosphate buffer (pH 4.92) containing 0.05% of sodium 3-(trimethylsilyl)-2,2,3,3-$^2H_4$ propionate (TSP). The honey-buffer mixture was left to stand for 5 min at room temperature and then centrifuged at 8200 *g* for 10 min. The supernatant solution (500 μL) was then transferred into a 5 mm NMR tube for measurement.

2.1.2. Tea samples

A total of 20 Tieguanyin (TGY) tea samples were collected from two different growing places with official certifications, i.e., Xianghua town (25°11′N, 117°44′E) and Xiandu town (25°03′ N, 117°38′ E), which are with a long history of TGY tea planting and manufacturing. Average temperature, total rainfall and sun exposure time are the three major climatic factors considered to contribute directly to the growth of tea plant and biochemical composition of tea leaves, largely deciding the quality of the finished tea. These tea samples are originated from the same batch of tea samples investigated by Meng et al. (Meng et al., 2017), obeying the same treated procedure which is summarized briefly as follows.

The finely grounded and accurately weighed tea powder 400 mg was brewed with 1.5 mL of deuteroxide and placed in a water bath at 85 ± 2 °C for 30 min. After cooling to room temperature, a volume of 600 μL supernatant was mixed with 100 μL phosphate buffer solution (100 mM, pH 5.29, 99.9% D_2O) to reduce pH variation across tea samples. Finally, the mixture was left to stand for 5 min at room temperature and centrifuged at 8200 *g* at 4 °C for 20 min. A volume of 500 μL was transferred into a 5 mm NMR tube and kept at 4 °C before experiments.

2.2. NMR experimental methods

All samples were run on a 500 MHz NMR spectrometer (Agilent Technologies, Santa Clara, CA, USA) equipped with three-dimensional gradient coils and a 5 mm switchable broadband detection probe with a maximum nominal gradient strength of 60 $G\,cm^{-1}$ at 298 K.

2.2.1. PSYCHE spectroscopy

The pulse sequence for PSYCHE and its description are shown in Fig. S1 (provided as Supplementary Data). The parameters for PSYCHE experiments on honey samples were as follows: the spectral windows in F_1 and F_2 were set to 50 Hz and 8 kHz respectively. The number of t_1 increments (number of chunks) and number of scans were set to 50 and 1, respectively. Durations for chirp pulses were set to 30 ms each and a flip angle β of 8°. G_1 and G_2 gradients were used for selection of desired coherence transfer pathways with the amplitudes of 16 and 24 $G\,cm^{-1}$ respectively and duration of 1.5 ms each. The G_3 gradient pulse was aligned with the center of the pulse element consisting of two chirp pulses with the amplitude of 1.0 $G\,cm^{-1}$. All gradient pulses were followed by a recovery delay of 0.5 ms. A recycle delay of 2.0 s and 2 prior dummy scans were used in our experiments. Because of the high-concentration of solutes in honey samples, water signals were weak enough

and did not disturb the solute signals in PSYCHE experiments. Therefore, there was no presaturation module applied.

Most of the PSYCHE parameters for tea were the same as those of honey experiments, except that the spectral windows in F_1 and F_2 were set to 80 Hz and 8 kHz respectively. The number of t_1 increments (number of chunks) and number of scans were set to 20 and 8, respectively. Due to faster decay for tea signals, a shorter evolution time ($1/SW_1$), less t_1 increments and more scan numbers were chosen. Besides, because of the low-concentration of tea samples, the presaturation module was employed before acquisition and the continuous-wave pulse with a certain saturation power and duration of 1 s was used.

2.2.2. NOESY spectroscopy

Conventional ^{1}H NMR spectra used for resonance identification and comparison with pure shift spectra were acquired using a standard water suppressed TNNOESY pulse sequence (NOESYpresat, Agilent). The primary parameters for honey samples were as follows: spectral width of 8 kHz, an acquisition time of 2.5 s, and a recycle delay time of 3.0 s using 64 scans and 16 prior dummy scans. A selective irradiation on water peak during fixed interval t_1 of 4 μs and a mixing time t_m of 100 ms was used for water suppression. Most of NOESY parameters for tea were the same as those of honey experiments, except that spectral width of 10 kHz, an acquisition time of 2.2 s, and 128 scans due to the lower sensitivity and 16 prior dummy scans. In order to weaken the water signals, the presaturation module was also employed in NOESY spectroscopy of both tea and honey samples.

2.3. Data preprocessing

All FIDs (pure shift FIDs are the processed FIDs after data chunking) were weighted by an exponential function with a 1.0 Hz line-broadening factor and a Gaussian function with a 0.3 Hz line-broadening factor before fast Fourier transformation. All spectra were manually phased and baseline-corrected using MestReNova (version 9.0). Both of the NOESY and PSYCHE spectra were calibrated to TSP at δ 0.00. The sensitivity was calculated as $\frac{\text{SNR}}{\sqrt{\text{acquisition time}}}$ (Ernst, Bodenhausen, & Wokaun, 1987), in which SNR is the signal-to-noise ratio calculated by dividing the intensity of the strongest resonance (the sugar peak δ 3.71 for honey and the Caffeine peak δ 3.84 for tea) by the standard deviation (SD) of noise signals in the region between δ 9.0–10.0.

The spectral region of δ 0.5–9.0 for NOESY and PSYCHE spectra was binned using adaptive binning algorithm (De Meyer et al., 2008), which reduces the effects of the peak position variation due to physicochemical environment differences (pH, ionic concentration), so that each peak is integrated into one bin as possible as we can. Also, peak-free regions and water resonance (δ 4.76–4.86) were removed from the spectra to reduce the effect of noise and residual water signal. Data normalization for tea samples was carried out using probabilistic quotient normalization (PQN) (Dieterle, Ross, Schlotterbeck, & Senn, 2006) to make overall concentrations of all samples being at a same level. For PQN standardization method, there is an assumption that intensities (concentrations) of most variables are stable or remain unchanged in pairwise samples. Because honey spectra are dominated by a few high-concentration content compounds such as Glc and Fruc, and both of them contribute to the discrimination of honey. It is obvious that the honey samples do not meet this assumption and therefore cannot be standardized by PQN methods. Therefore, we normalized honey spectra to the peak area of their own TSP at δ 0.00. Conversely, there are more compound signals detected in tea samples. The analysis results also show that the differential compounds account for a small proportion of all signals in the whole spectrum. Therefore, the PQN method is applicable. Finally, the normalized binned data were exported to an Excel file for further analysis.

2.3.1. Multivariate statistical modeling

Multivariate statistical analysis was performed on the binned data of honey and tea samples with the SIMCA-P software (version 14.0, Umetrics AB, Umeå, Sweden). To overview the data distribution and potential outliers, PCA models were built on mean-centered binned data. Then OPLS-DA models were built on the unit variance (UV) scaling data to identify the adulterated honey samples and discriminate the geographic origin of tea samples. Specifically, 19 bins by integrating 19 clearly-identified resonances from saccharides are identified in PSYCHE spectra of honey, with other low-intensity resonances excluded due to the low sensitivities. In NOESY spectra, 43 counter parts resonances within the same chemical shift range were identified and integrated into 43 bins. For tea, all bins were used to build the multivariate models. Model validations were carried out using both of seven-fold cross-validation and permutation test (200 permutations). The overall quality of fitting (R^2) and predictability (Q^2) were used to assess the robustness and effectiveness of the OPLS-DA model, respectively.

2.3.2. STOCSY analysis

STOCSY (statistical total correlation spectroscopy) (Cloarec et al., 2005), which takes advantage of the multicollinearity of the intensity variables in a set of spectra to generate a pseudo-two dimensional TCOSY (total correlation spectroscopy) NMR spectrum that displays the correlation among the various peaks of the whole sample set, was used to assess the anti-interference and reproducibility of PSYCHE, i.e., robustness of PSYCHE against the effect of the resonance overlapping. According to the above obtained 19 bins and 43 bins, matrixes with the dimensions of 27 × 19 and 27 × 43 were obtained for PSYCHE and NOESY spectra of honey samples, respectively. STOCSY were calculated and visualized in heat maps using house-writing software with MATLAB.

3. Results and discussion

3.1. Honey samples

3.1.1. Resonance assignment

Pure shift ^{1}H NMR spectra of two typical adulterated and pure honey samples via PSYCHE method are given in Fig. 1a and c, respectively. Corresponding conventional ^{1}H NMR spectra are shown in Fig. 1b and d. Resonance assignments of conventional ^{1}H NMR spectroscopy were performed according to the existing literature (Zheng, Zhao, Wu, Dong, & Feng, 2016) and confirmed by HMDB database (http://www.hmdb.ca) and in-house NMR database. The detailed spectral assignment of characteristic peaks is listed in Table 1. For PSYCHE spectra dominated by sugar regions (δ 3.0–5.5), according to the assignment conventional ^{1}H NMR spectra, α-glucose, β-glucose and fructose make major contributions to the intensive resonances. It is worth to note that there are a few residual multiplicities are still shown in PSYCHE spectra. The limit in strong coupling systems like saccharides of PSYCHE technique is probably the main reason. Besides, there are some rhythmic and irregular noises appeared on the baseline, which are probably originated from the data chunking process, resulting in difficulties in assignment of weak signals (Zangger, 2015). Since there are complex resonance patterns for these three saccharides, PSYCHE spectra are still crowded and overlapped to some extent after decoupling. Therefore, PSYCHE and NOESY spectra of the chemical standards of glucose and fructose were obtained to assist the resonance assignment of PSYCHE spectra (Fig. S1, provided as Supplementary Data).

It can be found from Fig. 1 that most of spin-spin couplings in PSYCHE spectra of honey are removed. For instance, the triplet at δ 3.25 and the doublet at δ 4.65 are decoupled and presented as singlets. Except one overlapped resonance from both α-glucose and fructose, nearly all resonances in PSYCHE spectra are clearly identified and assigned. However, due to the low SNR, signals from other low-content sugars and amino acids in aliphatic regions (δ 0.5–3.0) and aromatic

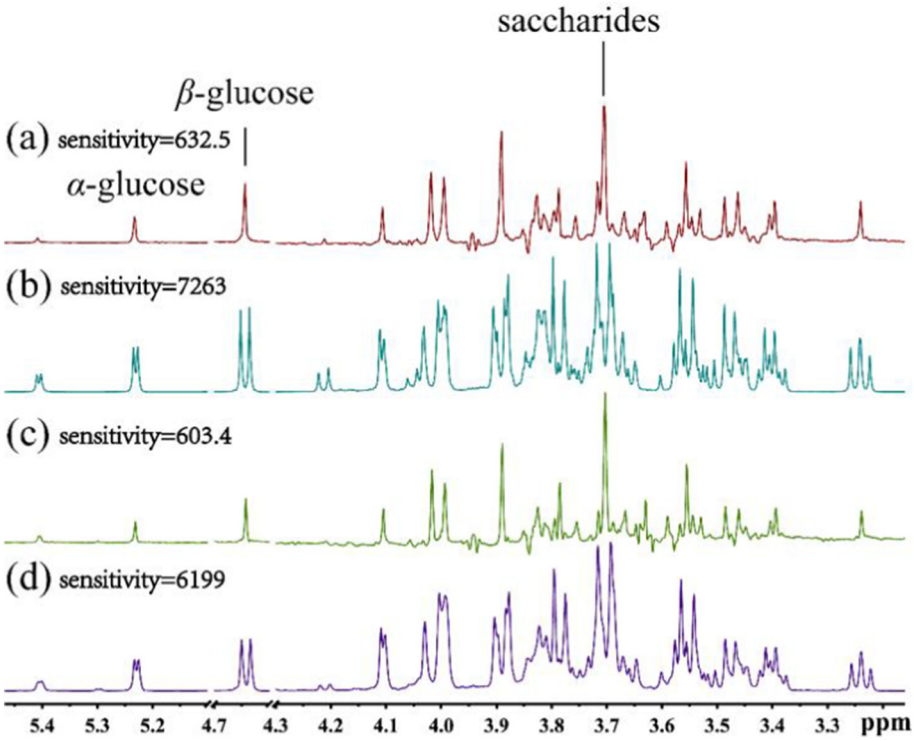

Fig. 1. Selected regions of typical adulterated honey spectra obtained by PSYCHE (a) and NOESY (b) and pure honey spectra obtained by PSYCHE (c) and NOESY (d).

Table 1
Peak assignment in NMR spectra of honey.

Component	Characteristic signals	
	Conventional spectrum	PSYCHE spectrum
α-Glucose	3.41 (t[a])	3.41 (s)
	3.54 (s)	3.54 (s)
	3.71 (t)	3.71 (s)
	3.82 (t)	3.84 (s)
	5.23 (d)	5.23 (s)
β-Glucose	3.25 (dd)	3.25 (s)
	3.40 (t)	3.40 (s)
	3.46 (m)	3.46 (m)
	3.49 (t)	3.49 (s)
	3.90 (d)	3.90 (s)
	4.64 (d)	4.64 (s)
Fructose	3.56 (t)	3.56 (s)
	3.71 (m)	3.71 (s)
	3.81 (m)	3.81 (m)
	4.00 (m)	4.00 (d)
	4.02 (d)	4.02 (s)
	4.11 (d)	4.10 (s)
Formic acid	8.45 (s)	
Hydroxymethyfrufural	7.54 (d)	
	6.68 (d)	
Succinic acid	2.51 (s)	
N-Acetylglutamic acid	1.98 (m)	
Lactic acid	1.32 (d)	
Rhamnose	1.23 (m)	
Ethanol	1.18 (t)	

[a] Multiplicity: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet.

regions (δ 6.0–9.0) such as, formic acid, rhamnose and *N*-acetylglutamic acid shown in conventional spectra cannot be observed in PSYCHE spectra. Sensitivities of the corresponding spectra presented in Fig. 1 indicate that sensitivities of pure shift spectra are only about 10% of that of conventional ones. By comparing the assigned pure shift resonances of two group samples, it can be found that the same components (α-glucose, β-glucose and fructose) are assigned in both adulterated and pure honey samples. It is difficult to recognize componential differences and determine whether it is adulterated or pure only by visual observation. Therefore, multivariate statistical analysis was then carried out using chemometric tools for adulteration identification and componential difference analysis.

3.1.2. Multivariate statistical analysis

PCA models of PSYCHE and NOESY spectra are shown in Fig. 2, in which the relationship of compositional patterns between two groups of honey is displayed. Pure and adulterated groups of honey samples are well separated in PCA score plots. The explained variation (R^2) of PCA models with two PCs were 83.67% and 81.43% for PSYCHE and NOESY respectively, which implies that both PCA models of PSYCHE and NOESY are reliable in representing their whole data sets. In Fig. 2, it can be found that the sample distributions of adulterated and pure groups are different between PSYCHE and NOESY, which is probably because the variables correlations are influenced by overlapped resonances in NOESY spectra.

To identify adulteration of honey and get an insight into the components responsible for the separation of pure and adulterated honey, OPLS-DA models were built on both PSYCHE and NOESY data, as shown in Fig. 3. As expected, a clear separation between two groups was observed on the OPLS-DA score plots. The overall quality of fit (R^2) and cross-validation coefficient (Q^2) values are used to assess the amount of variation represented and robustness of the model, respectively. Specifically, Q^2 can represent the model's ability to identify new samples to some extent. The high Q^2 value of the model indicates that PSYCHE technology can be used to identify adulterated samples. Permutation tests (Fig. S2) were also carried out to validate these OPLS-DA models, showing their validity, robustness and without overfitting.

Furthermore, individual peak contributions to group classification are presented by enhanced volcano plots of pairwise groups and shown in Fig. 4. In volcano plots, the horizontal ordinates represent the $\log_2$(fold-change) of the peak median areas (Cui & Churchill, 2003), which is used to evaluate the logarithm of the peak area ratio between

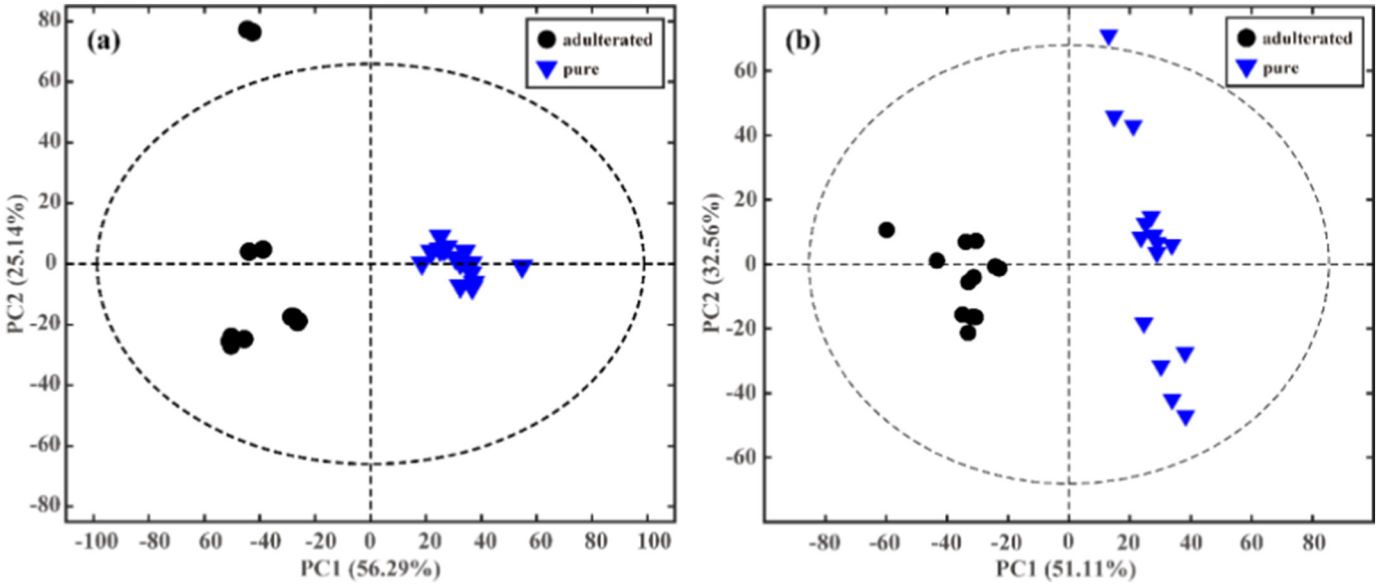

Fig. 2. PCA score plots for the binned data of NOESY (a) and PSYCHE (b) spectra.

two groups. The vertical ordinates indicate the corresponding *p*-values (i.e., Student's *t*-test with a Bonferroni correction between two groups), which is used to determine the significance of change for a peak in two groups. Each point with a distinct color and size represents one bin (i.e., one peak), and colors represent the absolute correlation coefficients |r| of corresponding peaks between the sample scores and the peak areas. The dot size reflects the variable importance projection (VIP, i.e. the degree of contribution to the model) of corresponding peaks in OPLS-DA models. It can be found that contents of sugar including fructose, *α*-glucose and *β*-glucose are higher in adulterated ones, which makes the major contribution to the group separation in both models. As we know, syrup is rich in saccharides but lack of amino acids and organic acids. When adulterating syrup in honey, the relative content of sugar including fructose and glucose increases. Besides, VIP values from the same resonances, are different in two models. For PSYCHE, the resonance at δ 3.72 from *β*-glucose with the largest VIP value makes the greatest contribution, but for NOESY, the resonance at δ 3.88 from fructose makes the greatest contribution. Besides, different resonances from the same compound make different contribution to the separation in each volcano plots. These differences are possibly resulted from the different relaxation decays of protons generated during the evolution time in PSYCHE sequence and the mixing time in NOESY sequence. Furthermore, for NOESY, contributions of single resonances cannot be well identified due to the overlapping. For example, the major contributions to the group separation are originated from overlapped peaks (such as fructose/*α*-glucose). When the ratio of two overlapped resonances changes, the identification of the model could be affected due to mutual counteracting or unilateral overwhelming domination.

3.1.3. Quantification analysis

Since both of PSYCHE and NOESY are dominated by *α*-glucose , *β*-glucose and fructose, we tried to quantify contents of three sugars from two kinds of spectra of both adulterated and pure honey samples. First, spectra of glucose and fructose are obtained using standard samples.

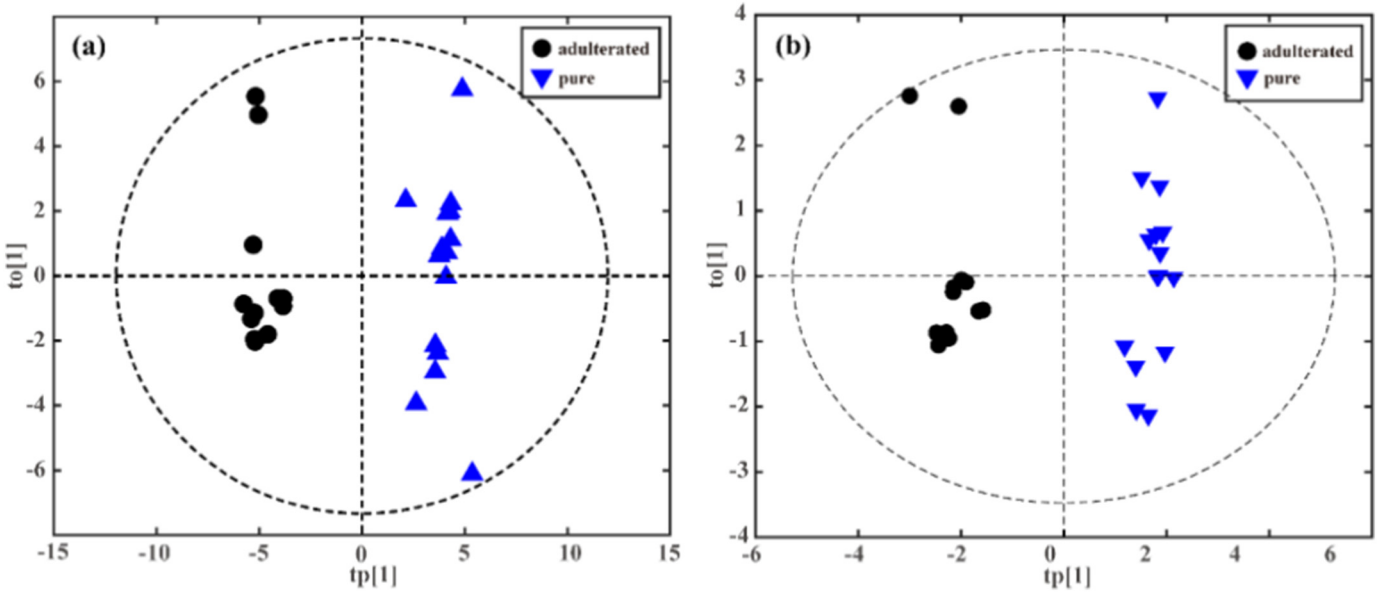

Fig. 3. OPLS-DA score plot of NOESY, R^2 = 97.5%, Q^2 = 0.95 (a) and PSYCHE, R^2 = 97.0%, Q^2 = 0.97 (b). Red and black dots represent the pure and adulterated honey samples. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

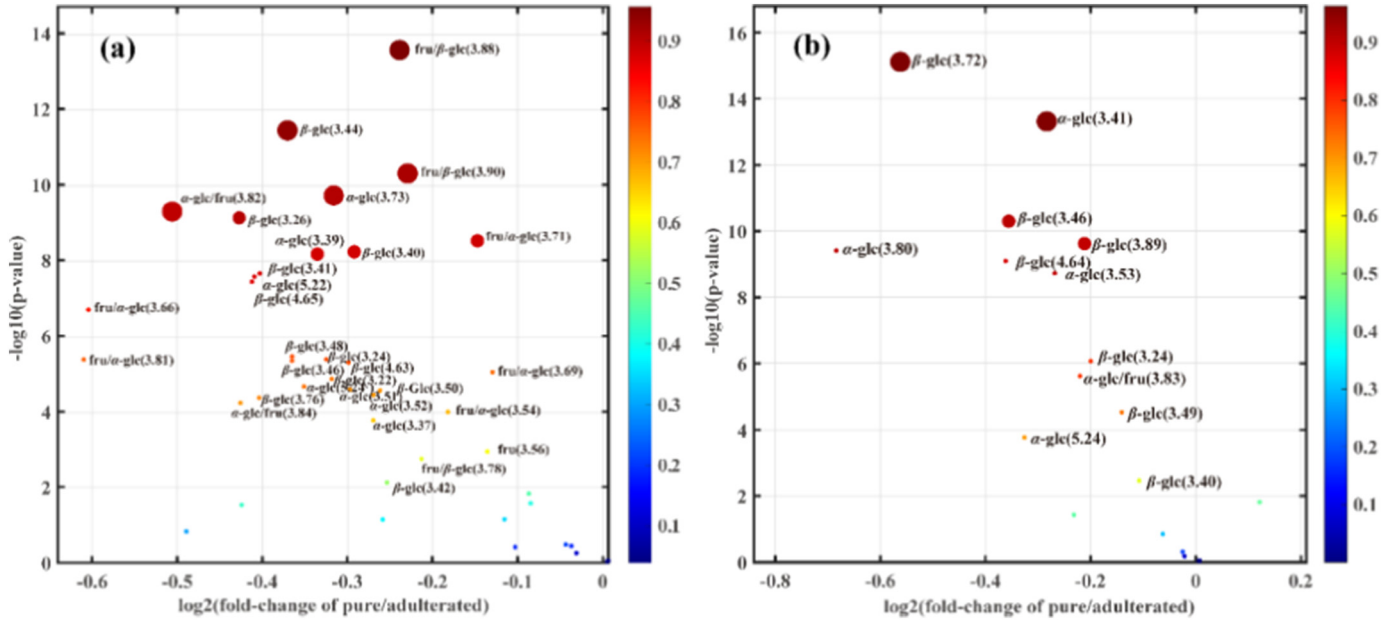

Fig. 4. Enhanced volcano plots of OPLS-DA Model. NOESY (a) and PSYCHE (b).

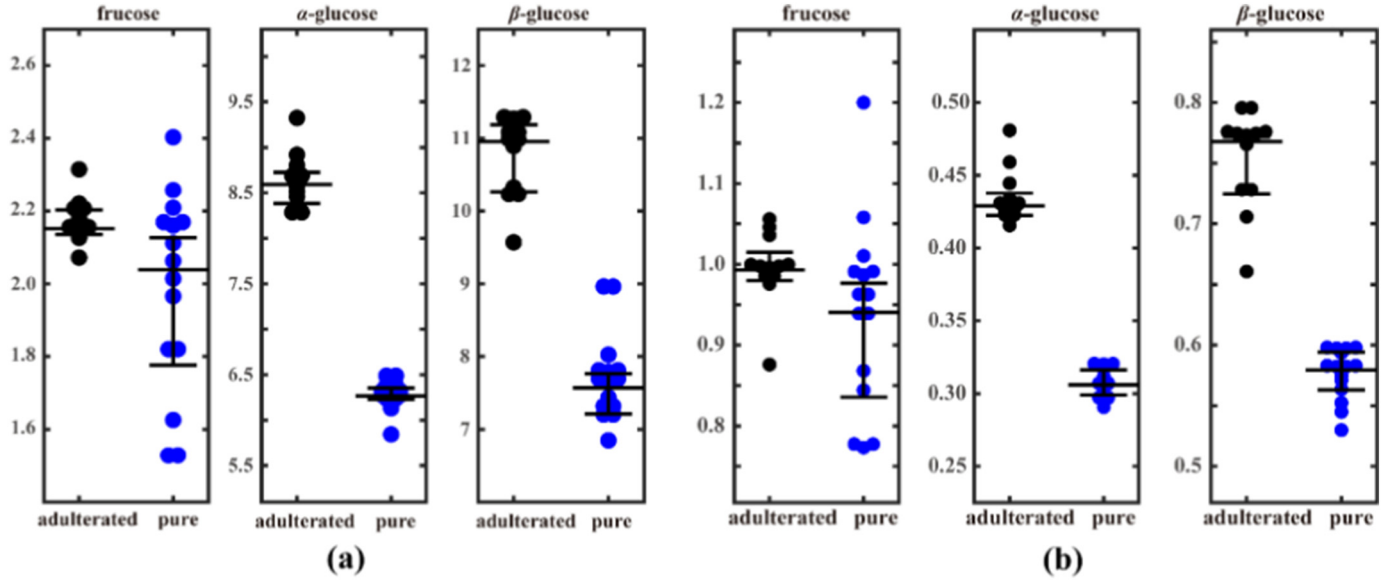

Fig. 5. Beeswarm plots for quantification results. NOESY (a) and PSYCHE (b). Three solid black lines represented the upper quartile, the median, and the lower quartile in statistics, respectively.

For glucose, we distinguished parts of α-glucose and β-glucose peaks according to the HSQC spectrum in HMDB. Then, the in-house standard spectra of three sugars are obtained. Finally, all honey spectra were fitted by the above-mentioned standard spectra of three sugars. The overall intensities for single compound are illustrated by Beeswarm plots (Fig. 5). In PSYCHE spectra, the intensity ratios of β-glucose to that of α-glucose for both two groups approximately equal to 1.8:1.0, which is close to its' natural abundance ratio of 64:36. On the contrary, the NOESY spectra cannot give out the correct answer, which is probably because of the serious overlapping in conventional spectra. This indicates that based on the accurate assignment after decoupling, PSYCHE is advantageous in accurate quantification of the compound concentrations, while the quantification of NOESY is easily interfered effected by overlapped or adjacent resonances due to the spin-spin coupling.

3.1.4. STOCSY analysis

As mentioned above, the intensity ratio of β-glucose to that of α-glucose in NOESY spectra is deviated from the actual proportion probably due to serious overlapping. Besides, in volcano plots, different resonances from the same compound make different contributions to the separation. The spectral instability and non-reproducibility are possible reasons. Reproducibility, an important trait of NMR spectroscopy, which is one of its significant manifestations that different resonance intensities from the same compound exhibit fixed proportional relationships equivalent to the ratio of numbers of protons, is an essential prerequisite for quantitative analysis. However, if there is overlapping among different signals, always for biological samples or natural products, the fixed proportional relationships could be disturbed, affecting the reproducibility. In this section, the STOCSY analysis was used to assess the anti-interference of PSYCHE, i.e., robustness of PSYCHE against the effect of the resonance overlapping.

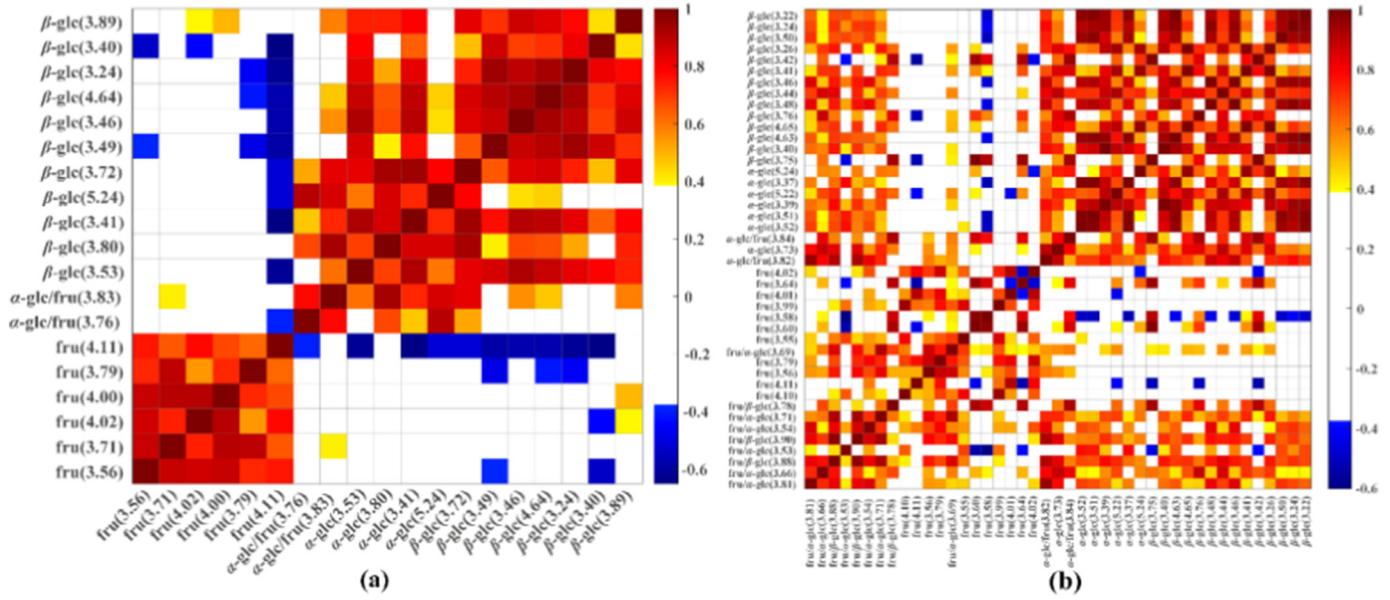

Fig. 6. Heatmaps of STOCSY for PSYCHE (a) and NOESY (b). Colors are set according to the correlation coefficient *r*: the larger the correlation coefficient, the hotter the color, and vice versa. White indicates that the correlation between corresponding peaks is not significant. The number in brackets after the name of the compound is the chemical shift of the corresponding peak.

It can be found in Fig. 6a that good correlation between various peaks from one compound is presented. But, for peaks from different compounds, it is not so. The overlapped *α*-glucose/fructose peaks present stronger correlation with *α*-glucose, but weaker correlation with fructose and *β*-glucose. It hints that the overlapped peak mainly originated from *α*-glucose. In addition, the correlation between the peaks of *α*-glucose and *β*-glucose is significant, because *α*-glucose and *β*-glucose always appear simultaneously in natural world. When *α*-glucose is dissolved in water, it will slowly convert into *β*-glucose. In addition, it can be seen from Fig. S3(a) that the correlation among peaks only from pure honey samples is further enhanced compared to the results from whole honey samples. However, for adulterated honeys, the correlation is chaotic, which is weaker among peaks from the same compound but stronger among peaks from different compounds (Fig. S3(b)). This is possibly resulted from that the adulterated sample contains some unknown signals.

From Fig. 6b, it is suggested that, unlike the clear correlation among resonances from the same compound shown in STOCSY of PSYCHE, strong correlations among the peaks from different compounds, such as fructose and *α*-glucose, fructose and *β*-glucose, are presented. In addition, for overlapped peaks, such as *α*-glucose/*β*-glucose, *α*-glucose/fructose, etc., they have a certain correlation with the characteristic peaks of *α*-glucose, *β*-glucose and fructose. We believe that the serious signal overlapping in NOESY spectra contributes to the irregular correlation. Similarly, if only performing the STOCSY of pure honey samples, the correlation among peaks of the same compound is enhanced, as shown in Fig. S4(a). Conversely, if only performing STOCSY of adulterated honey samples, the correlation is also irregular, similar to that shown in the STOCSY of PSYCHE, as shown in Fig. S4(b). The above STOCSY analyses show that compared to NOESY, PSYCHE presents better correlation due to the improved resolution after decoupling, which supports more stable analytical model and results.

3.2. Tea samples

Pure shift and conventional ^{1}H NMR spectra of tea are shown in Fig. 7. A wealth of spectral information is provided in the conventional spectrum. According to published literatures (Meng et al., 2017), HMDB database (http://www.hmdb.ca), and an in-house NMR database, peaks were assigned to various kinds of polyphenol (catechin, epigallocatechin, epicatechin, epicatechin-3-gallate, epigallocatechin-3-gallate, etc.), caffeine, amino acids (theanine, valine, isoleucine, leucine, threonine, etc.), organic acids (quinic acid, acetate, gallic acid), monosaccharides (including *α*-glucose, *β*-glucose, fructose) and polysaccharides. The detailed information is listed in Table 2. The assignment of pure shift spectra relied much on that of conventional spectra through artificial judgment and comparison. By carefully examining these spectra, the overlapping of signals located at δ 3.0–4.2 can be easily observed in conventional spectra. As the same as that of honey spectra, with the resolution improved, some overlapped signals in conventional spectra can be distinguished in pure shift spectra after decoupling. For instance, multiplets from sucrose (δ 3.40 and δ 3.70, etc.) and theanine (δ 1.12 and δ 3.22) were decoupled and turned into singlets. Similarly, the sensitivity of PSYCHE is still lower, which also results in resonances such as fatty acid (δ 1.27) and quinic acid (δ 4.00) presented in NOESY being absent in PSYCHE. Besides, the coupling of one resonance (δ 3.22) from theanine was not completed decoupled either.

PCA was also performed on PSYCHE to find an overall relationship of compositional patterns between two geographical-origin tea types (Xiandu and Xianghua) (Fig. 8). The unsupervised PCA scores plot suggests that all samples are obviously separated into two clusters. OPLS-DA model was also constructed to further explore differential components contributing to the group separation (Fig. 9). High explained variation R^2 (0.950) and prediction capability Q^2 (0.868) of the OPLS-DA model suggest that the model is valid and robust, implying significant differences in absorption patterns of the groups. Besides, it can be concluded that signals from caffeine, sucrose, epicatechin-3-gallate and epicatechin, fructose, alanine and theanine are the most responsible for the classification. Specifically, a higher level of sucrose, fructose, theanine, alanine and caffeine and a lower level of glucose and epicatechin/ epigallocatechin in tea from Xiandu than those from Xianghua can be observed. Meng researched the same TGY tea samples using conventional spectra together with multivariate statistical analysis and came to a detailed conclusion that theanine, alanine, isoleucine, and caffeine are of higher content in Xiandu TGY tea while epicatechin/ epigallocatechin, and quinic acid, gallic acid, glucose are of higher content in Xianghua TGY tea (Meng et al., 2017). In our work, the observed differential compounds are mostly in accordance with those of Meng's work. However, the contribution to the group

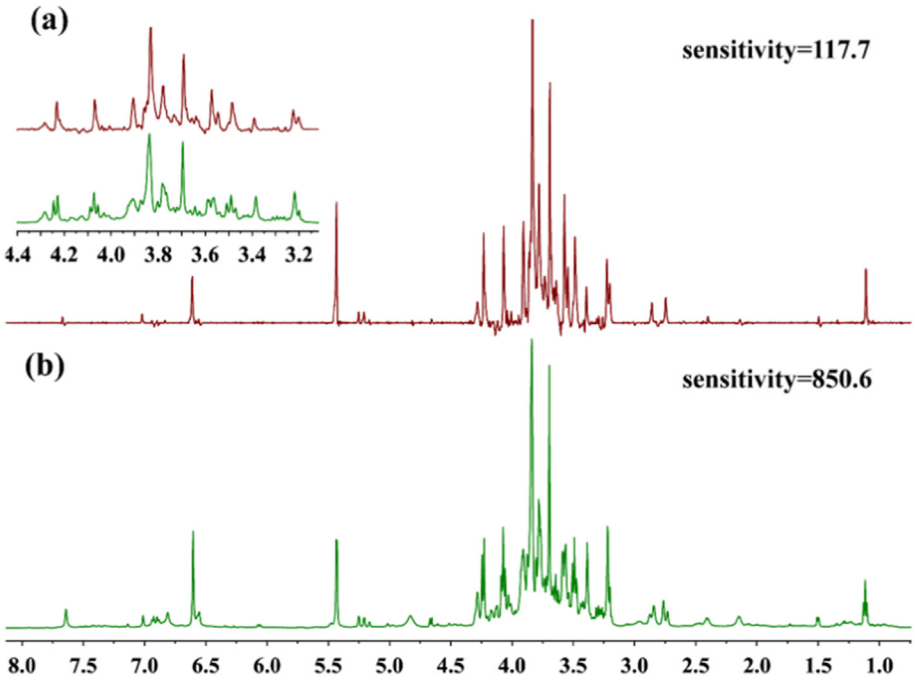

Fig. 7. ^{1}H NMR spectra by PSYCHE (a) and NOESY (b) of tea.

Table 2
Peak assignments in NMR spectra of tea.

Component	Characteristic signals	
	NOESY spectrum	PSYCHE spectrum
Sucrose	3.40 (m[a])	3.40 (s)
	3.64 (t)	3.65 (s)
	3.70 (s)	3.70 (s)
	3.78 (t)	3.78 (s)
	4.07 (t)	4.07 (s)
	4.23 (d)	4.23 (s)
	5.43 (d)	5.43 (s)
Theanine	1.12 (t)	1.12 (s)
	2.14 (m)	2.14 (s)
	3.22 (m)	3.22 (d)
Caffeine	3.50 (t)	3.50 (s)
	3.91 (m)	3.92 (s)
	3.84 (m)	3.84 (s)
Alanine	1.50 (d)	1.50 (s)
	3.84 (m)	3.84 (s)
Fructose	4.13 (t)	4.13 (s)
Epicatechin/epigallocatechin	2.75 (dd)	2.75 (s)
	4.28 (s)	4.28 (s)
Epigallocatechin-3-gallate	6.61 (s)	
Epicatechin/epicatechin-3-gallate	6.90 (m)	
	7.01 (s)	
Fatty acid	1.27 (m)	
Quinic acid	2.00 (m)	
β-Glucose	4.66 (d)	
α-Glucose	5.25 (s)	
Gallic acid	7.20 (m)	

[a] Multiplicity: s, singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet.

separation made by some compounds such as isoleucine, quinic acid, and gallic acid found in Meng's work was not shown in PSYCHE spectra due to the low sensitivity.

Experimental results indicate that resolution-enhanced decoupled spectra can be obtained from honey and tea by PSYCHE. Furthermore, PSYCHE spectra can be combined with multivariate statistical analysis, generating robust and reliable analytical models. With it, contents of sugar including fructose, glucose and sucrose which make the major contribution to the separation of honey are identified. Similarly, caffeine, sucrose, epicatechin-3-gallate and epicatechin, fructose, theanine and alanine contribute to the tea classification of Xiandu and Xianghua. However, because homonuclear decoupling increases the resolution at the cost of decreasing sensitivity, the low sensitivity of PSYCHE method compared to the conventional method, leads to information loss or indistinguishable signals for low concentration compounds. Besides, although it has been proved that PSYCHE technology has an advantage of being relatively tolerant of strong coupling, however, in PSYCHE spectra of honey and tea samples, there are still a few multiple splitting remained. It has been reported that in strongly coupled systems the chemical shift and scalar coupling behavior cannot be completely separated by any method (Zangger, 2015). Moreover, pure shift spectra are obtained based on data reconstruction that might cause rhythmic and hackly peaks, which could bring random errors when combined with multivariate statistical analysis.

In all, PSYCHE did not outperform greatly NOESY on the two datasets involved in this study. One of the main reasons is that PSYCHE provides relative lower sensitivity than NOESY, which results in some low-content compounds are difficult to be detected. The other reason is that both of the two datasets in this study are of relatively small sample size, and the statistical advantages cannot be fully reflected by such a small dataset. Nevertheless, PSYCHE can provide less overlapped spectra by decoupling the homonuclear coupling, which improves the spectral resolution and reduce the negative effects on quantitative analysis due to the spectral overlapping, such as, difficulty in metabolite assignment, quantitative inaccuracy, and so on. Overall, although PSYCHE does not perform better than the conventional methods in most of application areas, such as detection of compounds, sample discrimination, and so on, PSYCHE can serve as a supplementary technique to the standard methods, especially applying in certain agricultural products with a few high-content compounds.

4. Conclusions

pure shift ^{1}H NMR spectra of two natural products of honey and tea with complex composition have been acquired by PSYCHE method, providing the spectral information of high concentrations of

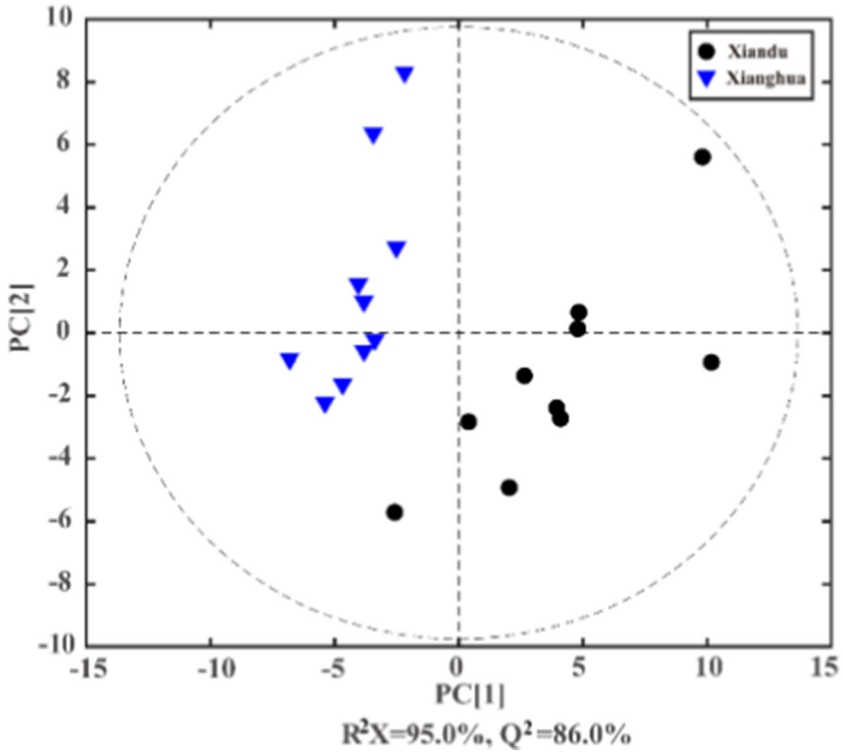

Fig. 8. PCA scores plot of tea samples derived from PSYCHE spectra. Blue triangles and black dots represent tea samples from Xiandu and Xianghua town, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

saccharides in honey and some of polyphenol and amino acids in tea. Compared to NOESY spectra, with resonances decoupled from multiplets to singlets, the spectral resolution of PSYCHE spectra is enhanced to some extent. Besides, combined with multivariate statistical analysis, reliable and robust analytical models are built and differential components based on PSYCHE spectra are further explored. Due to the well-resolved and accurate assignment of singlet resonances after decoupling, PSYCHE is advantageous in identification the differential components and accurate quantification of compound concentrations, while the analysis of NOESY may be subject to interference from overlapped resonances. It should be pointed out that the low sensitivity of pure shift ^{1}H NMR spectroscopy is the major shortcoming in detection of natural products with low-content components in honey, tea, etc. Although the spectral resolution has been improved in decoupled spectra, the resonance overlapping still exists due to the complex composition of natural products. However, an intended performance and promising applications of PSYCHE spectroscopy for food classification and quality control should be feasible on NMR spectrometers operating at higher magnetic fields.

Funding

This work was supported by funding from the National Natural Science Foundation of China (Grant Nos. 11874308, 81871445 and 11705068) and the Natural Science Foundation of Fujian Province of China (Grant Nos. 2016J01078 and 2017J05011).

Contributors

YB: NMR experiment measurement; JF: multivariable analysis; JX: tea sample collection; YH: guidance of PSYCHE experiments; HC: data check; XC: project set-up and management; JD: data evaluation and management; SD: manuscript correction and perfection; ZC: project administration.

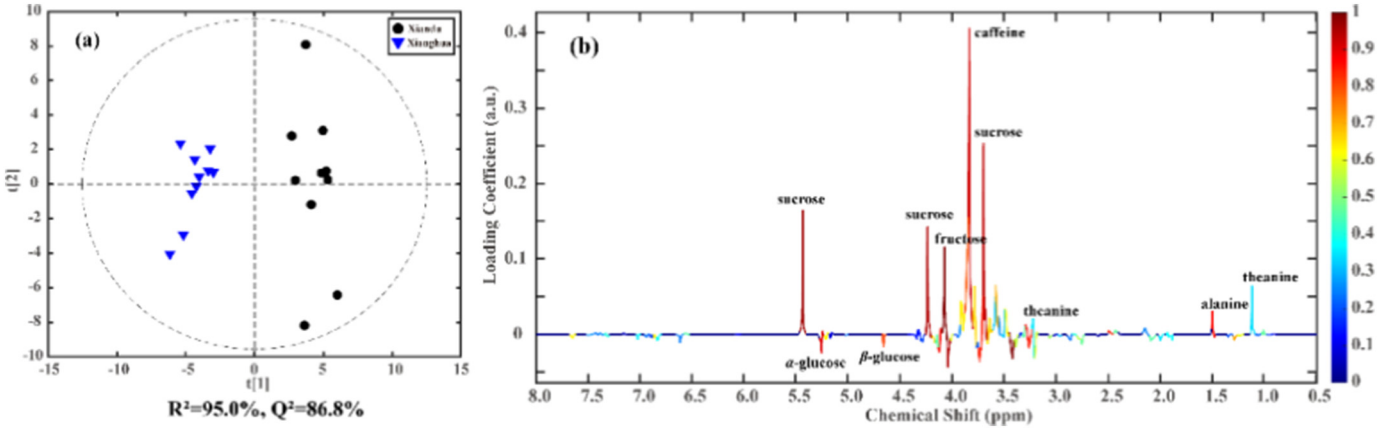

Fig. 9. OPLS-DA scores plots (left panel) and corresponding loadings plots (right panel) derived from PSYCHE spectra of tea samples.

Declaration of Competing Interest

None.

Appendix A. Supplementary data

PSYCHE sequence and its description, ^{1}H NMR spectra of standards samples of fructose and glucose, permutation test results for OPLS-DA models, and STOCSY heat maps of the PSYCHE and NOESY spectral data are provided (Figs. S1–5). Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodres.2019.108574.

References

Aguilar, J. A., Colbourne, A. A., Cassani, J., Nilsson, M., & Morris, G. A. (2012). Decoupling two-dimensional NMR spectroscopy in both dimensions: Pure shift NOESY and COSY. *Angewandte Chemie-International Edition, 51*(26), 6460–6463. https://doi.org/10.1002/anie.201108888.

Aguilar, J. A., Faulkner, S., Nilsson, M., & Morris, G. A. (2010). Pure shift ^{1}H NMR: A resolution of the resolution problem? *Angewandte Chemie-International Edition, 49*(23), 3901–3903. https://doi.org/10.1002/anie.201001107.

Aue, W. P., Karhan, J., & Ernst, R. R. (1976). Homonuclear broad-band decoupling and 2-dimensional *J*-resolved NMR spectroscopy. *Journal of Chemical Physics, 64*(10), 4226–4227. https://doi.org/10.1063/1.431994.

Bax, A., Mehlkopf, A. F., & Smidt, J. (1979). Homonuclear broadband-decoupled absorption-spectra, with linewidths which are independent of the transverse relaxation rate. *Journal of Magnetic Resonance, 35*(1), 167–169.

Cloarec, O., Dumas, M. E., Craig, A., Barton, R. H., Trygg, J., Hudson, J., ... Nicholson, J. (2005). Statistical total correlation spectroscopy: An exploratory approach for latent biomarker identification from metabolic ^{1}H NMR data sets. *Analytical Chemistry, 77*(5), 1282–1289. https://doi.org/10.1021/ac048630x.

Cui, X. Q., & Churchill, G. A. (2003). Statistical tests for differential expression in cDNA microarray experiments. *Genome Biology, 4*(4), 210. https://doi.org/10.1186/gb-2003-4-4-210.

Dai, W. D., Xie, D. C., Lu, M. L., Li, P. L., Lv, H. P., Yang, C., ... Lin, Z. (2017). Characterization of white tea metabolome: Comparison against green and black tea by a nontargeted metabolomics approach. *Food Research International, 96*, 40–45. https://doi.org/10.1016/j.foodres.2017.03.028.

Dal Poggetto, G., Castanar, L., Adams, R. W., Morris, G. A., & Nilsson, M. (2017). Relaxation-encoded NMR experiments for mixture analysis: REST and beer. *Chemical Communications, 53*(54), 7461–7464. https://doi.org/10.1039/C7CC03150E.

De Meyer, T., Sinnaeve, D., Van Gasse, B., Tsiporkova, E., Rietzschel, E. R., De Buyzere, M. L., ... Van Criekinge, W. (2008). NMR-based characterization of metabolic alterations in hypertension using an adaptive, intelligent binning algorithm. *Analytical Chemistry, 80*(10), 3783–3790. https://doi.org/10.1021/ac7025964.

Dieterle, F., Ross, A., Schlotterbeck, G., & Senn, H. (2006). Probabilistic quotient normalization as robust method to account for dilution of complex biological mixtures. Application in ^{1}H NMR metabonomics. *Analytical Chemistry, 78*(13), 4281–4290. https://doi.org/10.1021/ac051632c.

Ernst, R. R., Bodenhausen, G., & Wokaun, A. (1987). *Principles of nuclear magnetic resonance in one and two dimensions* (1st ed.). Oxford, UK: Clarendon Press.

Ernst, R. R., & Primas, H. (1963). Nuclear magnetic resonance with stochastic high-frequency fields - (a simple example to theory of systems with a stochastic hamiltonian). *Helvetica Physica Acta, 36*(5), 583–600.

Foroozandeh, M., Adams, R. W., Kiraly, P., Nilsson, M., & Morris, G. A. (2015). Measuring couplings in crowded NMR spectra: Pure shift NMR with multiplet analysis. *Chemical Communications, 51*(84), 15410–15413. https://doi.org/10.1039/C5CC06293D.

Foroozandeh, M., Adams, R. W., Meharry, N. J., Jeannerat, D., Nilsson, M., & Morris, G. A. (2014). Ultrahigh-resolution NMR spectroscopy. *Angewandte Chemie-International Edition, 53*(27), 6990–6992. https://doi.org/10.1002/anie.201404111.

Foroozandeh, M., Adams, R. W., Nilsson, M., & Morris, G. A. (2014). Ultrahigh-resolution total correlation NMR spectroscopy. *Journal of the American Chemical Society, 136*(34), 11867–11869. https://doi.org/10.1021/ja507201t.

Foroozandeh, M., Castanar, L., Martins, L. G., Sinnaeve, D., Dal Poggetto, G., Tormena, C. F., ... Nilsson, M. (2016). Ultrahigh-resolution diffusion-ordered spectroscopy. *Angewandte Chemie-International Edition, 55*(50), 15579–15582. https://doi.org/10.1002/anie.201609676.

Garbow, J. R., Weitekamp, D. P., & Pines, A. (1982). Bilinear rotation decoupling of homonuclear scalar interactions. *Chemical Physics Letters, 93*(5), 504–509. https://doi.org/10.1016/0009-2614(82)83229-6.

Grimes, J. H., & O'Connell, T. M. (2011). The application of micro-coil NMR probe technology to metabolomics of urine and serum. *Journal of Biomolecular NMR, 49*(3–4), 297–305.

Kakita, V. M. R., Shukla, V. K., Bopardikar, M., Bhattacharya, T., & Hosur, R. V. (2016). Measurement of ^{1}H NMR relaxation times in complex organic chemical systems: Application of PSYCHE. *RSC Advances, 6*(102), 100098–100102. https://doi.org/10.1039/C6RA20047H.

Kiraly, P., Foroozandeh, M., Nilsson, M., & Morris, G. A. (2017). Anatomising proton NMR spectra with pure shift 2D *J*-spectroscopy: A cautionary tale. *Chemical Physics Letters, 683*, 398–403. https://doi.org/10.1016/j.cplett.2017.01.031.

Kortesniemi, M., Slupsky, C. M., Ollikka, T., Kauko, L., Spevacek, A. R., Sjovall, O., ... Kallio, H. (2016). NMR profiling clarifies the characterization of Finnish honeys of different botanical origins. *Food Research International, 86*, 83–92. https://doi.org/10.1016/j.foodres.2016.05.014.

Marcone, M. F., Wang, S., Albabish, W., Nie, S., Somnarain, D., & Hill, A. (2013). Diverse food-based applications of nuclear magnetic resonance (NMR) technology. *Food Research International, 51*(2), 729–747. https://doi.org/10.1016/j.foodres.2012.12.046.

Meng, W. J., Xu, X. N., Cheng, K. K., Xu, J. J., Shen, G. P., Wu, Z. D., & Dong, J. Y. (2017). Geographical origin discrimination of oolong tea (TieGuanYin, Camellia sinensis (L.) O. Kuntze) using proton nuclear magnetic resonance spectroscopy and near-infrared spectroscopy. *Food Analytical Methods, 10*(11), 3508–3522. https://doi.org/10.1007/s12161-017-0920-4.

Meyer, N. H., & Zangger, K. (2013). Simplifying proton NMR spectra by instant homonuclear broadband decoupling. *Angewandte Chemie-International Edition, 52*(28), 7143–7146. https://doi.org/10.1002/anie.201300129.

Molinski, T. F. (2010). NMR of natural products at the 'nanomole-scale'. *Natural Product Reports, 27*(3), 321–329. https://doi.org/10.1039/b920545b.

Morris, G. A., Aguilar, J. A., Evans, R., Haiber, S., & Nilsson, M. (2010). True chemical shift correlation maps: A TOCSY experiment with pure shifts in both dimensions. *Journal of the American Chemical Society, 132*(37), 12770–12772. https://doi.org/10.1021/ja1039715.

Nilsson, M., & Morris, G. A. (2007). Pure shift proton DOSY: Diffusion-ordered ^{1}H spectra without multiplet structure. *Chemical Communications, 9*, 933–935.

Padovan, G. J., De Jong, D., Rodrigues, L. P., & Marchini, J. S. (2003). Detection of adulteration of commercial honey samples by the $^{13}C/^{12}C$ isotopic ratio. *Food Chemistry, 82*(4), 633–636. https://doi.org/10.1016/S0308-8146(02)00504-6.

Pan, R., Chen, H. P., Wang, C., Wang, Q. H., Jiang, Y., & Liu, X. (2015). Enantioselective dissipation of acephate and its metabolite, methamidophos, during tea cultivation, manufacturing, and infusion. *Journal of Agricultural and Food Chemistry, 63*(4), 1300–1308. https://doi.org/10.1021/jf504916b.

Shen, G. P., Huang, Y., Dong, J. Y., Wang, X. X., Cheng, K. K., Feng, J. H., ... Ye, J. D. (2018). Metabolic effect of dietary taurine supplementation on Nile Tilapia (Oreochromis nilotictus) evaluated by NMR-based metabolomics. *Journal of Agricultural and Food Chemistry, 66*(1), 368–377.

Venir, E., Spaziani, M., & Maltini, E. (2010). Crystallization in "Tarassaco" italian honey studied by DSC. *Food Chemistry, 122*(2), 410–415. https://doi.org/10.1016/j.foodchem.2009.04.012.

Zangger, K. (2015). Pure shift NMR. *Progress in Nuclear Magnetic Resonance Spectroscopy, 86-87*, 1–20. https://doi.org/10.1016/j.pnmrs.2015.02.002.

Zangger, K., & Sterk, H. (1997). Homonuclear broadband-decoupled NMR spectra. *Journal of Magnetic Resonance, 124*(2), 486–489. https://doi.org/10.1006/jmre.1996.1063.

Zheng, X., Zhao, Y. R., Wu, H. F., Dong, J. Y., & Feng, J. H. (2016). Origin identification and quantitative analysis of honeys by nuclear magnetic resonance and chemometric techniques. *Food Analytical Methods, 9*(6), 1470–1479. https://doi.org/10.1007/s12161-015-0325-1.