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Multivariate Analysis for ^{1}H-NMR Spectra of Two Hundred Kinds of Tea in the World

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NMR measurements coupled with pattern-recognition analysis offer a powerful mixture-analysis tool for latent-feature extraction and sample classification. As fundamental applications of this analysis for mixtures, the ^{1}H spectra of 176 kinds of green, black, oolong and other tea infusions were acquired by a 500 MHz NMR spectrometer. Each spectrum pattern was analyzed by a multivariate statistical pattern-recognition method where Principal Component Analysis (PCA) was used in combination with Soft Independent Modeling of Class Analogy (SIMCA). SIMCA effectively selected variables that contribute to tea categorization. The final PCA resulted in clear classification reflecting the fermentation and processing of each tea, and revealed marker variables that include catechin and theanine peaks.

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Introduction

Although the NMR spectroscopic technique has been now a powerful tool for molecular structure analysis, the target samples have been limited to purified compounds. However, the requirements for analyses of mixtures, such as biological fluids, cosmetics, food, beverages, supplements, additives, industrial materials, intermediate compounds of chemical reaction assays and polymers have been increasing.

NMR-based metabonomics has become focused on the rapid development of the bioscience frontiers of genomics and proteomics. Nicholson *et al*. have intensive research insight into the NMR spectroscopy of biological fluids, such as urine, spinal fluid, serum and intact tissue, and have established an integrated NMR-spectroscopic and chemometric method for metabolome.[1] Their investigation of various kinds of toxicity in animals has been combined with pattern-recognition methods for a metabolic change in an integrated biological system rather than individual cells.[2,3] We studied urinalysis using ^{1}H-NMR spectra of disease model rats to detect basic metabolisms, which resulted in clear classifications.[4]

For quality assessment or authentication, a metabolite profiling or some chemometric analysis using ^{13}C or ^{1}H NMR spectroscopy was carried out for several kinds of tea,[5] coffee,[6] juice,[7] beer,[8] olive oil,[9] crude oil,[10] and other mixtures.[11] The components of some of the kinds of teas were analyzed using mass spectroscopy,[12] HPLC[13] or ^{1}H NMR spectroscopy.[5] Gall *et al*. researched the metabolite profiling of 191 kinds of green teas, mainly Chinese tea, using ^{1}H NMR spectroscopy.[5] They discussed in detail the chemical compounds of the teas and carried out of PCA and a cluster analysis in connection with quality assessment and authentication. They identified about 30 kinds of organic chemical components in the tea. Four or more kinds of catechin, caffeine, six kinds of amino acids, including theanine, which is unique to tea, and some sugars were found. The kinds of catechin are different between each tea.[12-14] In Japan, many researchers have reported on the chemical compositions, processing, taste, aroma, and health-related properties of Japanese green tea.[15-17]

Their chemometric methods were still insufficient, and also provided unclear results for tea mixed with complex and multi-factorial components. For the successful analysis of mixtures, fundamental applications are needed to establish a suitable procedure.

Originally, chemometric methods including PCA, PLS, SIMCA had been developed in the field of IR and Raman spectroscopy to deconvolve the broad spectra.[18-20] It has subsequently been applied to the NMR spectra of bio samples for the analysis of overlapped peaks and for categorization of the metabolic functional profile.[21,22] However, these multivariate methods combined with NMR spectroscopy have not yet been well-established for standard use.

The object of this paper is to provide a detailed multivariate description based on NMR spectroscopy and a comprehensive assessment of this approach by examining a mixed sample. We used over 180 kinds of green, oolong, black and other teas, and measured their ^{1}H NMR spectra. In a ^{1}H spectral pattern the chemical compositions are reflected. It is not necessary to identify the proton signals of chemical compounds. Each spectrum is used to recognize as a whole pattern and the related chemometric solutions can be adequately applied to discern the significant features of the spectra.

Experimental

Materials

Most of the teas were purchased at shops selected as possible

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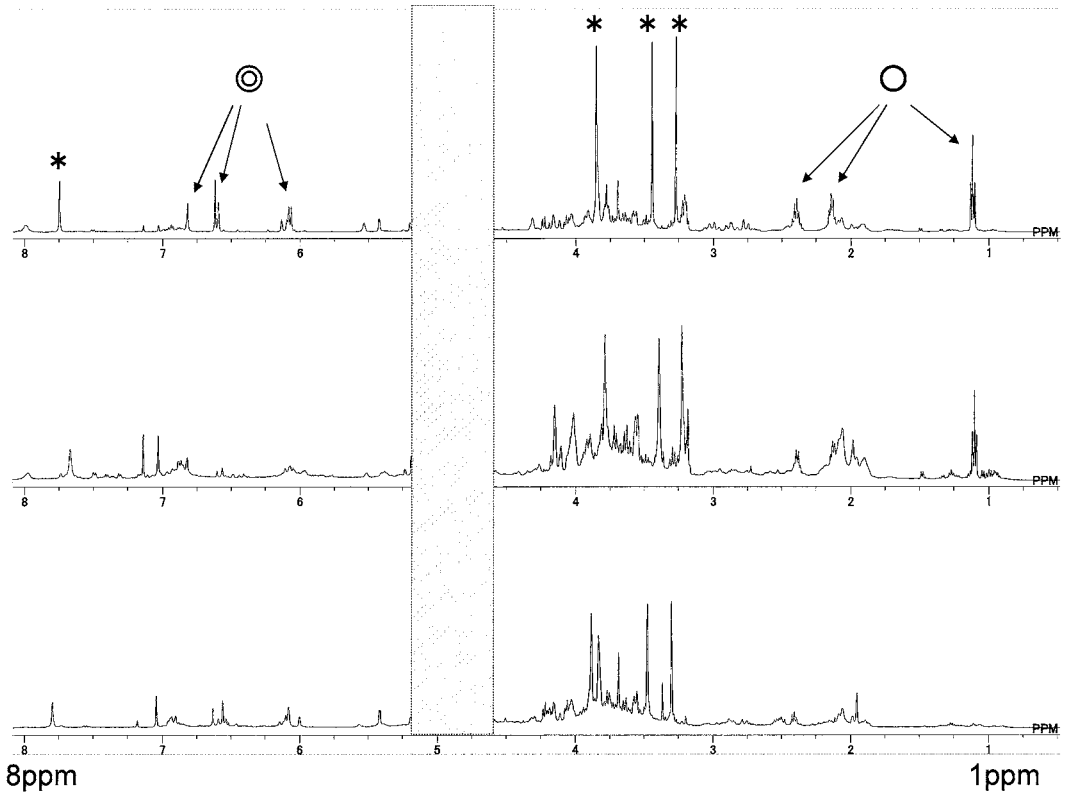

Fig. 1 Example spectra of teas. From the top; Japanese green tea (Yabukita), black tea (Assam), and hoji tea (Kofu). The grey box including water signals indicates the region removed from the analysis in each data set. On the top spectrum, ○, ◎ and ✻ indicate the peaks of theanine, caffeine, and catechin, respectively.

sources of pure species, and several other teas were supplied by the Vegetable and Tea Institute, and the University of Shizuoka. The genetic strain, season of picking, processing, *etc*. of the latter teas were clearly known, but the majority of commercial teas were mixed and identified only by the labels of the packages. One hundred seventy-six teas were available for this study: 49 were Japanese green teas, 6 were Chinese green teas, 51 were black teas, 52 were oolong teas, 10 were Japanese hoji teas, 2 were yellow teas, 2 were white teas, and 4 were puer teas. Some of the Chinese and black teas were mixed with jasmine, mushroom, vanilla or bergamot.

Extraction

Three grams of tea leaves were stirred with 5 mL of distilled water at 75˚C, allowed to cool for 3 min, and centrifuged at 10000 rpm for 10 min. Each sample consisted of 500 μL of the supernatant, and 50 μL of D_2O containing TSP as a reference in a 5-mm tube.

NMR spectroscopy

The ^{1}H NMR spectra were recorded at 25˚C using a 500 MHz JEOL ECA NMR spectrometer provided with an auto-sampler. D_2O was used as the internal field-frequency lock. Each spectrum consisted of 16 K complex data points with a spectrum width of 5 kHz, obtained by 128 scans with an acquisition time of 1.75 s and a recycle delay of 5 s per scan. The pulse angle was 45˚. The pre-saturation pulse sequence was used to suppress the water signal.

NMR data-reduction procedures and pattern-recognition analysis

Each NMR spectrum was segmented into 228 regions of 0.04 ppm width over a range of 0.40 to 9.52 ppm, and an integral calculation was performed for each spectral region. Any integrated regions from 4.52 to 5.20 ppm that contained a water signal were eliminated from the data table, and then the total data were reduced to 211 regions. The remaining integral values of each spectrum were normalized to the total of the summed integral values for 100 in order to compensate for any concentration difference between the tea samples.

The spectral processing mentioned above was performed by "ALICE2 for Metabolome" β version software, followed by multivariate statistical analysis using Sirius (PRS, Norway) version 6.5 software. We developed ALICE2 based on the whole trial analysis described in this paper, and released as a new software, "ALICE2 for Metabolome" version 1.0 (JEOL),[23] which integrates NMR spectroscopy and the multivariate pattern-recognition method of PCA and SIMCA in a single interface. The functionalities will be introduced elsewhere.

Results and Discussion

^{1}H spectra of teas

The ^{1}H NMR spectra were obtained for a total of 187 teas infused from the leaves. The metabolic profiling of *Camellia sinensis* is also an interesting problem. The detailed assignment of signals has been mentioned elsewhere.[24] Green tea is unfermented, oolong is semi-fermented, and black is fully fermented. Hoji tea is roasted green tea. Japanese green tea and Chinese green tea are processed by steaming and by baking, respectively. Fermentation is an oxidation process by peroxidase in the tea leaf, which produces theaflavins and

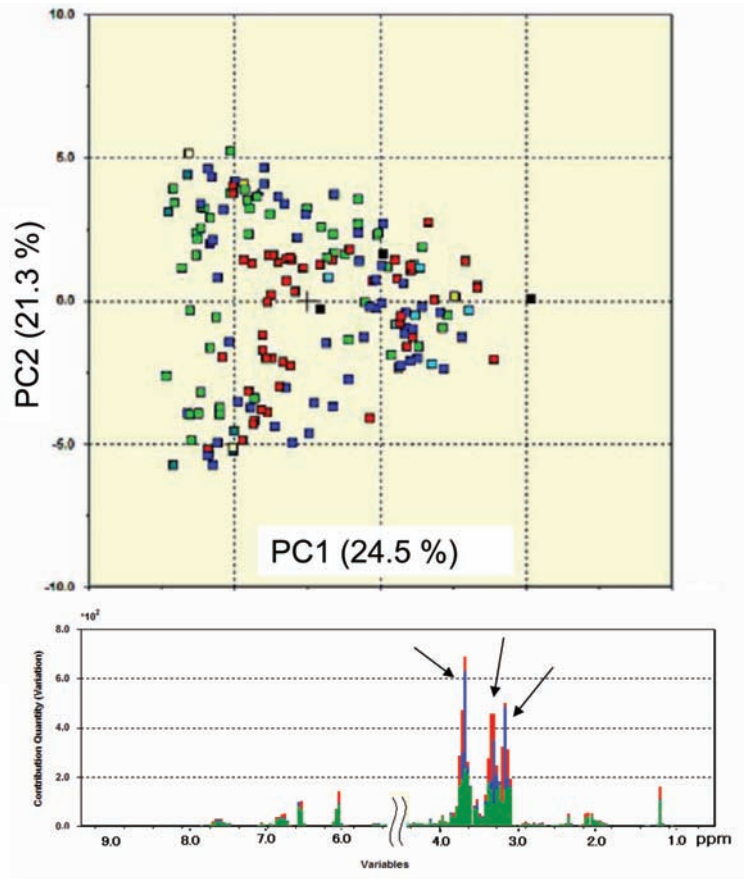

Fig. 2 PCA score plot of PC1-PC2, in which 211 variables were equally accounted for the data sets. Each spectrum is colored by the category of tea. Green, Japanese green tea; dark green, Chinese green tea; red, black tea; blue, oolong tea; sky blue, hoji tea; black, puer tea; yellow, yellow tea; white, white tea. The lower plot is the contribution quantity of 211 variables, showing that responsible for PCA classification. The red, blue and green bars correspond to the contribution to PC1, PC2 and the residuals, respectively. The arrows from the right correspond to the peaks of 3.30, 3.46 – 3.50, and 3.86 ppm, respectively.

thearubigins *etc*.[25,26] The theaflavins and thearubigins present in black and oolong teas, and those compositions might be reflected in the spectral patterns. Figure 1 shows the ^{1}H NMR spectra selected as a typical example of 3 type of tea, and in the top spectrum, the characteristic signals of 3 major components of teas mentioned in the Introduction section are indicated. All of the other chemical compositions, including polyphenols, differ between teas. The chemical compositions are influenced not only by the level of fermentation, but also by many other factors: the processing, genetic strain, growth altitude, picking season, storage, flavor-additives, *etc*. In our experiment, the tea infusions sampled were similar to the tea we drink in daily life, without the addition of any chemical buffer for controlling the pH or other modification.

Pattern recognition analysis: (I) PCA-method

Eleven spectra were recognized as outliers and discarded by a preliminary statistical analysis. Subsequently a total of 176 teas were used in the pattern-recognition analysis. The number of teas in each category is listed in the section of *Materials*. They were submitted to PCA in which all of the 211 variables, bucketed regions, were equally accounted for the data sets. The result of the PCA score plot of PC1 and PC2 is shown in Fig. 2. One point shows one spectrum of tea. This figure shows no clear classification between the tea categories from colored differently beforehand. The variance plot of each variable which explains the contribution to the first major two PC's and the residuals were added with the score plot. The plot shows that the 3 larger variance regions around the variables of 3.30, 3.46 – 3.50, and 3.86 ppm contain the 3 largest caffeine peaks. The grouping in the PCA score plot is influenced mostly by the largest quantities of variation. In this case, the caffeine peak intensities have the predominant effect.

The initial intention of this experimental analysis was to differentiate teas according to the categories we know in daily life, *e.g*. green tea, black tea and oolong tea. We did not predict a major contribution of the caffeine peak variance to the classification. The score sum of PC1 and PC2 is 46%, which was at a low level for explaining the characteristics of the samples. In the other case of mixed samples, employing simple PCA once may perform a well-defined classification.[4] For an advanced analysis to extract the target information from samples, the possible next steps are as follows:

1) Expand the higher order PCs by exploring the pairs of PC4-PC5 or PC1-PC6 and so on.
2) Standardize or scale each variable for submitting PCA.
3) Adapt the SIMCA method for adequate variable selection and to resubmit PCA.

Method 1) is not recommended in an exploration analysis because it is an artificial trick to apply higher principal components with lower contributions than PC1 or PC2, especially in the case where the number of samples is small for

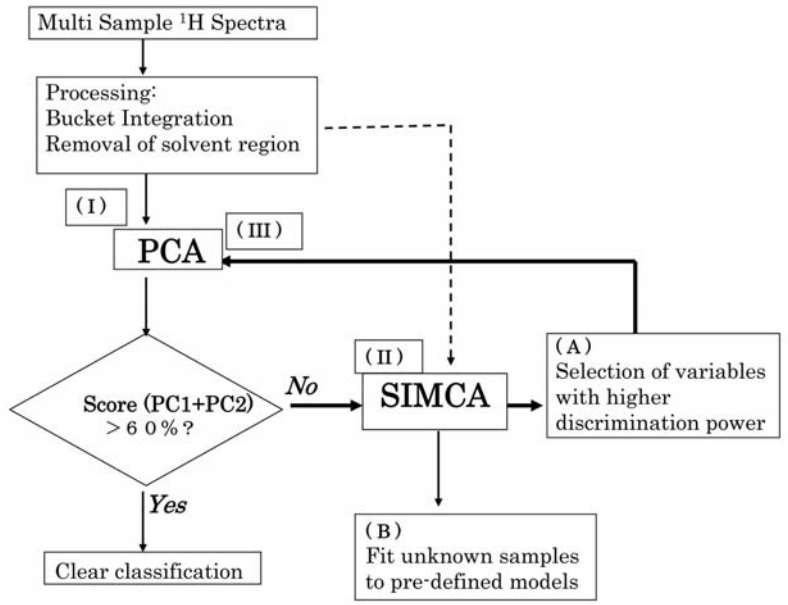

Fig. 3 Flow chart of the analysis. The flow shown as a bold line, from (I), (II) to (III) is the main one, however, other lines such as a dashed line are also available.

the statistical confidence. Method 2) is available essentially when they have a large dynamic range, which is not adequate in the ordinary case of NMR spectral analysis, except for special purposes, because the information on the spectral intensities drops out and noises are often emphasized. Consequently, method 3) will enable us a useful analytical procedure not only for NMR spectra datasets, but also for any other spectroscopic data sets.

A flow chart of the analysis is shown in Fig. 3, which contains the essential flow of (I) PCA → (II) SIMCA → (III) PCA. Following step (I), the next procedure of the (II) and (III) steps will be explained below.

Pattern-recognition analysis: (II) SIMCA-method

In most cases of mixture analysis, *a priori* rough knowledge of the sample, such as normal or abnormal, is available. It is noted that the SIMCA method is powerful at making effective use of knowledge and enabling us to conduct further analysis.

(II)-1) Preparatory step of SIMCA; making a mathematical model. First, the "training set" was selected as one type of tea, for example, the 51 black teas listed in the *Materials* section were selected and a "black tea model" was made in mathematical terms by applying PCA. Then, green tea samples were selected and a "green tea model" was made independently.

Figure 4 shows a Cooman's plot of the 2 independent models of black and green aligned on the *X* axis and *Y* axis, respectively. The two rigid lines show the class boundaries, *i.e*., 68% confidence, 1σ, of the classification based on each of the green and black tea models.[1] There are no samples in the mixed area of the 2 models in Fig. 4; thus, the Cooman's plot resulted in a well-characterized classification of the 2 models. The 2 models are considered to characterize by the fermentation level, since green tea is non-fermented and black tea is fully fermented.

(II)-2) Use of the SIMCA method. The SIMCA method has the following 2 advantages:

A) The modeling power of each predefined model and the discriminating power between the 2 models can be calculated.

B) Unknown samples are able to fit onto the predefined models (classes).

A) Selection variables by discrimination power: This is step A) in the flow of Fig. 3. In Fig. 4, Cooman's plot was added with 3 plots of 211 variables; the upper 2 plots are the modeling powers for green and black teas, respectively, and the lower plot is the discrimination power between the 2 tea models. Although the modeling power profiles, *i.e*. overall spectral patterns, resemble each other, there are some variables with distinct high values in the discrimination plot. The variables with higher discrimination power than 1.0 contribute to discerning the two. The cutoff level of discrimination power is not fixed, but depends on the characteristics of the samples. Here, the set V_{gb} of 28 variables with higher discrimination power than 2.0 was selected. In the same way as for the pair of models; green and hoji teas were employed SIMCA; the results are shown in Fig. 5. In this case, the 2 modeling power profiles do not resemble each other, and the discrimination powers are mostly higher than that of Fig. 4. The variables with discrimination power higher than 3.0 were selected as sets of V_{gh}. Likewise, SIMCA was also applied to hoji and black models, and the variables were selected as the set V_{hb}, not shown here in the figure. Thus, the total number of variables besides the overlapping ones was 46, $V_{gb} + V_{gh} + V_{hb}$, as summarized in Table 1. They are used in the following analysis of PCA, in the latter section (III).

B) Fitting a sample: Taking step B) in Fig. 3, 52 oolong samples were fitted to either model of green and black tea in Cooman's plot in Fig. 4. The result is shown in Fig. 6; each sample of oolong teas that have distances of horizontal and vertical vectors from each model was calculated and plotted in the Cooman's plot. For the oolong teas, where broad distributions appeared, some samples overlapped with the green tea model, the others with black tea model; the remaining samples were placed in between, or far from the 2 groups. Several oolong teas were taste tested by us, and were found to be matched well to this Cooman's plot. This shows the excellent presentation of classification reflecting the fermentation level. It is considered that there was no analytical method available to differentiate the fermentation, itself, until now.

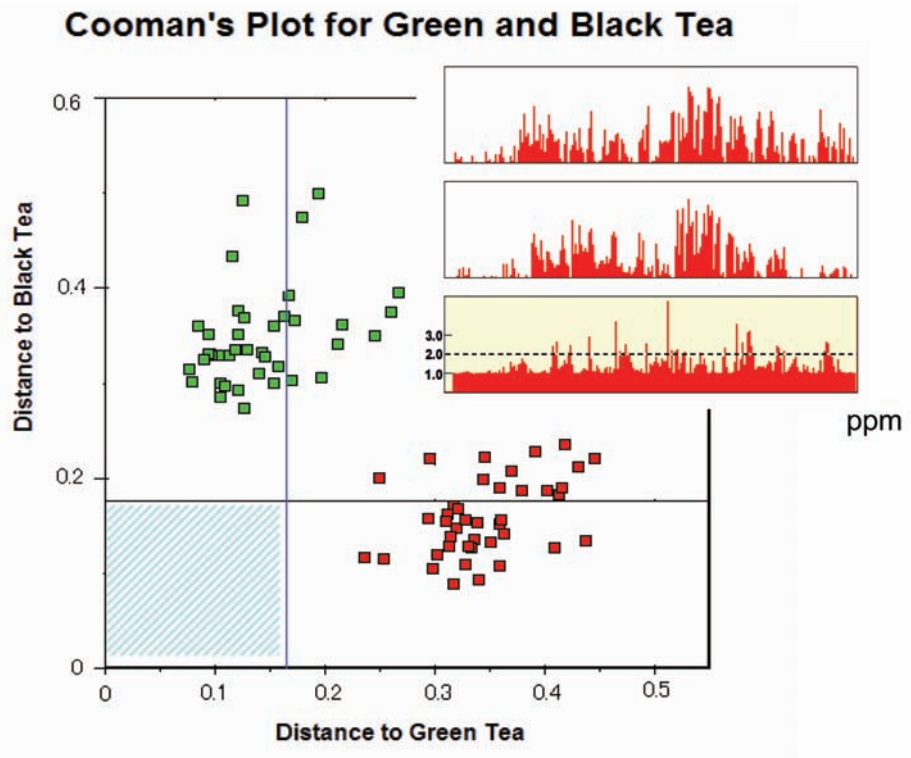

Fig. 4 Cooman's plot for 2 models of black and green tea. The symbol color indicates the same as Fig. 2. The Cooman's plot describes the confidence of each model by the vertical and horizontal solid lines. The shaded quadrant shows an unoccupied field that indicates a mixed area of both models' classification. The upper right plots show from the top: green and black modeling power plots, respectively and discrimination power plot of 211 variables.

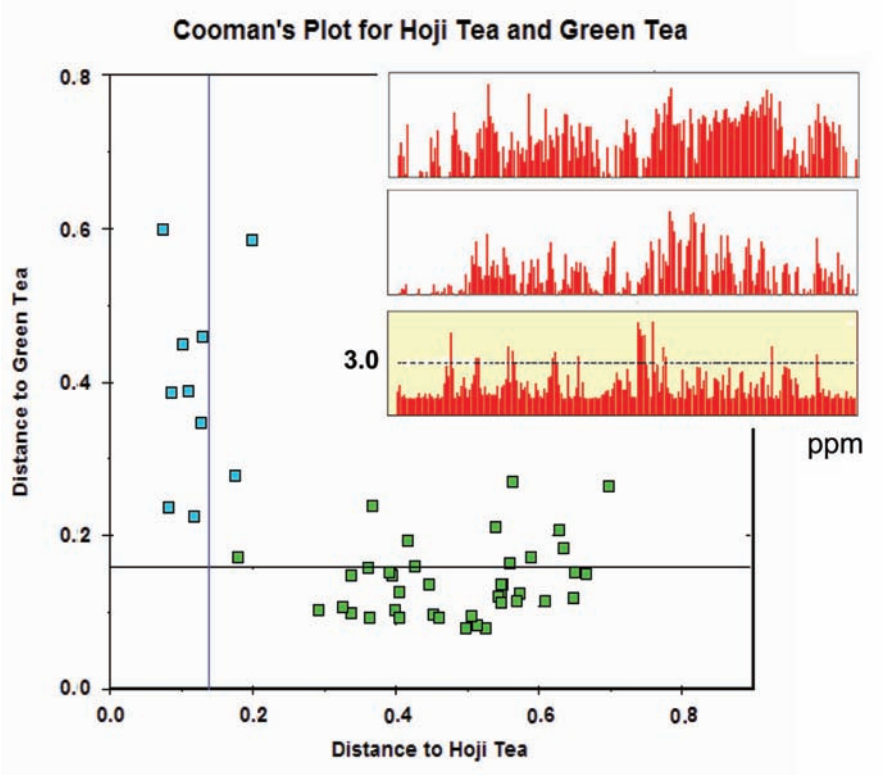

Fig. 5 Cooman's plot of the hoji and green tea models, and the both modeling power plots, from the top hoji and green tea, respectively and discrimination power plot of variables.

Table 1 Selected variable sets with higher discrimination power of each pair of teas, V_{gr}, V_{gh}, V_{rh}

	ppm (±0.02)	V_{gr}	V_{gh}	V_{rh}
1	7.98		*	
2	7.46			*
3	7.42	*	*	*
4	7.38			*
5	7.34	*	*	*
6	7.30		*	
7	7.14			*
8	7.06	*		
9	6.66	*		
10	6.58		*	*
11	6.54		*	*
12	6.50		*	
13	6.10	*	*	
14	6.02	*		
15	5.94	*		
16	5.90	*		
17	5.86	*		
18	5.46	*		
19	4.34	*	*	*
20	4.26	*		*
21	4.22		*	*
22	4.14	*	*	
23	4.02	*	*	
24	3.98	*	*	
25	3.66	*	*	
26	3.62		*	
27	3.34		*	
28	3.22			*
29	3.14	*		
30	2.90	*		
31	2.78	*	*	
32	2.66	*		
33	2.62	*		
34	2.58	*		
35	2.54	*	*	
36	2.14		*	
37	2.06	*		
38	1.98			*
39	1.74		*	
40	1.70		*	
41	1.66	*	*	
42	1.50			*
43	1.14		*	*
44	1.06	*		
45	1.02	*		*
46	0.98	*		
		28	23	15

Each number of variables is 28, 23, and 15, respectively. The total number omitting the overlap is 46.

Pattern recognition analysis: (III) PCA-method

Following step (A), the final step (III) in Fig. 3 is to submit the PCA again with the 46 adequate variables selected. In process (I) of the preliminary PCA, the overall variables equally accounted for the data sets consequently acted as noises against extracting the especially focused features in a multi-factorial complex mixture, such as this case of commercial teas.

The result is shown in Fig. 7. The green and black teas are classified into separate groups with least mutual overlapping; the oolong teas are distributed diffusely between the 2 groups and overlap with the other 2 groups. Yellow and white teas were located at the near marginal region between the green and black tea groups. Considering that green tea is non-fermented, black is fully fermented, yellow and white teas are slightly fermented and oolong ones are at various stages of fermentation, this classification expresses the important feature of tea fermentation. Hoji tea, which is roasted green tea of low grade,[14] was segregated from the green tea group in the PCA score plot. Roasting is considered to be thermal oxidation other than the enzymatic oxidation of fermentation, and hoji tea was reasonably classified. The puer tea was transformed Chinese green tea by a combination with microbial metabolism that contains complex fermentation processes; it was located at the exact marginal region between the black tea and hoji tea groups in the PCA score plot. This resulted in a very interesting classification; nevertheless, our SIMCA was not employed for puer tea.

A more detailed inspection shows the difference between the 2 green tea groups, Japanese and Chinese. The difference could be interpreted to reflect the processing between the 2 groups, as mentioned above in this section. To sum up, both PC1 and PC2 together indicate the responsibility for fermentation (oxidation) and other processing, of which the features are the initial aim of tea category classification. It was shown that the score sum of PC1 and PC2 was 73%, which is a good level.

In addition within the Japanese green tea group, we found Uji-cha and Shizuoka-sencha located in the near upper and central part, respectively, and Ban-cha was in the lower region, not indicated in the figure. These 3 teas are generally of higher, middle and lower grade, respectively. Hoji tea was also located in the lower region. These results may suggest that a detailed characterization or authentication could be possible with an augmented sample set, as suggested by our experimental PCA score plot.

Figure 8 shows the distribution of the contribution quantities that have the same meaning as Fig. 2. The major contribution variables are the consequent marker of resonance signals at 1.14, 3.14, 6.10, and around 6.6 ppm, which is a different set of variables from that of Fig. 2. The bucketed regions of 1.14 ppm surely correspond to theanine, and that of 3.14 ppm might correspond to one of the caffeine signals. However, it might identify a compound other than caffeine, because not all of the caffeine signals appeared together. To resolve the overlapped peaks for unambiguous assignment, further analysis is necessary, such as 2D NMR techniques. For example, the DOSY technique will add the correct dimension to resolve overlapped peaks in 3 – 4 ppm, and give additional information concerning catechin chemistry, as stated in the section *^1H spectra of teas*; black teas and puer teas are known to have oxidized catechins, such as theaflavin or the catechin dimmer.[25] The peaks of 6.10 and around 6.60 ppm seem to correspond to catechins, and it is worth mentioning that the latter regions of catechin appeared as a marker variable candidates, even though their intensities are very small in the original spectra, as shown in Fig. 1. As for our first screening stage, it showed that catechins and theanine characterize the tea categories as they appear in the literature.

Reproducibility of PCA for sample preparation

All of the tea samples were extracted at 75˚C for 3 min and measured within 3 h in our experiment. One of the Japanese green teas, Uji-cha, was used for a reproducibility test of spectra over temperature and time. The tea samples were infused at temperatures of 60, 75, and 90˚C, respectively. In each case, measurements at times of 1, 3, and 7 h while being sealed up in sample tubes after infusion were also carried out. The 9 spectra themselves were submitted to PCA, and the result showed good repeatability in times, but showed some spread by temperature. However, the spread with temperature is actually very small in the whole PCA score plot in Fig. 7. The 9 samples were submitted to PCA together with other teas and they fell into the green tea group; the whole PCA score plot was not very much different from Fig. 7, not shown here. Thus, the repeatability of the spectrum pattern for the sample in our experiments is ensured.

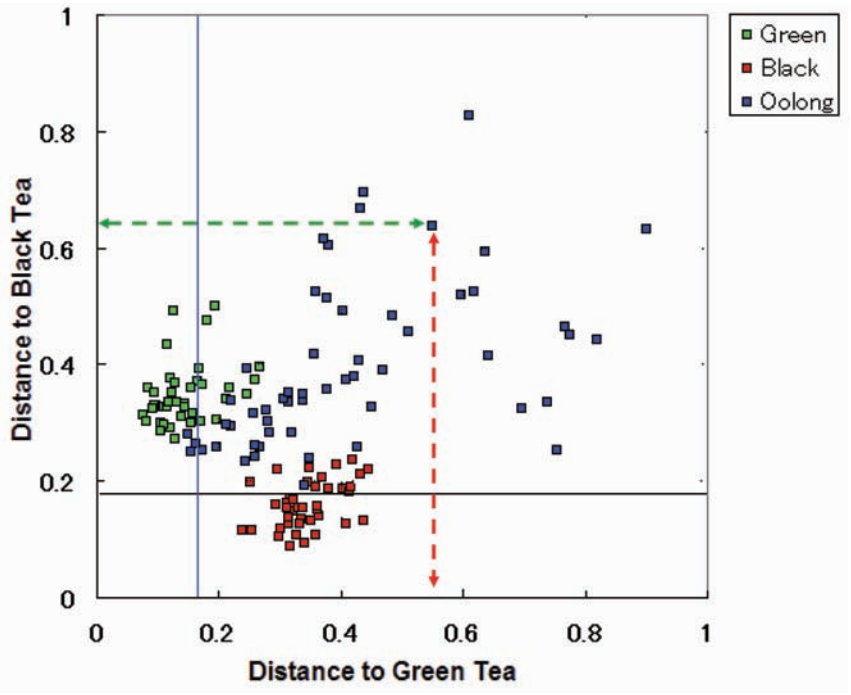

Fig. 6 Fitting oolong teas onto the two predefined black and green tea. The red and green dashed lines indicate the residual distances of one of the samples from the black and green tea models, respectively.

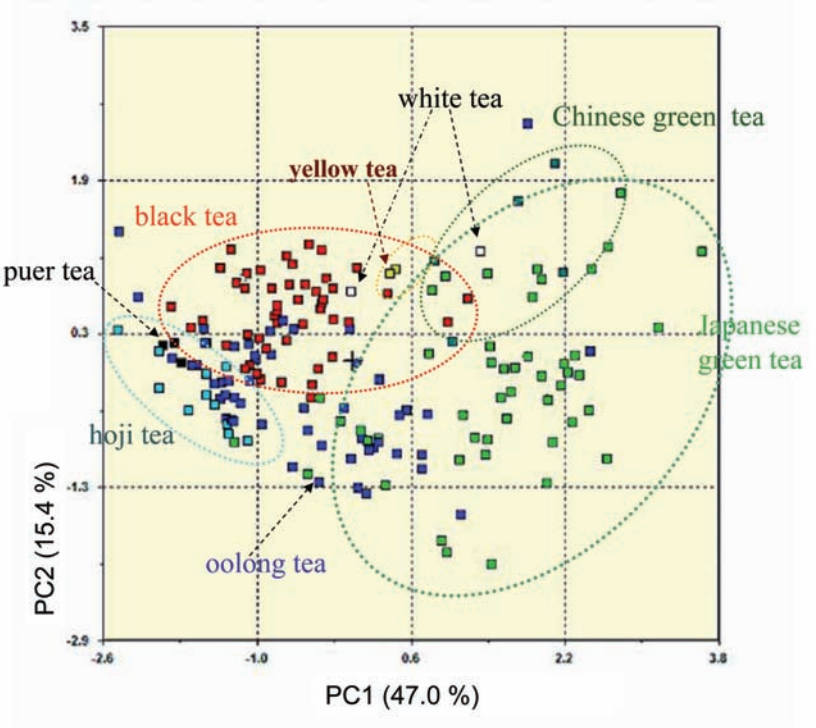

Fig. 7 Final PCA score plot, in which selected variables in the Table 1 are accounted for the data sets. The symbol colors indicate the same as in Fig. 2. The score of PC1 and PC2 is 47.0 and 15.4%, respectively.

Conclusion

^{1}H spectra of about 180 kinds of tea were measured and analyzed by a pattern-recognition method. In the first analytical step, PCA was used to survey the whole profile of the samples, and then SIMCA was submitted for modeling by effective use of *a priori* knowledge of the sample sets. We selected the variables contributing to discerning the tea categories by the SIMCA method, and using the variables subjected to PCA again, which resulted in clear classification. This procedure successfully extracted sample features and marker variables in

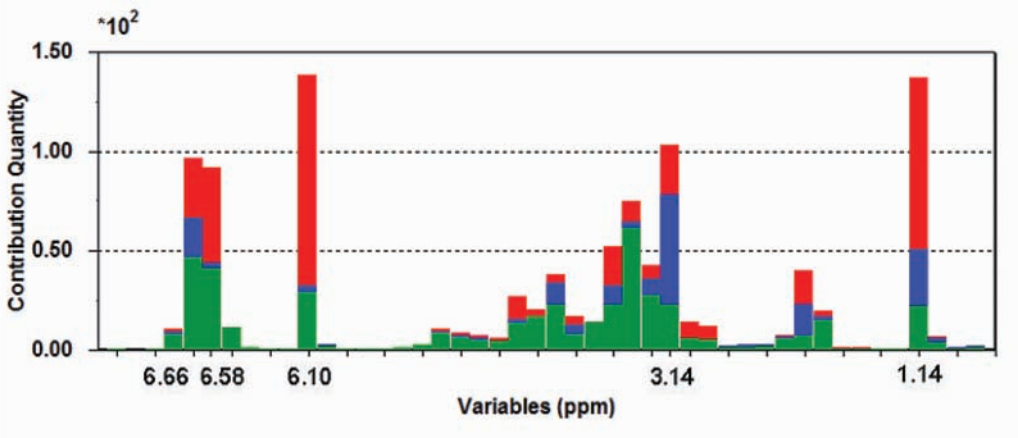

Fig. 8 Distribution of contribution quantities of 46 variables, responsible for the first 2 PCs and the residuals, which are in Table 1. The largest 5 quantities are added by the chemical shifts (ppm). The red, blue and green bars are the same as in Fig. 2.

the case of a multi-factorial complex system such as tea.

In conclusion, the integrated NMR-spectroscopy and the chemometric methods of PCA and SIMCA will provide a standard and robust procedure for mixture analysis.

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