Gas Chromatographic-Mass Spectrometry and Chemometric Analysis for Detection and Quantification of Paraffin in Beeswax

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GC-MS fingerprint analysis, principle component analysis (PCA), and partial least square (PLS) analysis were introduced for detection and quantification of paraffin in beeswax. Firstly, beeswax, paraffin and standard adulterated samples were prepared and analyzed by GC-MS. Secondly, seventeen chromatographic peaks were selected as characteristic peaks and their relative peak areas (RPAs) were calculated for quantitative expression of the GC-MS fingerprints. Then, the PCA is performed after a suitable data processing. The scores of PCA showed that two types of beeswax and paraffin could be clustered reasonably into different groups. Lastly, Seven PLS factors were selected to build the PLS model with cross-validation. The plot of the predicted concentration versus the actual concentration values appeared to show the high precision of model. Therefore, GC-MS fingerprint in combination with chemometric techniques provide a very flexible method for detection and quantification of paraffin in beeswax.

Keywords: gas chromatography-mass spectrometry (GC-MS), beeswax, paraffin, principal component analysis (PCA), partial least square (PLS)

Introduction

Beeswax is a most important bee product besides honey that widely used for centuries as a working material in different industries as well as in cultural and artistic applications. In recent years, it has been gradually used more and more in food, pharmacy and cosmetic fields which are very close to our lives. This continuing importance frequently requires a reliable quantity assurance of beeswax. However, due to a high demand for pure beeswax and limited resources available, this product has been increasingly adulterated with much cheaper hydrocarbons of alien origin. As a result, the quality of beeswax has been severely compromised, which makes it useless for the industries mentioned above. What's more, these industrial grade adulterants contain many carcinogens such as heavy metals and polycyclic aromatic hydrocarbons. It has been a latent chronic threat to the health of human being. Consequently, the authenticity of beeswax is becoming

progressively more important.

In fact, the current quality criteria for pure beeswax according several pharmacopoeia i.e. acid value, ester value, saponification value and drop point, tests for paraffin and other adulterant are inadequate (Ruoff and Bogdanov, 2004), since various factors could affect analytical values, and so also detection of adulteration (Tulloch, 1973). Ensuring product authenticity and implementing some of these strict standards and criteria has been very difficult. Advanced analytical methods based on gas chromatography (GC) (Jimenez *et al.*, 2006; Asperger *et al.*, 2001; Regert *et al.*, 2005), gas chromatography-mass spectrometry (GC-MS) (Asperger *et al.*, 1999; Asperger *et al.*, 1999; Aichholz and Lorbeer, 1999; Aichholz and Lorbeer, 2000; Garnier *et al.*, 2002) have been utilised to characterize the compositions of beeswax. Furthermore, the GC-MS analysis brought to some essential differences in beeswax composition between pure beeswax and some beeswax samples where mixture of pure beeswax with another substances is suspected by comparing chromatographic profiles (Jimenez *et al.*, 2004; Bonaduce and Colom-

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bini, 2004; Jimenez *et al.*, 2003). Identification of beeswax adulteration with hydrocarbons of alien origin by selection of three alkanes: $C_{20}H_{42}$, $C_{22}H_{46}$ and $C_{24}H_{50}$ was elaborated by Ewa *et al.* (Was *et al.*, 2010). Fingerprints and characteristic marker compounds were employed for discrimination and identification purposes. Considering that beeswax is a complex natural material, an appropriate characterisation of fingerprints requires the use of many variables. Thus, chemometric techniques can be introduced to find trends or associations among the analysed data, or to establish a subset of measured parameters allowing beeswax characterisation.

Chemometrics is a practical statistic approach that applied (a) to design or select optimal measurement procedures and experiments, and (b) to provide maximum chemical information by analyzing chemical data (Sharaf *et al.*, 1986). Minimalistic approaches, such as the principal component analysis (PCA) with the aim to reduce a multidimensional space to a lower dimensional planes, are regularly used to investigate complex problems. Partial least squares (PLS) is used to find the fundamental relations between two matrices (X and Y), i.e. a latent variable approach to modeling the covariance structures in these two spaces. PLS-regression is particularly suited when the matrix of predictors has more variables than observations, and when there is multicollinearity among X values (Latorre *et al.*, 1999). Chemometrics have been practiced effectively in food safety fields on traceability, authenticity (Oliveri *et al.*, 2011; Nicolaou *et al.*, 2011; Stanimirova *et al.*, 2010).

In this study, paraffin as a common adulterant, was going to be mixed with beeswax to prepare the standard adulterations. The standards adulterations, beeswax and paraffin were analyzed by GC-MS. Then, characteristic peaks and their RPAs were calculated for quantitative expression of the GC-MS fingerprints. The difference among beeswax, paraffin and adulteration were explored by Chemometrics tools, such as PCA which is to compress and visualize the data matrix. We also attempted to quantify the adulteration levels through the prediction model.

Materials and Methods

Materials and reagents Analytical grade petroleum ether was redistilled before use. Squalane (99% minimum purity) was obtained from AccuStandard Inc. (USA) and used as an internal standard for quantification. Normal alkane standard mixture (2000 mg L^{-1}) was also obtained from AccuStandard and used to identify different alkanes in the samples being studied.

Squalene (50 mg) was dissolved in petroleum ether (50 mL) using an ultrasonic bath and vortex mixing, and used as an internal standard stock solution (1000 mg L^{-1}).

Sample and preparation Six pure beeswax samples, mixed with the rest of the beehive, from *Apis cerana cerana* (*A. c. cerana*) and *Apis mellifera* (*A. mellifera*) was collected from different apiary. The pure beeswax was cleaned prior to analysis by adding it to a beaker with boiling water (100 g L^{-1} of wax) and heating until it melted completely. The mixture was then filtered with a 40 mesh screen and cooled at room temperature. Due to its lower density, the wax solidified over the water and the impurities were located on the bottom of the solidified wax, these were removed with a scraper. The clean-up procedure was repeated once more. The pure beeswax samples were allowed to air dry at room temperature. Pure paraffin was purchased commercially. Standard adulteration samples were mixed pure beeswax (*A. c. cerana* and *A. mellifera*) with the concentration of paraffin of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, and 90%. The mixtures were melted in a beaker with boiling water (100 g L^{-1}) and then cooled at room temperature. The adulterated samples were allowed to air dry at room temperature.

All samples of wax were kept at $-18^{\circ}C$ and then ground into a powder. The powder samples were then kept in darkness at room temperature until analysis. For analysis, wax (50 mg) was dissolved in petroleum ether (10 mL) using an ultrasonic bath and vortex mixing to achieve a concentration of 5000 mg L^{-1} . One milliliter of sample was pipetted into a vial and internal standard stock solution (50 μL) was added and mixed thoroughly.

GC-MS analysis A Hewlett-Packard 6890 gas chromatograph (USA) was directly coupled to a Hewlett-Packard 5973 mass spectrometer. The chromatograph was equipped with a 30 m $\times$ 0.25 mm $\times$ 0.25 μm HP-5MS column from Hewlett-Packard. The oven temperature was held at $50^{\circ}C$ for 1 min, and then programmed at $20^{\circ}C min^{-1}$ to $180^{\circ}C$ and held there for 1 min, and then to $280^{\circ}C$ at a rate of $15^{\circ}C min^{-1}$ where it was held for 20 min. The carrier gas (helium) flow was kept constant at 1 mL min^{-1} . A splitless injection (1 μL) was performed with a HP7673A automatic sampler, and the injection port temperature was $280^{\circ}C$. The pressure of inject was 5.23×10^4 Pa. The transfer line temperature was $290^{\circ}C$. The MS conditions were as follows: ion source, $250^{\circ}C$; quadrupole, $150^{\circ}C$; and solvent delay, 4 min. The electron multiplier voltage was maintained at 200 V above autotune. The mass spectrometer was operating in the EI positive mode (70 eV) and mass scan range was 50–550 m/z . The other conditions of MS are in Table 1.

Data processing Relative peak area (RPA) of different components were obtained by dividing the peak areas of internal standard. Components that were absent in the beeswax were recorded as zeroes. Centering and autoscaling were selected to preprocess the data. In centering, data were scaled

Table 1. Compounds Identified by GC-MS.

Rt (min)	Components	Molecular formula	Similarity	molecular ion	Qualitative ion-1	Qualitative ion-2	Quantitative ion
11.79	Nonadecane	C ₁₉ H ₄₀	95	268	109	79	72
12.50	Eicosane	C ₂₀ H ₄₂	97	282	105	85	78
13.17	Heneicosane	C ₂₁ H ₄₄	99	296	144	92	80
13.80	Docosane	C ₂₂ H ₄₆	99	310	141	99	80
14.40	Tricosane	C ₂₃ H ₄₈	98	324	128	70	79
14.97	Tetracosane	C ₂₄ H ₅₀	99	338	153	75	81
15.54	Pentacosane	C ₂₅ H ₅₂	96	352	116	66	76
16.16	Hexacosane	C ₂₆ H ₅₄	99	366	142	70	80
16.89	Heptacosane	C ₂₇ H ₅₆	99	380	138	72	80
17.70	Octacosane	C ₂₈ H ₅₈	98	394	112	81	79
18.71	Nonacosane	C ₂₉ H ₆₀	99	408	153	88	81
19.90	triacontane	C ₃₀ H ₆₂	99	422	145	86	80
21.39	Hentriacontane	C ₃₁ H ₆₄	95	437	98	76	72
23.20	Dotriacontane	C ₃₂ H ₆₆	99	451	154	88	80
25.44	Tritriacontane	C ₃₃ H ₆₈	99	465	152	81	81
28.18	Tetratriacontane	C ₃₄ H ₇₀	87	479	123	99	54
31.56	Pentatriacontane	C ₃₅ H ₇₂	95	493	101	76	72

by subtracting their mean values, while in autoscaling, data were performed by subtracting their mean values and dividing into the standard deviation. Principle component analysis (PCA) was applied to data compression and visualise the possible differences among samples. If there is a strong correlation between the two inputs, i.e. the RPA of n-alkanes and the adulteration levels, it is then possible to employ a multivariate regression model to predict the adulteration levels. Whilst supervised methods are very powerful, it is possible to over-fit the model; therefore, cross-validation of PLS was undertaken. We achieved this using an independent test set for data matrix.

Results and Discussion

General The chromatograms of beeswax and paraffin are showed in Fig. 1. For beeswax, the number of carbon in n-alkanes covers from C₁₉ to C₂₉ for *A. c. cerana* and from C₂₁ to C₃₁ for *A. mellifera*, in the absence of mostly even-numbered carbon. The RPA of C₂₇ was the highest, followed by that of C₂₉, and C₂₅. The RPAs of C₂₇, C₂₉ and C₂₅ are more than 80% of the sum of RPAs. For paraffin, the n-alkanes occur at range C₁₉ and C₃₅, and odd- and even- numbered carbons are alternated. The RPAs of C₃₁ and C₃₂ are most abundant. The total RPAs of odd-numbered carbons approximately equals to even-numbered ones. The profile of adulterations mixed with beeswax and paraffin were also determined by GC-MS. As there are nine chromatograms of

adulterations in different mixture ratio ranged from 10% to 90%, the chromatograms of adulterations were not showed in Fig. 1. The chromatograms of adulterations are similar to that of paraffin. Furthermore, the RPAs of main constituents present a certain changing tendency. Seeing from the profile of chromatograms, there were significant differences among the beeswax, paraffin and adulterations. Moreover, the significant differences not only derived from one or two special components, but also from numerous variables, which could be certified by the result of collinearity diagnostics. The stepwise regression result (see in Table 2) shows 9 variables were chose in this model and 6 variables which the VIF is more than 10, were in collinearity, such as C₂₅, C₂₇, C₂₈, C₂₉, C₃₃, C₃₅. So, it's complicated to describe the chemical properties of each groups without proper chemometric processing.

Data reduction Prior to chemometric processing, methods of data preprocessing were compared. Centering and autoscaling, which are most commonly employed as data pre-treatment methods were performed using MatLab software. Fig. 2a and 2b show the loading plots of PC1 vs PC2 in two pre-treatments. In centering treatment, the loading feature in first principle component (PC1), C₂₂, C₂₄, C₂₈ and C₃₀–C₃₅, are the dominated variables that represent 86.84% of the total variability. It illustrates that the information described by PC1 is closely related to paraffin. While, C₁₉–C₂₁, C₂₃, C₂₅–C₂₇, and C₂₉ dominate in second principle component

Fig. 1. Three representative total ion chromatograms, (a) pure beeswax from *A. c. cerana*, (b) pure beeswax from *A. mellifera*, (c) paraffin.

(PC2) that represents 6.71% of the total variability, which indicates the character extracted by PC2 mostly comes from beeswax. The accumulated contribution of first two principle components is 93.55% of the total variability. In autoscaling, the first two principal components (PCs) accounted for 76.73% of the total variance, the first accounted for 65.80%, and the second for 10.93%. The PC1 was strongly associated with the RPAs of C_{22} , C_{24} , $C_{26}-C_{28}$ and $C_{30}-C_{35}$, and the PC2 could be defined by levels of $C_{19}-C_{21}$, C_{23} , C_{25} and C_{29} . By comparing the results of two preprocessing, we found that centering is more suitable for this study, being attributed to the higher accumulated contribution.

Examining the score plot of PCA (Fig. 3), a nature separation among beeswax from *A. c. cerana*, *A. mellifera* and paraffin was found. It illustrated that there are essential differences between beeswax and paraffin, and also between two types of beeswax. Likewise, it also revealed that the samples could be clustered reasonably into different groups by PCA. In additional, the score plot shows adulterated standards are roughly linearly arranged between two types of beeswax and paraffin. It is possible that there is a strong correlation between the profiles of n-alkanes and the levels of adulteration.

Quantification of paraffin As above-mentioned, lots of variables are collinearity. So we chose the PLS to built the predicting model. The PLS was also implemented in the

Table 2. The coefficients of variables of stepwise regression.

Model	Standardized Coefficients (Beta)	t	Sig.	Collinearity Statistics	
				Tolerance	VIF
(Constant)		3.81	0.00		
C27	0.47	13.95	0.00	0.04	26.00
C20	0.09	7.52	0.00	0.32	3.17
C21	-0.08	-6.98	0.00	0.37	2.73
C28	-0.73	-7.35	0.00	0.01	220.00
C35	0.25	2.45	0.02	0.00	225.00
C25	0.15	7.08	0.00	0.10	10.40
C33	-0.28	-2.07	0.05	0.00	397.00
C29	0.32	4.11	0.00	0.01	139.00
C19	0.03	2.84	0.01	0.31	3.28

a. Dependent Variable: level of adulterations;

b. the number of 11.00 stands for the number of times in stepwise regression.

Fig. 2. Loading plots of PCA based on two different data processing methods, (a) column centering, (b) autoscaling.

statistical software Matlab. During calibration of the PLS model, sub-sampling of the training data took place with the Kennard and Stone algorithm, leaving one set out so that a cross-validation set could later be generated; this allowed for the selection of the optimum number of PLS factors for calibration. According to the lowest RMSECV rules, Seven PLS factors were selected to build the PLS model, which account for almost all of the variation in the responses (99.84% for *A. c. cerana*, 99.65% for *A. mellifera*). PLS regression results for the paraffin and beeswax from *A. c. cerana* and *A. mellifera* mixture combinations are shown in Fig. 4. The plots of the predicted concentration versus the actual concentration values show that two models have the high precision, since the root mean square error (RMS) is close to the root mean squared error of cross-validation (RMSECV). The RMS and RMSECV of *A. c. cerana*-paraffin mixture are 0.128 and 0.131, and those of *A. Mellifera*-paraffin mixture are 0.180, 0.181, respectively. But the predictability of model in *A. c. cerana*-paraffin mixture is better than in *A. Mellifera*-paraffin mixture, due to the root mean squared error of predictive (RMSEP) of the former (0.148) is lower than the latter (0.440).

The most discriminative features used by the PLS regression, which models the adulteration levels of the paraffin, can be found by inspecting the plots of PLS regression coefficient (Fig 5). They generally have higher magnitudes than the non-discriminative ones in the plots. The most discriminative peaks and coefficient of *A. c. cerana*-paraffin mixture and *A. Mellifera*-paraffin mixture are $C_{20}(5.02)$, $C_{21}(-2.41)$, $C_{22}(-2.39)$, $C_{25}(2.45)$, $C_{28}(-2.24)$, $C_{33}(-2.43)$, $C_{35}(3.10)$, and $C_{24}(-1.54)$, $C_{26}(-2.29)$, $C_{28}(-4.48)$, $C_{29}(2.09)$, $C_{31}(1.38)$,

Fig. 3. The score plot of PCA analysis of beeswax, paraffin, and the adulterated standards.

$C_{33}(-1.51)$, $C_{34}(2.33)$, respectively. PLS suggest that despite small magnitude of some characteristic peaks, they have a significant influence on quantification of paraffin.

We can use the PLS model to predict the level of paraffin adulterated in beeswax sample. Firstly, we analyzed the beeswax sample by GC-MS. Secondly, characteristic peaks and their RPAs were calculated for quantitative expression of the GC-MS fingerprints. Then, put the RPAs of n-alkanes into PLS model, we get the variation Y. In this study, the variation Y is the concentration of paraffin adulterated in beeswax.

Accuracy and precision In order to identify the accuracy and precision of the method, a recovery test was added. Three levels of n-alkanes (C_{19} – C_{35}) and blank control were set for beeswax from *A. c. cerana*, beeswax from *A. mellifera* and paraffin respectively before samples were tested

Fig. 4. Plots showing the predicted levels of paraffin adulteration estimated from PLS model (a) *A. c. cerana*-paraffin mixture, (b) *A. Mellifera*-paraffin mixture; The black dots represent the training data set and the square, the test set.

Fig. 5. Plots of PLS regression coefficient (a) *A. c. cerana*-paraffin mixture, (b) *A. Mellifera*-paraffin mixture.

on GC-MS after pretreatment. Concentration and recovery was calculated with internal standard quantification. The recovery test result shows that upon above three levels, average recovery ranged from 80% to 110% and relative standard deviations is below 10% for three types of wax (Table 3), which means the method is reliable in terms of accuracy and precision.

Conclusions

The centering is a better data pre-treatment in this study as the higher accumulated attribution. Two PCs that represents 93.55% of the total variability are achieved. The PCA results show two types of beeswax and paraffin could be clustered reasonably into different groups. Seven PLS factors are extracted to building the predictive model using PLS analysis with crossing-validation. the root mean square error (RMS) is 0.128 and 0.180, respectively. The root mean squared error of predictive (RMSEP) is 0.148 and 0.440. the root mean squared error of cross-validation (RMSECV) is 0.131 and 0.181. The most discriminative peaks of *A. c. cerana*-paraffin mixture and *A. Mellifera*-paraffin mixture are $C_{20}H_{42}$, $C_{35}H_{72}$, $C_{25}H_{52}$. and $C_{28}H_{58}$, $C_{34}H_{70}$ and $C_{26}H_{54}$, respectively. PLS suggest that despite small magnitude of some characteristic peaks, they have an significant influence on quantification of paraffin. We can use the PLS model to predict the level of paraffin adulterated in beeswax sample. Of course, the PLS model should be validated and improved in future researches, owing to preliminary explore the possibility of application. In conclusion, GC-MS and chemometric analysis can be used to detect and quantify the paraffin in beeswax.

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Table 3. Analytical recoveries and relative standard deviations (%) of beeswax and paraffin (n=5).

Sample	Different levels (mg/g)	recovery (%)	R.S.D. (%)
beeswax from <i>Apis ceranacerana</i>	5	91.3 ~ 101.6	7.4
	10	87.5 ~ 97.8	5.2
	100	87.6 ~ 97.5	4.6
beeswax from <i>Apis mellifera ligustica</i>	5	95.8 ~ 99.7	4.9
	10	89.4 ~ 97.3	6.8
	100	90.1 ~ 96.5	8.3
paraffin	5	95.4 ~ 101.4	4.9
	10	85.2 ~ 98.3	8.7
	100	88.7 ~ 98.2	6.2

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