A Box-behnken Design for Characterizing Chinese Truffles (*Tuber indicum*) Aroma by HS-SPME-GC-MS

Sanping Fang[1], Biao Pu[1], Anjun Chen[1], Kangzhou[1], Xiaolin Ao[1] & Danping Xu[1]

[1] Sichuan Provincial Key Laboratory of Agricultural Products Processing and Preservative, College of Food Science, Sichuan Agricultural University, China

Corresponding author: Biao Pu, Sichuan Provincial Key Laboratory of Agricultural Products Processing and Preservative, College of Food Science, Sichuan Agricultural University, Ya'an 625014, China. Tel: 86-835-288-2875. E-mail: pubiao2012@gmail.com

Abstract

The aim of the present investigation is to fully characterize the aroma of Chinese truffles (*Tuber indicum*) by headspace solid phase microextraction (HS-SPME). To develop an objective method to extract aroma compounds, four different fibers were studied and a Box-Behnken design (BBD) was applied. From the statistical analysis of the experimental result, it was able to determine that the most important factor was the extraction temperature and the optimum extraction conditions were as follows: extraction time 20.6 min, extraction temperature 52.4 $^{\circ}$C and equilibrium time 6.8 min, By using gas chromatography mass spectrometry (GC-MS) analysis under the optimal conditions, it identified 24 compounds, three of which were reported for the first time in the Chinese truffle: 2-methylpropanal, 2,3-butanedione, 2-nonanone. And we found that the highest content compound was dimethyl sulfide, followed by 3-methylbutanal, 2-methylbutanal, 2-butanol and 1-pentanol, 1-octen-3-ol, all of those compounds were previously described as characteristic aroma of truffle.

Keywords: Chinese truffles, Box-Behnken design, optimization, HS-SPME-GC-MS

1. Introduction

Truffles are subterranean fungi, belonging to the family Tuberaceae (Gao, Hu, & Liu, 2001; Kües & Martin, 2011), which grow in symbiosis with certain trees such as oaks, poplars and hazels (Kües & Martin, 2011). About 60 different kinds of truffles are distributed in the world, most of which grow in Europe, particularly in Italy, France, Spain (Wang & Marcone, 2011). They are known as "diamonds", with a high economic value, which are the most expensive mushrooms in the world (Wang & Marcone, 2011; García-Montero, Díaz, Di Massimo, & García-Abril, 2010).

There are about 25 kinds of truffles in China (Gao, Zhang, Chen, & Liu, 2004). Chinese truffle (*Tuber indicum*) is the most important truffle in Asian regions, distributing mainly in the provinces of Sichuan and Yunnan of China (García-Montero et al., 2010). About 100 tons of Chinese truffles are produced annually and most of them are exported to Europe and Japan (Lin, 2008). Truffles are highly appreciated due to their unique aroma (Culler et al., 2010). In the past few decades, most research has primarily focused on studying aroma in the white truffle (*Tuber magnatum*), the black truffle (*Tuber melanosporum*) and summer truffle etc., and more than 200 aroma compounds have been identified by GC-MS analysis (Culler et al., 2010; March, Richards, & Ryan, 2006; Gioacchini et al., 2005; Falasconi et al., 2005; Piloni, Tat, Tonizzo, & Battistutta, 2005; Díaz, Ibáñez, Reglero, & Señoráns, 2009; Díaz, Ibáñez, Señoráns, & Reglero, 2003; Bellesia, Pinetti, Bianchi, & Tirillini, 1998; Tirillini et al., 2000; Splivallo, Bossi, Maffei, & Bonfante, 2007; Gioacchini et al., 2011; Díaz, Francisco, & Reglero, 2002). However, Chinese truffle aroma by was characterized in very few studies.

HS-SPME is widely used to extract food aroma. This extraction technique is an ideal method for aroma isolation, because it is solvent-free, simple and sensitive (Ho, Wan Aida, Maskat, & Osman, 2006; Lord & Pawliszyn, 2000). Generally, in most the published papers, each of factors were evaluated separately in optimizing SPME conditions. So it appears to be very time-consuming and the interactions between parameters are not considered. In order to overcome these disadvantages, BBD should be applied which allows evaluating the effect of the interactions and, at the same time, reducing the number of experiments (Ferreira et al., 2007). In the case of the analysis of truffle aroma compounds, the SPME technique has been used by different authors (Culler et al., 2010;

March et al., 2006; Gioacchini et al., 2005; Falasconi et al., 2005; Piloni et al., 2005; Bellesia et al.,1998; Tirillini et al., 2000; Splivallo et al., 2007; Gioacchini et al., 2011), but few performed the optimization of the method. And to our knowledge, there are virtually no reports on applying BBD to characterize Chinese truffle aroma.

GC-MS is one of the most widespread routine technologies applied to determine aroma compounds. For truffle aroma, many papers identified aroma compounds only based on a comparison with mass spectral databases (Culler et al., 2010; March et al., 2006; Gioacchini et al., 2005; Falasconi et al., 2005; Piloni et al., 2005; Díaz et al., 2009; Díaz et al., 2003; Bellesia et al., 1998; Splivallo et al., 2007; Díaz et al., 2002). However, no retention indices (RI) and no reference compounds are given to support the identification experiments, all aroma compounds have to be regarded as tentative identification and remain unidentified.

Therefore, the aim of the work is to develop an objective method to fully characterize Chinese truffle aroma by HS-SPME-GC-MS. An ideal fiber was selected among four different polarity fibers to reduce discrimination toward volatile compounds. To optimize the extraction conditions, we have applied BBD considering the interactions between variables. In order to support the identification results, the reference compounds are used and retention indices are compared.

2. Materials and Methods

2.1 Materials and Chemicals

Truffles used in this work belong to the species *T. indicum* and were bought in Panzhihua (Sichuan Province, China). These truffles were deeply frozen until extraction. A mixture of aliphatic hydrocarbons (C_5–C_{25}) (Sigma) was to calculate the retention index (RI) of each compound. The aroma standards, dimethyl sulfide, 3-methylbutanal, 2,3-butanedione, 2-nonanone, 2-methylbutanal, 1-pentanol, hexanal, 2-methylpropanal, 1-octen-3-ol, 1-propanol were purchased from Sigma.

2.2 HS-SPME

A SPME holder (Supelco, Bellefonte, PA, USA) was used to do the experiments. Approximately 5 g of sample was placed in a 15 ml vial. Once the desired temperature was reached, the vial was placed inside the water-bath (no fiber exposition). After the equilibrium time, the fiber was exposed to the headspace of the truffle during the corresponding extraction time.

2.3 GC-MS Analysis

An Agilent-6890 GC system coupled to an Agilent-5973 mass spectrometer was used. The injector, in splitless mode for 2 min, was set at 250 °C. A helium carrier gas was used at a constant flow of 1.0 mL/min with a column HP-5 (Agilent, CA, US) 30 m × 0.25 mm i.d. × 0.25 um film thickness. The GC oven temperature program was 30 °C, hold 1 min, 2 °C/min to 100 °C, hold 5 min, 5 °C/min to 170 °C, hold 5 min (total run 57.5 min). Quadrupole detector in autotune mode, ion M+, operating at 70 eV (MS source 150 °C, MS quad 230 °C, scan range from *m/z* 40-400). The compounds were identified by comparison of the spectra with those in a mass spectrometry library (NIST 05s. LIB). The identification was confirmed by comparison of RI reported in the literature, and in some cases comparison with reference standards.

2.4 Box–Behnken Design and Statistical Analysis

On the basis of single-factor experiment, proper ranges of extraction time, equilibrium time and extraction temperature were preliminarily determined. A BBD with three independent variables at three levels was performed. For the design, the total number of experiments was 15 (Tables 1 and 2), Triplicate extractions were carried out for each run.

Table 1. Factors and levels in Box-Behnken design

variables	symbols		levels		
	uncoded	coded[a]	-1	0	1
equilibrium time (min)	x_1	X_1	5	8	11
extraction temperature (°C)	x_2	X_2	40	50	60
extraction time (min)	x_3	X_3	20	25	30

[a] Code level reflect what is done in practice. [$X_1 = (x_1-8)/3$, $X_1 = (x_2-50)/10$, $X_3 = (x_3-25)/5$].

Table 2. Box-Behnken design with three independent variables

run	X_1	X_2	X_3	peak area	
				experimental	predicted
1	0	-1	-1	10523200	10911651
2	0	-1	1	11689247	11940777
3	0	1	-1	12259524	12007961
4	0	1	1	12907739	12519255
5	-1	0	-1	13580002	13462005
6	1	0	-1	11610992	11629915
7	-1	0	1	10367685	10348727
8	1	0	1	13603269	13721237
9	-1	-1	0	9932745	9662247
10	-1	1	0	11159016	11528531
11	1	-1	0	10549700	10180159
12	1	1	0	9718190	9988663
13	0	0	0	13495245	13484300
14	0	0	0	13418522	13484300
15	0	0	0	13539193	13484300

The experimental data (Table 2) was analyzed by BBD to fit the following second-order polynomial model:

$$Y = \beta_0 + \Sigma\beta_i X_i + \Sigma\beta_{ij}X_i X_j + \Sigma\beta_{ii}X_i^2 \tag{1}$$

Where Y is response (peak area); β_0, β_i ($i = 1, 2, 3$) and β_{ij} ($i = 1, 2, 3; j = 1, 2, 3$) are the model coefficients; X_i and X_j are the coded independent variables.

Design-Expert (Version 8.0) software package was used to perform the analysis of variance (ANOVA). The P values of less than 0.05 were considered to be statistically significant.

3. Results and Discussion

3.1 Choice of Fiber

Fiber is an important factor (Díaz et al., 2002; Pelusio et al., 1995). SPME extraction was performed with four different fibers: divinylbenzene/Carboxen/polydimethylsiloxane (DVB/CAR/PDMS, thickness 50/30 mm), polydimethylsiloxane (PDMS, thickness 100 mm), Polyacrylate (PA, thickness 85 mm) and polydimethylsiloxane/divinylbenzene (PDMS/DVB, thickness 65 mm). Those fibers were supplied by Supelco (Bellafonte, PA, USA). They were conditioned under the specifications of the producer before use. Other conditions were as follows: extraction temperature 30 ºC, extraction time 10 min, equilibrium time 5 min. As be shown in Figure 1, the peak area is largest when DVB/CAR/PDMS fiber is used. Therefore, a DVB/CAR/PDMS fiber was adopted in our work. This result was similar to that reported by Gioacchini et al. (2005), which seemed to indicate that a certain coating fibers should be selected in accordance with the character of aroma compounds analyzed (Ferreira et al., 2007; Lord & Pawliszyn, 2000; Ho, Wan, Maskat, & Osman, 2006)
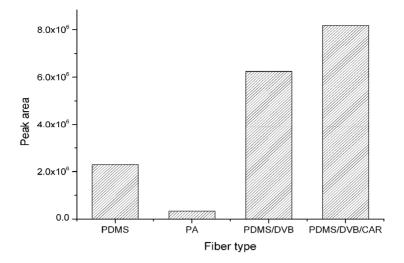
Figure 1. Influence of different fiber coatings on the peak area

3.2 Influence of Extraction Temperature

According to the researches of Díaz et al. (2002) and Gioacchini et al. (2005), extraction temperature will significantly affect the peak area. In this work, extraction temperature was set at 20, 30, 40, 50, 60, and 70 ^{o}C when other conditions were as follows: DVB/CAR/PDMS fiber, extraction time 10 min, equilibrium time 5 min. As be shown in Figure 2, the peak area increases with increasing extraction temperature, and reaches the highest value when temperature is 50 ^{o}C. When extraction temperature is higher than 60 ^{o}C, the peak area is decreased significantly. A possible explanation is that the use of high temperatures may induce the degradation of volatiles (Díaz et al., 2009). For this case, 50 ^{o}C was chosen as extraction temperature.
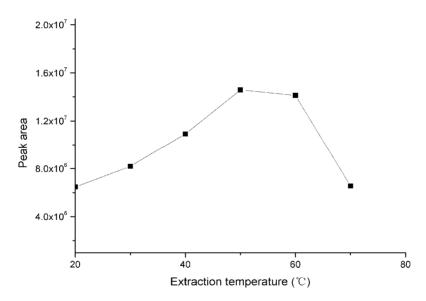

Figure 2. Influence of extraction temperature on the peak area

3.3 Influence of Extraction Time

The extraction time plays an important role on the peak area (Gioacchini et al., 2005; Falasconi et al., 2005). In this process, extraction time was set at 10, 15, 20, 25, 30, and 35 min when other extraction conditions were as follows: DVB/CAR/PDMS fiber, extraction temperature 50 ^{o}C, equilibrium time 5 min. The peak area affected by different extraction times is seen in Figure 3, the results indicates that the peak area obviously increases as the

extraction time increases. However, the peak area changes little after 20 min. Under this condition, 20 min was adopted in the experiment.
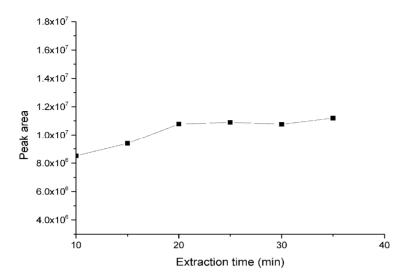

Figure 3. Influence of extraction time on the peak area

3.4 Influence of Equilibrium Time

In order to make the sample release more aroma and maintain them equilibrium, the sample should be stood for some time before the fiber is exposed. In this work, equilibrium time was set at 2, 5, 8, 11, and 14 min (Figure 4) when other extraction conditions were as follows: DVB/CAR/PDMS fiber, extraction temperature 50 $^{\circ}$C, extraction time 20 min. The results show that the peak area has little increase with increasing equilibrium time and reaches the highest value when equilibrium time is 8 min. As equilibrium time is higher than 8 min, the peak area has a small decrease. While there are no significant difference for different equilibrium time in the experiment, such behavior is accordance with the one reported by Diaz et al. (2009). This may suggest that it is easy to achieve the equilibrium between stationary phases and aroma. Consequently, the equilibrium time was fixed at 8 min.
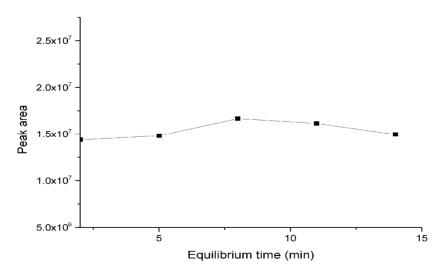

Figure 4. Influence of equilibrium time on the peak area

3.5 Box-Behnken Design and Analysis of Variance (ANOVA)

The BBD for Design Expert 8.0 was employed to fit the second-order polynomial to the experimental data,

represented as the peak area in Table 3. From the Design Expert 8.0 output of BBD, the second-order polynomial equation is given below:

$$
\begin{aligned}
Y = &+1.34843 \times 10^7 + 3.85105 \times 10^5\, X_1 + 4.18697 \times 10^5\, X_2 - 2.55489 \times 10^5\, X_3 \\
&- 1.29458 \times 10^5\, X_1 X_2 + 1.30115 \times 10^6\, X_1 X_3 - 5.14445 \times 10^5\, X_2 X_3 \\
&+ 1.55591 \times 10^5\, X_1{}^2 - 1.79498 \times 10^6\, X_2{}^2 - 1.34942 \times 10^6\, X_3{}^2
\end{aligned}
\tag{2}
$$

Where Y is the peak area of aroma; X_1, X_2 and X_3 are the coded values of the equilibrium time, extraction temperature and extraction time, respectively.

Table 3. Optimum value of the process parameters for maximum response

parameters	optimum value		predicted response
	coded values	real values	
X_1 (equilibrium time, min)	-0.40	6.8	
X_2 (extraction temperature, $^\circ$C)	0.24	52.4	13684015
X_3 (extraction time , min)	-0.88	20.6	

Table 4. ANOVA table for the RSM model

source	sum of squares	degree of freedom	mean square	F value	p value [b]
X_1	1.186E+012	1	1.186E+012	2.95	0.1463
X_2	1.402E+012	1	1.402E+012	7.93	0.0373
X_3	5.222E+011	1	5.222E+011	6.71	0.0488
$X_1 X_2$	6.704E+010	1	6.704E+010	0.38	0.5650
$X_1 X_3$	6.772E+012	1	6.772E+012	38.31	0.0016
$X_2 X_3$	1.059E+012	1	1.059E+012	5.99	0.0581
X_1^2	8.939E+010	1	8.939E+010	0.51	0.5088
X_2^2	1.190E+013	1	1.190E+013	67.30	0.0004
X_3^2	6.723E+012	1	6.723E+012	38.04	0.0016
model	2.890E+013	9	3.211E+012	18.16	0.0026
residual	8.838E+011	5	1.768E+011		
lack-of-fit	8.763E+011	3	2.921E+011	78.32	0.0126
total	2.978E+013	14			

[b] $p < 0.01$, highly significant; $0.01 < p < 0.05$, significant; $p > 0.05$, not significant.

ANOVA is shown in Table 4. We can find that the model is highly significant. The value of determination coefficient ($R^2 = 0.9703$), a very small p value of total model (p = 0.0026) and no significant lack of fit (p = 0.0126) indicate good relation between the predicted and experimental values. Figure 5 shows that the predicted values obtained are quite close to the experimental values. All the results indicate that the model developed is successful.
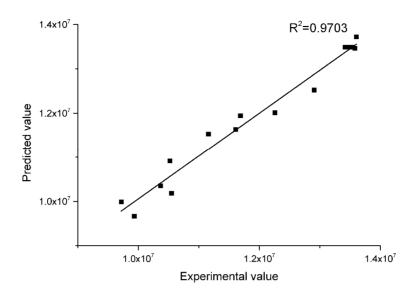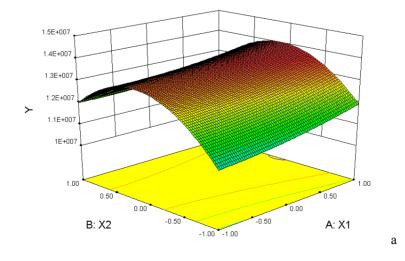
Figure 5. Comparison between the experimental values and the predicted values of BBD

3.6 Three Dimentional (3D) Response Surfaces Model (RSM)

The best way to express the effect of each variable on the peak area is to generate surface response plots. Figures 6a and 6c show that extraction temperature has a greater effect on the response than equilibrium time and extraction time, and the highest value is obtained at ~53 ^{o}C. The highest F value (Table 4) of 7.93 also indicates that extraction temperature is the most important factor. This result is consistent with the observations made by other investigators (Gioacchini et al., 2005; Díaz et al., 2009; Díaz et al., 2002).

The results show that the peak area obviously increases accompanying the increase of extraction time, but there is no significant change after ~20 min (Figure 6a and 6b). Our results seem to contrast with the results of Díaz et al. (2009) who, focusing on optimization of summer truffle aroma by SPME, found the maximum peak area obtained at extraction time 30 min. The differences may be caused by the varied truffle species and fibers examined by the researchers (March et al., 2006; Pelusio et al., 1995). The effect of the equilibrium time on the respond is not very significant in the tested experimental region (Figure 6a and 6c). The result is in accordance with Díaz et al. (2002).
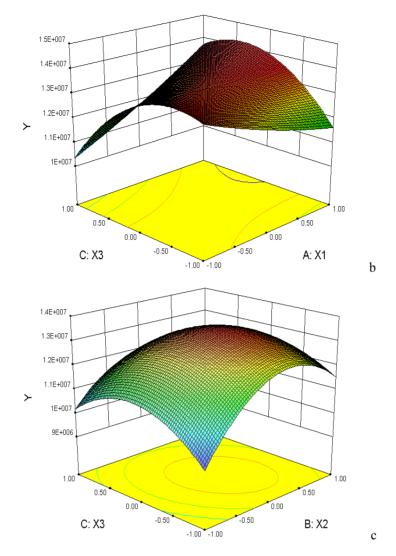

a

Figure 6. Response surface plots showing the effects of factor (X1, equilibrium time; X2, extraction temperature; X3, extraction time) on the response Y

3.7 Optimum Condition and Verification

The analysis of response surface was performed with Design Expert 8.0. As shown in Table 5, the optimal level of them is determined as extraction time 20.6 min, extraction temperature 52.4 ^{o}C and equilibrium time 6.8 min, with a predicted the peak area of 13684015. In order to confirm the predicted results, Triplicate extractions under the optimum extraction conditions were performed and the mean value 13296624 of the peak area was obtained (Table 5). The result that the predicted value and experimental value have a good agreement verified the validity of the model.

Table 5. Predicted and experimental value for the responses at the optimum conditions

equilibrium time , min	6.8
extraction temperature, ^{o}C	52.4
extraction time, min	20.6
peak area:	
predicted	13684015
experimental	13296624

3.8 Identification of Unknown Compounds

The compounds in Chinese truffle aroma extracted under the optimal conditions were identified by GC-MS analysis, literature data (RI) (Culler et al., 2010; Tirillini et al., 2000; Splivallo et al., 2007; Liu, Li, & Tang, 2012) and reference standards were used to support the identification. Figure 7 shows the total ion current (TIC) mass chromatogram of summer truffle. The 24 identified compounds are listed in Table 6 along with their relative percentages, three of which are reported for the first time in the Chinese truffle: 2-methylpropanal, 2-Nonanone, 2,3-butanedione. Among the compounds identified in the present work, the amount of dimethyl sulfide which is considered to be the most characteristic compound of truffle (Wang et al., 2011; March et al., 2006; Gioacchini et al., 2005; Splivallo et al., 2007; Splivallo et al., 2011) is the highest. This is an important finding, because it is generally believed that dimethyl sulfide is very small in Chinese truffle (Splivallo et al., 2007). A possible explanation is that the extract conditions used by the previous researchers are not optimal. From the quantitative point of view, the contents of 3-methylbutanal, 2-methylbutanal, 2-methylbutanol, 2-butanol, 1-Pentanol, 1-octen-3-ol, are also very high in Chinese truffle. And these compounds have been found in most truffles, which are described as responsible for truffle aroma by many investigators (Culler et al., 2010; Gioacchini et al., 2005; Tirillini et al., 2000; Splivallo et al., 2007).

Table 6. Identified compounds and relative percentages (Area %) of Chinese truffle aroma compounds extracted by HS-SPME under the optimal conditions

n	RI	Compound	area %	Mode of identification[c]
1	502	acetaldehyde	0.851	RI, MS
2	511	dimethyl sulfide	19.63	RI, MS, Std
3	526	propanal	2.180	RI, MS
4	532	2-methylpropanal	9.897	RI, MS, Std
5	591	2,3-butanedione	5.164	RI, MS , Std
6	608	1-propanol	1.318	RI, MS , Std
7	614	2-methyl-1-propanol	2.750	RI, MS
8	622	2-butanone	1.183	RI, MS
9	633	ethyl ethanoate	6.160	RI, MS
10	648	2-butanol	9.177	RI, MS
11	669	3-methylbutanal	12.824	RI, MS, Std
12	681	2-methylbutanal	8.435	RI, MS, Std
13	695	2-pentanone	1.495	RI, MS
14	735	3-methylbutanol	2.008	RI, MS
15	751	1-pentanol	6.367	RI, MS, Std
16	758	2-methylpentanal	0.308	RI, MS
17	798	hexanal	1.463	RI, MS, Std
18	807	ethyl butanoate	0.093	RI, MS
19	979	1-Octen-3-ol	1.167	RI, MS, Std
20	987	3-octanone	0.517	RI, MS
21	1043	benzeneacetaldehyde	1.236	RI, MS
22	1104	2-nonanone	0.545	RI, MS, Std
23	1421	1,2,3-trimethoxy-5-methylbenzene	4.262	MS
24	1739	1-methylethyltetradecanoate	0.967	MS

[c] comparison of their RI (HP-5 column) to literature data (RI); comparison of their MS data to the NIST05 database (MS); in some cases direct GC-MS comparison with authentic standards.
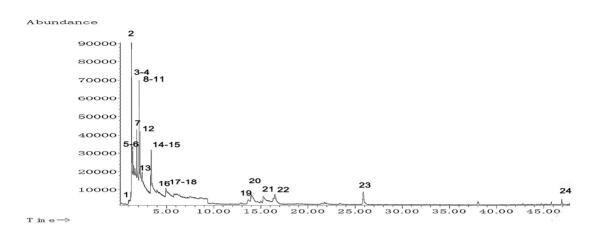
Figure 7. TIC mass chromatogram of Chinese truffle aroma

4. Conclusions

In this study, a Box–Behnken design was adopted to optimize the extraction of Chinese truffle aroma. Through the statistical analysis, the extraction temperature was found to have the most significant effects on the extraction of aroma and the optimum extraction conditions were as follows: extraction time 20.6 min, extraction temperature 52.4 $^{\circ}$C and equilibrium time 6.8 min. By using HS-SPME-GC-MS analysis under this optimal conditions, it identified 24 compounds, three of which are reported for the first time in the Chinese truffle: 2-methylpropanal, 2,3-butanedione, 2-nonanone. And we found that the highest content compound was dimethyl sulfide, followed by 3-methylbutanal, 2-methylbutanal, 2-butanol and 1-pentanol, 1-octen-3-ol, all of those compounds are previously described as characteristic aroma of truffle. Therefore, we has demonstrated an effective method to extract and objectively characterize the aroma of Chinese truffle. The method developed could be extended to other truffle species and to different ripening stage.

Acknowledgements

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