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Identification of key odor compounds in the burnt smell of upper tobacco leaves through the molecular sensory science technique

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ABSTRACT: Silica gel column chromatography, gas chromatography-olfactometry (GC-O) combined with GC-mass spectrometry (GC-MS) and recombination-omission tests led to the identification of the key odor compounds in the burnt smell of upper tobacco leaves. The results showed that a total of 16 phenolic compounds were identified from the extracts of the burnt smell components (EBSC), and 10 of which were identified as important odorants in EBSC with odor activity values (OAVs) greater than 1. In addition, the recombination tests showed that there was no significant difference in the five odors attributes between the original EBSC and the recombinant model, proving the successful simulation of the typical odors of EBSC. The omission tests demonstrated that the key odor compounds in EBSC ($p < 0.05$) were o-cresol, p-cresol, 2,6-dimethylphenol, 2-ethylphenol, 2,4-dimethylphenol, 4-ethylphenol, 3,5-dimethylphenol, and 2,4,6-trimethylphenol. Further studies on characterization of key odor compounds in EBSC will be helpful to the directional elimination of the burnt smell from upper tobacco leaves.

KEYWORDS: burnt smell, Silica gel column chromatography, gas chromatography-olfactometry, odor activity value, recombination and omission tests

INTRODUCTION

Tobacco is an important agricultural crop and a major source of the raw materials for cigarette production [1, 2]. The growing position of tobacco affects the processing characteristics of raw materials and the quality of flue gas [3]. Among them, the upper tobacco leaves account for one third of the whole tobacco leaves and 30%–40% of the total tobacco leaves in China. The upper leaves of flue-cured tobacco can be used as main raw material for the production of advanced cigarettes in foreign countries, but the industrial availability of domestic upper leaves is relatively low due to its physical and chemical properties [4]. Meanwhile, the yield of tobacco in the middle part is limited, which restricts the development of high-grade cigarette products in China. The main reasons for the low availability of domestic upper tobacco leaves can be summarized into two aspects. Firstly, the relative high thickness of tobacco leaves lead to the poor compatibility during processing [5]; and secondly, the upper tobacco leaves is prone to produce an unpleasant smell that cannot be well coordinated with the aroma of overall smoke during the combustion and pyrolysis process [6]. Specifically speaking, the unpleasant smell is mainly perceived as dry, burnt, and irritates offensive taste by the sensory organs, and it is usually defined as the burnt smell. The generation of the burnt smell may be related to the microstructure and chemical compositions of the upper leaves.

Many previous studies focused on the method to reduce the burnt smell. Li et al [7] developed a tobacco leaf pretreatment method using multienzyme complex. Yang et al [8] found that protease, starch-1,4-glycosidase enzymes, and lipoxygenase can improve the burnt smell and pungency of tobacco leaf in different degrees. Chi et al [9] designed a compound enzyme formulation to improve the sensory quality of expanded tobacco produced in Liao, and the sensory evaluation results showed that wood gas and burnt smell decreased significantly. Almost all of these studies employed sense evaluation approach to characterize the burnt smell, and the key odor compounds that responsible for the burnt smell is unclear. Therefore, it is highly desirable to determine the key odor compounds in burnt smell in order to purposefully improve the smoking quality of upper leaves.

The development of aroma analysis techniques such as gas chromatography-mass spectrometry (GC-MS) and gas chromatography-olfactometry (GC-O) has supplemented sensory analysis and greatly improved the identification of odor compounds [10]. Many previous studies have proven that the key odor compounds in various foods, such as yeast [11], cocoa [12], watermelon juice [13], beer [14], can be identified by using aroma analysis techniques. This paved the way for the separation and identification of key odor compounds in burnt smell during the smoking of upper tobacco leaves.

Therefore, the aim of this study was to identify key

odor compounds of burnt smell by using the molecular sensory science technique. First, silica gel column chromatography, GC-O and frequency detection analysis (FDA) was used to identify odor compounds in EBSC. Then, the odor activity values (OAVs) of important active compounds identified by FDA were determined by using quantitative measurements. Finally, the key odor compounds were identified by recombination - omission tests. A better understanding of the odor compounds in the burnt smell of upper leaves will be beneficial for the quantitative evaluation of this offensive taste and for the directional reduction of burnt smell during the new product development, which would be more accepted by consumers.

MATERIALS AND METHODS

Samples and chemicals

The upper leaves of flue-cured tobacco collected from Bijie, Guizhou Province in 2019 with grade of B2F (Classified according to the national standard of GB2635-1992 Flue-cured tobacco) were selected as the raw materials. Then, cigarette samples made of the upper leaves were produced by the Inner Mongolia Kunming Cigarette Limited Liability Company. The cigarette samples were evaluated and verified the obvious burnt smell by 10 sensory experts.

Chemical were purchased from various sources: petroleum ether, ethyl acetate and ethanol from Fuyu Fine Chemical Co., Ltd. (Tianjin, China); dichloromethane from Dikema Technology Co., Ltd. (Beijing, China); phenethyl acetate, o-cresol, p-cresol, 2,6-dimethylphenol, 2-ethylphenol, 2,4-dimethylphenol, 2,5-dimethylphenol, 4-ethylphenol, 3,4-dimethylphenol, 3,5-dimethylphenol, and 2,4,6-trimethylphenol, catechol from Maclin Biochemical Technology Co., Ltd. (Shanghai, China); N-alkane standards (C_9-C_{30}) from Sigma-Aldrich (St. Louis, MO, USA); pure water from Wahaha Group Co., Ltd. (Hangzhou, China); and petroleum ether, ethyl acetate and ethanol were redistilled before use. All other chemical were of GC quality.

Preparation of particulate extracts from mainstream flue gas

In preparation for smoking, 200 cigarette samples were conditioned at (22 ± 1) °C and (60 ± 2)% humidity for 48 h. Mainstream cigarette smoke was generated on an RM20H smoking machine (Borgwaldt KC, Germany) under ISO 3308 (35 ml puff volume, 2 s puff duration, 60 s puff interval, no ventilation block). The particulate phase of mainstream cigarette smoke was captured on a Cambridgefilter pad. Twenty cigarettes were smoked, and a Cambridgefilter pad was used to capture particulate matters from mainstream cigarette smoke. After smoking, the Cambridgefilter pads were transferred in a 1000 ml screw-capped plastic bottle, then 600 ml dichloromethane solution was added. The sample solution was ultrasonically extracted at 20 °C for 30 min. After that, dichloromethane in the sample solution was removed by water bath at 40 °C, and the mainstream flue gas granular extracts was obtained.

Separation of the extracts by column chromatography

A silica gel (60 g, 100-200 mesh, Qingdao Ocean Chemical Co., Ltd.) column (60 cm long × 2.5 cm in diameter) packed in petroleum ether was prepared, and 0.6 g mainstream flue gas granular extracts was charged onto the column. The flow rate was 2.5 ml/min, and the effluent was collected by volume, 10 ml for each fraction. The elution was performed with 200 ml petroleum ether (100%), 800 ml petroleum ether/ethyl acetate (83:17 by volume), 400 ml ethyl acetate/ethanol (6:94 by volume), and finally 600 ml ethanol (100%). A total of 200 fractions were collected. Fractions without obvious odor characteristics were removed. Fractions with similar odor characteristics were combined to obtain a total of 14 portions. Eight sensory experts evaluated the separated fractions by direct smelling. The evaluation results shall be subjected to the unanimous approval of at least 6 experts, and the sensory perceptions were exhibited as dry, burnt and irritate offensive taste. The 14 fractions were combined, and the volume was reduced by vacuum distillation to obtain 1.0 ml EBSC for GC-O analysis.

GC-O analysis

For the detection of odor compounds in EBSC, an Agilent 7890B chromatograph equipped with an ODP-2 olfactory detection port (Gerstel, MÃijlheim an der Ruhr, Germany) was used. The carrier gas (helium, purity 99.999%) flowed at a rate of 1 ml/min. The sniffing port was pumped with moist air at 40 ml/min to elute odor compounds. The experiment was conducted by 8 evaluators who had been trained in sensory perception. During the experiment, evaluators recorded the odor retention time and its description. Each evaluator repeated the evaluation twice. The total number of times odorants were smelled by the evaluators was used as their detection frequency (DF). The odorants with DF $\geqslant$ 4, detected at least once by each of the 4 evaluators were identified as potent odorants.

GC-MS analysis

The GC-MS was performed on an Agilent 7890B GC with an Agilent 5977A mass selective detector (MSD). The separation was performed with an HP-5MS column (60 m length, 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) and a DB-WAX column (60 m length, 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA); inlet temperature, 250 °C; carrier gas (He) flow rate, 1.0 ml/min; sample volume, 1 μl; and non-split injection mode.

The heating procedures were as follows: HP-5MS, held at 50 °C (the initial temperature) for 2 min, then heated to 200 °C at 2 °C/min, and the finally heated it at 10 °C/min to 250 °C for 5 min; DB-WAX, held at 50 °C (the initial temperature) for 2 min, then heated it at 10 °C/min to 160 °C for 15 min, and the finally heated it at 1.5 °C/min to 250 °C for 5 min. Mass spectrometry conditions: EI ionization source and the electron energy was 70 eV; the ion source temperature was set at 230 °C; and the scanning range was 35–450 amu. For the accurate quantitative measurement of odor compounds in EBSC, GC-selected-ion monitoring (GC-SIM) was used.

Identification and quantitation analysis

For the identification of odor compounds in EBSC, the retention indices (RIs) of unknown odor compounds were calculated from the retention times of n-alkanes (C_9-C_{30}) according to the improved Kovats method [15]. Comparing the mass spectrum of the odor compounds with that of a standard spectrum in the NIST 17 database (Agilent Technologies, Palo Alto, California, USA), the odor compounds were tentatively identified if the mass spectrometric information of each chromatographic peak corresponds to the NIST 17 mass spectrum library by at least 80%. By comparing the RIs, mass spectra with pure standards, and odor descriptions, positive identification could be ensured. The RI values were calculated using a standard solution of alkanes (C_9-C_{30}). Accurate amount of odor compounds in EBSC was measured using the GC-selected-ion monitoring (GC-SIM) with HP-5MS type capillary chromatographic column. The odor compounds with DF $\geqslant$ 4 in GC-O were quantitatively analyzed by standard curve. The standard compound of each odorant was configured into mother liquor with dichloromethane, and then configured into seven mixed standards with different mass concentrations successively. The internal standard was phenethyl acetate. The horizontal coordinate was the ratio of the concentration of each component to the concentration of the internal standard, and the vertical coordinate was the ratio of the peak area of each component to the peak area of the internal standard. The internal standard curve was established to calculate the content of individual odor compounds in EBSC.

Calculation of OAVs

Referring to the threshold value of compounds in water [16], the OAVs of each compound was calculated according to Eq. (1):

$$\mathrm{OAV} = \frac{\mathrm{C}}{\mathrm{T}}, \tag{1}$$

where C is the concentration of the odor compounds in EBSC, and T is the odor threshold of the odor compound.

Table 1 Odor characteristics of eluted fractions from Silica gel column chromatography.

No.	Fraction	Odor description
Part 1	16–20	Weak aroma, solvent
Part 2	21–35	Green, floral, sweet
Part 3	36–43	Fatty, soapy, floral
Part 4	44–51	Caramel-like, creamy
Part 5	52–61	Sweet, creamy, weak bitter
Part 6	62–77	Sweet, Nutty, sour
Part 7	78–97	Dry, burnt, weak medicine
Part 8	98–105	Dry, burnt, medicine, weak smoky
Part 9	106–113	Dry, burnt, smoky, pungent
Part 10	114–119	Weak dry and smoky, sweet
Part 11	120–137	Roast, honey-like, beeswax
Part 12	138–158	Chenmical, honey, stink
Part 13	159–181	Sun-dried, fishy
Part 14	182–200	Sweet, solvent

Recombination and omission tests

A 75% (w/v) ethanol-water solution was used as the matrix for recombination. According to the quantitative analysis, odor compounds with OAVs $\geqslant$ 1 were added to the matrix of recombination. Then, five odor attributes were evaluated by 10 sensory experts to describe the overall odors, which were burnt, woody, smoky, pungent, and musty/dusty. In order to evaluate odor intensity, the sensory experts were asked to rate each attribute on a scale of 0 to 9; where 0 was no scent, and 9 was very strong. Each test was repeated three times, and the average was taken as the final sensory score.

To determine the significance of certain compounds, triangle tests were performed using the ISO 4120: 2004. To accomplish this, 10 omission models were prepared in the same manner as the recombination model, a compound at a time was omitted in order of OAVs from small to large. Two completely recombination models and one omission model were presented simultaneously for the sensory evaluation. In the recombination test, the same sensory expert panel was used, and they were asked whether differences could be detected. The statistical difference between recombination (Fig. 1) and omission (Table 4) were compared with a one-way analysis of variance (ANOVA) using SPSS statistics 22.0.

RESULTS AND DISCUSSION

Sensory-oriented separation of particulate extracts from mainstream flue gas

Due to the complexity of flue gas and the very low concentration of some compounds, it is difficult to accurately identify the compounds that produce burnt smell from the gas directly. Therefore, after the sample is extracted, it is necessary to separate the extract in advance to reduce the complexity of the sample and obtain a sufficient number of related compounds.

Silica gel column chromatography is a very effective method to extract and separate the specific flavor components from complex system [17]. Silica gel column chromatography was used to separate different compounds based on polarity differences. By mixing different gradient mobile phase to obtain different polarity of solvent, and further on the chromatographic column elution in order to realize the separation of complex samples, it has been widely used in separation of volatile compounds, such as black tea [18] and coffee [19].

The sensory expert panel conducted odor evaluation on each of the 200 fractions collected by silica gel column chromatography. Fractions without obvious odor characteristics were removed. Fractions with similar odor characteristics were combined to obtain a total of 14 portions (Table 1). As can be seen from the Table, Parts 7 to 10 were considered as the burnt smell fractions due to their similar burnt characteristic smell. The burnt smell fractions were then combined and reduced to 1.0 ml by vacuum distillation to obtain EBSC.

Odor compounds in EBSC

A total of 16 odor compounds were identified in EBSC by GC-O and GC-MS (Table 2), all of which were phenolic compounds. Phenolic compounds are a kind of important harmful components in mainstream cigarette smoke, which usually exhibit an adverse effect on cigarette smoke and could produce an unpleasant smell [20]. They are mainly generated from the pyrolysis of lignin in tobacco leaves [21]. Actually, most of the phenolic compounds in mainstream smoke cannot be assigned to aroma substance. Kazuhisa et al [22] reported the compounds of 2,5-dimethyl-4-hydroxy-3(*2H*)-furanone, 2-hydroxy-2-cyclopenten-1-one and 2,3-dihydro-3,5-dihydroxy-6-methyl-*4H*-pyran-4-one generated from LC fraction 1 contributed greatly to the smoke aroma. These important aroma compounds were not detected in EBSC, which also indicated the feasibility of burnt smell from the particulate extracts by using separation method of column chromatography. Phenolic compounds are mostly regarded as the main source of flavor in smoked food product [23]. The smoked flavor is not confined to a single characteristic, but by a variety of odor attributes together, such as smoky, woody, burnt, pungent, and others [24]. In our experiments, similar results were found.

Among them, o-cresol (8), p-cresol (8), and 4-ethylphenol (8) were probably the most important odor compounds in EBSC with the highest detection frequency, followed by 2-ethylphenol (7) and 2,4-dimethylphenol (7). O-cresol and p-cresol gave smoky, woody, pungent, burnt, and musty/dusty odor. It was reported that o-cresol and p-cresol played the most important role in the smoke-curing process [25]. 4-ethylphenol produced a smoky odor and affects product quality, such as cocoa [12], wine [26]. 2-ethylphenol and 2,4-dimethylphenol contributed to smoky odor. 2-ethylphenol was one of the main odor compounds of Pixian doubean sauce, and studies found that it could give raw pepper and greenly odors [27]. 2,4-dimethylphenol has been reported to have a pungent, smoky odor in smoked salmon [28], which was similar to the experimental results. In addition, odor compounds with DF $\geqslant$ 4 include 2,6-dimethylphenol (6), 2,5-dimethylphenol (6), 3,4-dimethylphenol (5), 3,5-dimethylphenol (6), 2,4,6-trimethylphenol (5), and catechol (6) played an important role in EBSC. Phenol derivatives were in the majority, and these compounds may have synergistic effect to strengthen the burnt smell [29]. However, different concentrations of odor compounds contribute differently to overall flavor [30]. Therefore, the quantitative result of each odor compound and the OAVs will be combined to analyze their contribution to EBSC in the future.

Quantitation of odor compounds and calculation of OAVs

OAVs represent the contribution of a single odorant to the overall odors. A total of 10 odor compounds containing OAVs $\geqslant$ 1 were found in EBSC (Table 3), suggesting that these compounds were important contributors to the overall characteristic odor in EBSC. In this study, p-cresol was the most important contributor with the highest OAV of 3528. P-cresol was also reported as an important odorant in liquid smoked salmon [28]. While 4-ethylphenol and 2-ethylphenol (with relatively high OAVs of 1025 and 733, respectively), 4-ethylphenol was also reported to be the main odorant of cocoa smoke odor [23]. The thresholds of o-cresol (11.33 mg/kg), 3,4-dimethylphenol (16.84 mg/kg), 3,5-dimethylphenol (18.59 mg/kg), and 2,4,6-trimethylphenol (18.59 mg/kg) were high, but their OAVs were greater than 1; hence they also contributed greatly to the burnt smell. Although the DF value of catechol (6) was large, its threshold (198 mg/kg) was very high, resulting in OAV $<$ 1 and small contribution to the burnt smell. In order to verify the key odor compounds identified in EBSC, recombination and omission tests were used.

Recombination and omission tests

Based on the quantitative results of EBSC, recombination test was conducted to verify the contribution of 10 odor compounds with OAVs $\geqslant$ 1 to the overall odors in EBSC. As shown in Fig. 1, the performance of the recombination system showed good similarity with EBSC, and there was no significant difference among the five odor attributes ($p < 0.05$). This suggested that the identification and quantitative experiments were accurate, and that the important odor compounds were

Table 2 Odor compounds in EBSC.

No.	RI[a]		Compound	Odor description	DF[b]	Identification[c]
	HP-5MS	DB-WAX				
1	981	1987	Phenol	Phenolic, chemical	2	MS, odor, RI, Std
2	1060	2000	O-cresol	Smoky, woody, pungent, burnt, musty/dusty	8	MS, odor, RI, Std
3	1075	2079	P-cresol	Smoky, woody, pungent, burnt, musty/dusty	8	MS, odor, RI, Std
4	1090	1889	Guaiacol	Smoky,sweet	3	MS, odor, RI, Std
5	1117	1918	2,6-Dimethylphenol	Smoky, woody, musty/dusty, medicinal	6	MS, odor, RI, Std
6	1148	2063	2-Ethylphenol	Smoky, woody, burnt	7	MS, odor, RI, Std
7	1181	2070	2,4-Dimethylphenol	Smoky, woody, pungent, musty/dusty	7	MS, odor, RI, Std
8	–	2072	2,5-Dimethylphenol	Smoky, woody, pungent, musty/dusty	6	MS, odor, RI, Std
9	1198	2209	4-Ethylphenol	Smoky, phenolic, burnt, animal	8	MS, odor, RI, Std
10	–	2189	3,4-Dimethylphenol	Smoky, woody, musty/dusty	5	MS, odor, RI, Std
11	1181	2174	3,5-Dimethylphenol	Smoky, woody, musty/dusty	6	MS, aroma, RI, Std
12	1192	1956	2-Methoxy-4-methylphenol	Smoky, woody, sweet	2	MS, odor, RI, Std
13	1204	–	2,4,6-Trimethylphenol	Smoky, woody	5	MS, odor, RI, Std
14	1219	2661	Catechol	Chemical, phenolic, medicinal,	6	MS, odor, RI, Std
15	1247	–	4-Isopropylphenol	Smoky, phenolic, medicinal	3	MS, odor, RI, Std
16	1315	2213	2-Methoxy-4-vinylphenol	Smoky, woody, sweet	2	MS, odor, RI, Std

[a] RI: HP-5MS, the retention index of the compound on the non-polar column HP-5MS; RI: DB-WAX, the retention index of the compound on the polar column DB-WAX.
[b] Detection frequency (DF) determined in EBSC using an HP-5MS column.
[c] MS, compounds were identified by MS spectra; odor, compounds were identified by the odor description; RI, compounds were identified by a comparison to the pure standard; Std, confirmed by comparison with authentic standards.

Table 3 Quantitative Ion, calibration equation, relative content, olfactory threshold, and OAVs of odor compounds in EBSC.

No.	Compound	Quantitative Ion[a]	Calibration equation	R^2	Relative content[b] (mg/kg)	Olfactory threshold (mg/kg)	OAV
1	O-cresol	108	$y = 0.5688x + 0.0013$	0.9999	387.8 ± 5.82	11.33	34
2	P-cresol	107	$y = 0.6336x - 0.0393$	0.9990	1023.1 ± 12.28	0.29	3528
3	2,6-Dimethylphenol	122	$y = 0.4905x + 0.0014$	0.9999	99.7 ± 1.10	1.23	81
4	2-Ethylphenol	107	$y = 0.5159x + 0.0011$	0.9997	220 ± 4.62	0.3	733
5	2,4-Dimethylphenol	122	$y = 0.4541x + 0.0160$	0.9992	252.7 ± 5.81	6.9	37
6	2,5-Dimethylphenol	107	$y = 0.6523x - 0.0089$	0.9991	110.8 ± 1.66	4.65	24
7	4-Ethylphenol	107	$y = 0.5073x + 0.0111$	0.9998	307.6 ± 7.38	0.3	1025
8	3,4-Dimethylphenol	107	$y = 0.6300x - 0.0114$	0.9991	98.2 ± 1.77	16.84	6
9	3,5-Dimethylphenol	122	$y = 0.5245x - 0.0056$	0.9994	96.3 ± 1.35	18.59	5
10	2,4,6-Trimethylphenol	121	$y = 0.2227x + 0.0090$	0.9992	68.1 ± 1.77	18.59	4
11	Catechol	110	$y = 0.4047x + 0.0135$	0.9991	63.4 ± 0.76	198	< 1

[a] Selected ions for quantification.
[b] The quantitative results were shown as means ± standard deviation ($n = 3$).

accurately identified. The subtle differences may be due to the inadequate recognition of compounds with lower activity values, matrix effects, or the omission of compounds assisting in recombination test [31].

In order to determine the importance of a single odor compound to the overall odors, a total of 10 omission models were prepared (Table 4), and the models missing a single compound were evaluated by omission tests. Triangle tests were used to determine the differences between the omission model and the recombination model, as shown in Table 4. When p-cresol (model 2), 2-ethylphenol (model 4), 2,4-dimethylphenol (model 5), and 4-ethylphenol (model 7) were omitted, at least nine sensory experts correctly judged the odor differences from the three samples, showing the highest significant differences ($p < 0.001$). This result revealed the significant role of the 4 compounds in EBSC's overall odors. In addition, the lack of o-cresol (model 1) and 2,6-dimethylphenol (model 3) showed significant differences ($p < 0.01$), which agreed with the high DF and OAVs. As a result, these two compounds also had a large impact on the overall odors of EBSC. The complete recombinant showed significance ($p < 0.05$) when 2,4,6-trimethylphenol (model 10) was omitted. In spite of having higher DF and OAVs, there were no significant differences when the recombination was conducted without the 2,5-dimethylphenol

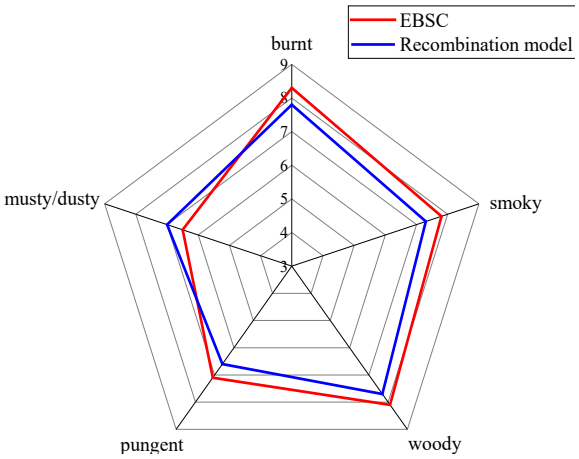

Fig. 1 Contrast chart of feature description contours of EBSC and recombination model.

Table 4 Omission tests from complete recombinant.

No.	Compound omitted	N[a]	Significance[b]
1	O-cresol	8	**
2	P-cresol	10	***
3	2,6-Dimethylphenol	8	**
4	2-Ethylphenol	10	***
5	2,4-Dimethylphenol	9	***
6	2,5-Dimethylphenol	3	–
7	4-Ethylphenol	10	***
8	3,4-Dimethylphenol	4	–
9	3,5-Dimethylphenol	7	**
10	2,4,6-Trimethylphenol	6	*

[a] Number of correct sensory experts, (from the total of 10) assessing the difference of odors by triangle test.
[b] Significance: *, significant ($p < 0.05$); **, highly significant ($p < 0.01$); ***, very highly significant ($p < 0.001$).

and 3,4-dimethylphenol ($p > 0.05$). Consideration of significance differences of omission tests, DF, and OAVs, the strongest odorants in EBSC were identified as o-cresol, p-cresol, 2,6-dimethylphenol, 2-ethylphenol, 2,4-dimethylphenol, 4-ethylphenol, 3,5-dimethylphenol, and 2,4,6-trimethylphenol.

CONCLUSION

In this work, 16 kinds of phenolic compound were successfully separated and identified in EBSC by using silica gel column chromatography separation in combination with GC-O analysis. Among them, 10 important phenolic compounds with OAVs $\geqslant 1$ were contributed significantly to the burnt smell of EBSC through GC-SIM-MS quantitative and OAVs analysis. Furthermore, the typical odors of EBSC can be simulated successfully by using the 10 phenolic compounds and through the recombination tests. The omission and recombination tests results showed that o-cresol, p-cresol, 2,6-dimethylphenol, 2-ethylphenol, 2,4-dimethylphenol, 4-ethylphenol, 3,5-dimethylphenol, and 2,4,6-trimethylphenol were the key odor compounds contributing to the burnt smell of EBSC. The results obtained in this study can provide theoretical support to directionally eliminate the burnt smell from upper tobacco leaves.

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REFERENCES

1. Ren X, He XF, Zhang ZF, Yan ZQ, Jin H, Li XZ, Qin B (2015) Isolation, identification, and autotoxicity effect of allelochemicals from rhizosphere soils of flue-cured tobacco. *J Agric Food Chem* **63**, 8975–8980.
2. Vesna R, Nermina D, Marija S (2015) Influence of mineral matter content on static burning rate of Virginia tobacco from different production areas in Serbia. *Ind Crops Prod* **67**, 381–386.
3. Long MH, Hua YK, Wang XG, Wang YK, He C, Huangfu DY, Zi WH (2017) Effect of different combined moistening and redrying treatments on the physicochemical and sensory capabilities of smoking food tobacco material. *Drying Technol* **36**, 52–62.
4. Anja T, Hubert K, Lutz M (2008) Tobacco smoke: Unraveling a controversial subject. *Exp Toxicol Pathol* **60**, 141–156.
5. Chen YJ, Ren K, He X, Gong JSQ, Hu XD, Su JE, Jin Y, Zhao ZX, et al (2019) Dynamic changes in physiological and biochemical properties of flue-cured tobacco of different leaf ages during flue-curing and their effects on yield and quality. *BMC Plant Biol* **19**, 555.
6. Zhang L, Zhang XT, Ji HW, Wang WW, Liu J, Wang F, Xie FW, Yu YJ, et al (2018) Metabolic profiling of tobacco leaves at different growth stages or different stalk positions by gas chromatography-mass spectrometry. *Ind Crops Prod* **116**, 46–55.
7. Li HT, Guan SS, Qiu CY, Liu J (2017) Study on using the compound enzyme preparation for improving the tobacco burnt gas. *Shipin Gongye* **38**, 56–60. [in Chinese]
8. Yang B, Shen J, Yang JF, Shu JS, Zhang FJ, Wang SL, Chen J, Ma S (2021) Response surface methodology to optimize compound enzymes to improve the availability of upper tobacco leaves. *Hunan Agric Sci* **51**, 75–79. [in Chinese]
9. Chi GJ, Ning W, Xu HN, Huang LT, Dai G (2021) Effect of adding complex biological enzymes online on the sensory quality and applicability of expanded cut tobacco. *Chin Tob Sci* **42**, 91–97. [in Chinese]
10. Edgar CIV, Kadri K (2013) Associations of volatile compounds with sensory aroma and flavor: The complex nature of flavor. *Molecules* **18**, 4887–4905.
11. Zhang Y, Song HL, Li P, Yao J, Xiong J (2017) Determination of potential off-flavour in yeast extract. *LWT Food Sci Technol* **82**, 184–191.
12. Füllemann D, Steinhaus M (2020) Characterization of odorants causing smoky off-flavors in cocoa. *J Agric Food Chem* **68**, 10833–10841.
13. Yang X, Yang F, Liu Y, Li J, Song HL (2020) Identification of key off-flavor compounds in thermally treated watermelon juice via Gas Chromatography-Olfactometry-

Mass Spectrometry, aroma recombination, and omission experiments. *Foods* **9**, 227.
14. Jiang JK, Tian JY, Li WX, Yu G (2020) Analysis of volatile components in beer containing *Cordyceps militaris* extract by electronic nose and GC-MS. *ScienceAsia* **46**, 323–329.
15. Cates VE, Meloan CE (1963) Separation of sulfones by gas chromatography. *J Chromatogr* **11**, 472–478.
16. Van Gemert LJ (2011) Compilations of odour threshold values in air, water and other media. In: Van Gemert LJ (ed) *Odour Threshold Values in Water, Part II*, Utrecht: Oliemans Punter & Partners BV, The Netherlands, pp 207–359.
17. Ray M (2010) *Sensory-Directed Flavor Analysis*, Taylor and Francis, CRC Press, Florida, USA.
18. Robin J, Ashu G (2015) Fractionation and identification of minor and aroma-active constituents in Kangra orthodox black tea. *Food Chem* **167**, 290–298.
19. Hironari M, Michiaki N, Seiji H, Shuichi H (2013) Identification of the odour-active cyclic diketone cis-2,6-dimethyl-1,4-cyclohexanedione in roasted Arabica coffee brew. *Food Chem* **138**, 2346–2355.
20. Carruthers W, Johnstone RA (1960) Some phenolic constituents of cigarette smoke. *Nature* **185**, 762–763.
21. Guo GN, Liu X, Li R, Li Q, Yu HB, Li MJ (2019) Characterization of tobacco stalk lignin using nuclear magnetic resonance spectrometry and its pyrolysis behavior at different temperatures. *J Anal Appl Pyrolysis* **142**, 104665.
22. Kazuhisa M, Frank D, Emmie D, Nobuo O, Hirotoshi T, Pat S (2015) LC fractionation followed by pyrolysis GC-MS for the in-depth study of aroma compounds formed during tobacco combustion. *J Anal Appl Pyrolysis* **116**, 68–74.
23. Parker M, Osidacz P, Baldock GA, Hayasaka Y, Black CA, Pardon KH, Jeffery DW, Geue JP, et al (2012) Contribution of several volatile phenols and their glycoconjugates to smoke-related sensory properties of red wine. *J Agric Food Chem* **60**, 2629–2637.
24. Wang HW, Edgar C (2018) Sensory characteristics of various concentrations of phenolic compounds potentially associated with smoked aroma in foods. *Molecules* **23**, 780.
25. Eliza K, Nina BP (2005) Volatiles composition and flavour profile identity of smoke flavourings. *Food Qual Prefer* **17**, 85–95.
26. Milheiro J, Filipe RL, Vilela A, Cosme F, Nunes FM (2017) 4-Ethylphenol, 4-ethylguaiacol and 4-ethylcatechol in red wines: Microbial formation, prevention, remediation and overview of analytical approaches. *Crit Rev Food Sci Nutr* **59**, 1367–1391.
27. Lin HB, Liu Y, He Q, Liu P, Che ZM, Wang XM, Huang JQ (2019) Characterization of odor components of Pixian Douban (broad bean paste) by aroma extract dilute analysis and odor activity values. *Int J Food Prop* **22**, 1223–1234.
28. Varlet V, Serot T, Cardinal M, Knockaert C, Prost C (2007) Olfactometric determination of the most potent odor-active compounds in salmon muscle (*Salmo salar*) smoked by using four smoke generation techniques. *J Agric Food Chem* **55**, 4518–4525.
29. Song HL, Liu JB (2018) GC-O-MS technique and its applications in food flavor analysis. *Food Res Int* **114**, 187–198.
30. Loureiro V, Malfeito-Ferreira M (2003) Spoilage yeasts in the wine industry. *Int J Food Microbiol* **86**, 23–50.
31. Gao WJ, Fan WL, Xu Y (2014) Characterization of the key odorants in light aroma type chinese liquor by Gas Chromatography-Olfactometry, quantitative measurements, aroma recombination, and omission studies. *J Agric Food Chem* **62**, 5796–5804.