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A rapid analysis of antioxidants in *Sanghuangporus baumii* by online extraction-HPLC-ABTS†

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In the present study, a simple and efficient approach based on the online extraction-high performance liquid chromatography coupled with ABTS antioxidant assay (OLE-HPLC-ABTS) was established to quickly and directly analyze the antioxidants in *S. baumii*. Through this system, the HPLC mobile phase *via* a guard column packed with a *S. baumii* sample was used for online extraction (OLE). The separation was performed on an Agilent Poroshell EC-C18 column with a gradient elution using 0.1% formic acid (A) and 0.1% formic acid–acetonitrile (B) as mobile phase systems and detected at a wavelength of 254 nm. Then, the separated compounds were reacted with the antioxidant solution (ABTS), and the response was recorded at a wavelength of 400 nm. The developed analytical method was successfully applied to *S. baumii* samples, and eight antioxidants were identified. The established system integrated the online extraction, separation and online antioxidant detection, which is rapid, efficient, and suitable for the rapid screening of antioxidant compounds from solid sample mixtures.

1. Introduction

Sanghuangporus baumii is the dry fruit body of *Sanghuangporus baumii* (Pilat) L. W. Zhou and Y.C. Dai (Basidiomycota, Agaricomycetes, Hymenochaetales, Hymenochaetaceae, and *Sanghuangporus*), a kind of precious and functional mushroom historically used for food and medicine.[1] *S. baumii* was first recorded in Shennong's Classic of Material Medical and mainly distributed in oriental countries (China, Korea, and Japan). It is commonly used in the treatment of amenorrhea, dysentery and diarrhoeal diseases.[2,3] Modern researches have confirmed that *S. baumii* mainly contains polysaccharides, flavonoids, triterpenoids and pyranone compounds with various biological activities, such as anti-tumor, anti-oxidation, anti-inflammatory, immunomodulatory and hypoglycemic.[4–7] In the human body, reactive oxygen free radicals participate in numerous biological activities (immunity and signal transduction processes, *etc.*). The overproduction of active oxygen free radicals has a destructive effect, leading to a variety of diseases, such as heart disease, Alzheimer's disease, and tumors, by causing an oxidative damage to the normal cells and tissues.[4,9] Therefore, the exploitation of natural and efficient antioxidants not only has far-reaching significance for the treatment of diseases caused by the excessive free radicals but also promotes anti-tumor, immune regulation or other pharmacological effects. At present, it has been reported that *S. baumii* possesses antioxidant activity.[8] However, up to date, the specific antioxidant active ingredients of *S. baumii* are still unclear. The quantitative analysis of active ingredients in Chinese medicine is an important part for the evaluation of its quality.[10,11] Therefore, screening the antioxidants from *S. baumii* is helpful in improving the quality evaluation of its products.

Traditionally, the commonly used method for the discovery of active compounds from complex mixtures basically contains the procedure of separation–purification–activity verification or activity-guided isolation, with the disadvantages of complicated steps, long time-consumption, high energy consumption and low efficiency.[12] Recently, some online antioxidant screening methods such as HPLC-ABTS/DPPH/FRAP has been widely used in the rapid screening of antioxidants from Chinese herbal medicines, such as *Fallopia sachalinensis*, *Morchella angusticeps*, and Du-zhong brick tea.[13–15] However, the above-mentioned methods with extracted sample solutions for the HPLC injection analysis still require offline sample preparation programs, such as ultrasonic extraction and heating reflux extraction, often causing the large consumption of organic reagents and insufficient extraction. Fortunately, the online extraction high

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performance liquid chromatography (OLE-HPLC) analysis technology was successfully developed and applied to the discovery of antioxidants from traditional Chinese medicine.[16,17] Through this technology, the sample was extracted by a liquid phase eluent, and the extracted compounds were separated in a single run, avoiding the complex sample processing.

Therefore, in the present study, the approach of online extraction-HPLC-ABTS (OLE-HPLC-ABTS) was established by the combination of the online extraction and HPLC-ABTS, and it was successfully applied to analyze the antioxidants in *S. baumii*. As a result, eight compounds with antioxidant activities were identified *via* Q-TOF-MS/MS. This method provides a basis for the improvement of the basic research of the medicinal substances of *S. baumii* and their related product development.

2. Materials and method

2.1 Fungi materials, chemicals and reagents

The sample collected from Jinzhai (Anhui, China) was identified as *S. baumii* by Dr. Z. M. Qian (Sunshine Lake Pharma Co., Ltd). Voucher specimens were deposited at the Sunshine Lake Pharma Co., Ltd (Dongguan, China).

Protocatechuic acid (99.20%) and protocatechualdehyde (94.30%) were purchased from the Nature Standard (Shanghai, China). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) was purchased from Aladdin (Shanghai, China). Acid-washed diatomaceous earth was purchased from Sigma-Aldrich (Shanghai, China). The deionized water used for the experiments was purified using a Milli-Q purification system (Millipore, USA).

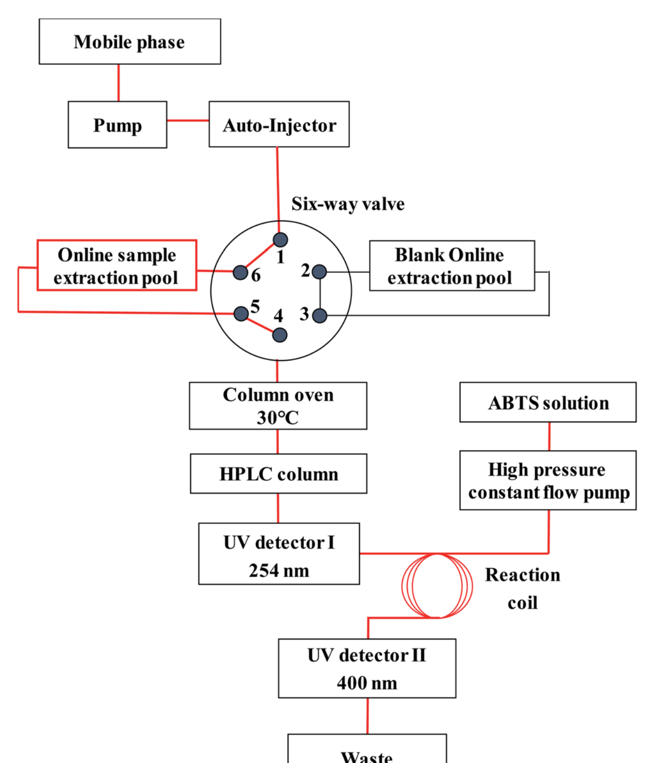

Fig. 1 The schematic diagram of the OLE-HPLC-ABTS system.

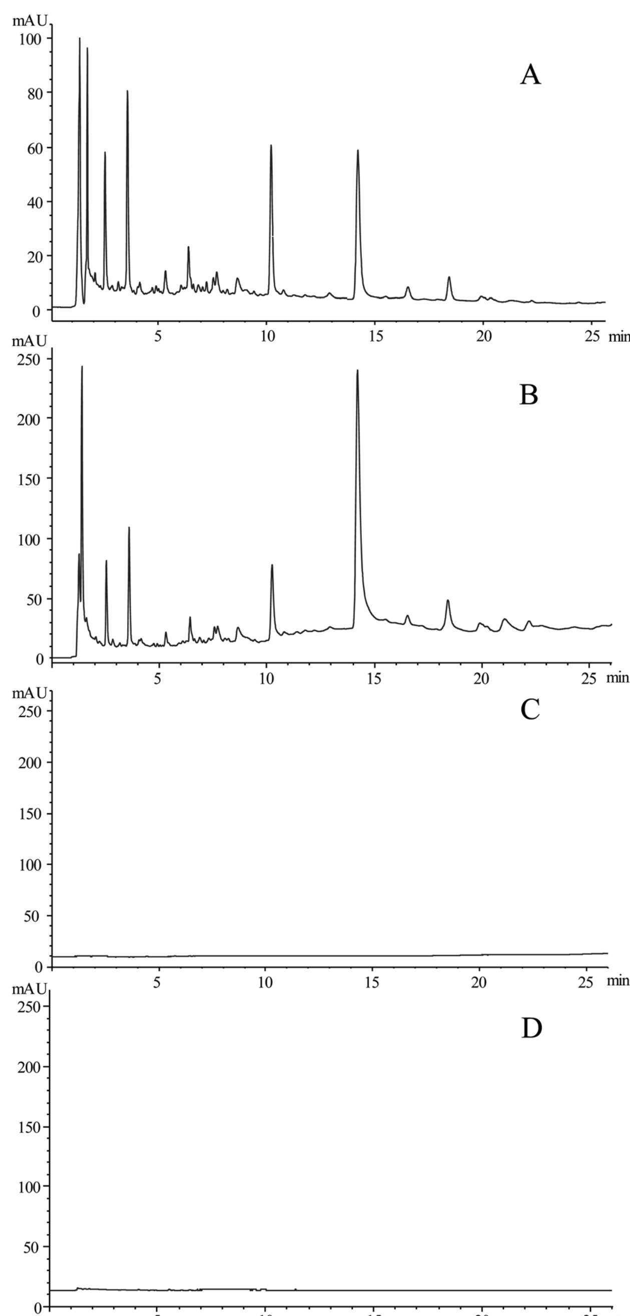

Fig. 2 The chromatograms of the OLE-HPLC of *S. baumii*. The pretreated sample by ultrasound extraction with 50% methanol (A), the OLE sample (B), the secondary OLE sample (C), and the negative sample (D).

2.2 Preparation of standard stock solutions and working solutions

The mixed standard stock solution at a concentration of 1 mg mL^{-1} was prepared with 50% methanol, and the working solution was further diluted to the intended concentration with 50% methanol.

The stock ABTS solution was prepared by mixing ABTS (7 mM dissolved in deionized water) and potassium persulfate (5 mM dissolved in deionized water) in equal volume (v/v = 1 : 1) and

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stored in a flask protected from light using an aluminum foil at 4 °C for at least 12 h. The working ABTS radical solution was prepared by diluting with absolute ethanol to an absorbance of 0.95–1.05 AU. A UV detector was set at 400 nm according to the UV scanning result of the ABTS working reagent showing a maximum absorption at this value. This liquor was protected from light with an aluminum foil and drawn out by pump to react with the separated sample through a buffer coil.

The working FRAP radical solution was prepared by mixing TPTZ (10 mM in 40 mM HCl), $FeCl_3$ (20 mM in water) and acetate buffer (300 mM, adjusted pH to 3.6 with 1 mM NaOH solution) in the ratio of 1 : 1 : 50. Then, the reagent solution flask was covered with an aluminum foil until further use. The FRAP working solution was prepared freshly for each run.

2.3 Sample preparation

2.3.1 Preparation of *S. baumii* extract by ultrasound extraction. A *S. baumii* sample was first ground and then filtered through a sieve. 0.5 g of powder was weighed, placed in a centrifuge tube to which methanol (5 mL) was added at a concentration of 10%/50%/100%, respectively. The sample was extracted for 30 min by ultrasound, and the lost weight was compensated after cooling the extract to the room temperature. The turbid extracted solution was centrifuged for 10 min (14 000 rpm), and the supernatant was filtered through a 0.22 μm microporous membrane (JinTeng, China) prior to HPLC analysis.

2.3.2 Preparation of online extraction pool. The dried powder of the *S. baumii* sample (0.1 g) was mixed with clean, acid-washed diatomaceous earth (1 g). Then, the mixture (approximately 20 mg) was accurately weighed and packed into a hollow guard column (3 × 4 mm). The two ends of the guard column were sealed with a filter film (pore size of 0.22 μm) and a metal ring, respectively. The guard column containing the sample powder was placed inside a matched column holder to form the whole online extraction cell of the *S. baumii* sample. Moreover, the online extraction cell of the negative sample was prepared wherein the mixture of *S. baumii* and clean, acid-washed diatomaceous earth was replaced by the clean, acid-washed diatomaceous earth.

For investigating the dispersion and extraction effects of different dispersants, dispersants such as silica gel, Florisil, neutral alumina and polyamide were mixed with the *S. baumii* sample for online extraction.

2.4 The OLE-HPLC and post-column antioxidant system conditions

An Agilent 1260 Series high performance liquid chromatograph (HPLC) (Agilent Technologies, USA) was equipped with a quaternary pump, an online degasser, an auto-sampler, a column oven and a diode array detector (DAD). A Phenomenex hollow guard column (3 × 4 mm) with its fitted holder was used for the online extraction module.

An Agilent Poroshell EC-C18 column (4.6 × 100 mm, 2.7 μm) was used for the sample separation. The column temperature was set at 30 °C. The mobile phase consisted of 0.1% formic acid–water (A) and acetonitrile contained 0.1% formic acid (B) at a flow rate of 0.8 mL min^{-1}. The gradient elution program was as follows: 0–5 min, 15–23% B; 5–12 min, 23–25% B; 12–35 min, 25–35% B. The detection wavelength was set at 254 nm. All of the online extraction pools were connected with HPLC column through a cutting six-way valve (Fig. 1). The blank online extraction pool was connected to the liquid phase flow path for equilibrating the column, while the online sample extraction pool was connected to the flow path of the OLE-HPLC analysis. The extraction process was triggered by an auto-injector extraction of 0 μL.

Online post-column antioxidant activity was detected by combining a high pressure constant flow pump (Wufeng, China) and a 230 II UV-detector (Elite Analytical Instruments, China) to the main online extraction-HPLC system. After the online HPLC analysis, the separated sample was mixed to react with the antioxidant working solution pumped at a constant flow rate of 0.4 mL min^{-1} ($f_{HPLC} : f_{ABTS/FRAP} = 2 : 1$) by a high pressure constant flow pump through a reaction coil of 3 m long. Then, the antioxidant profiles of separated compounds were recorded by an UV detector (Fig. 1).

2.5 Q-TOF-MS/MS conditions for identification analysis

HPLC-Q-TOF-MS was used to identify the compounds in the separated sample. The chromatographic conditions were the

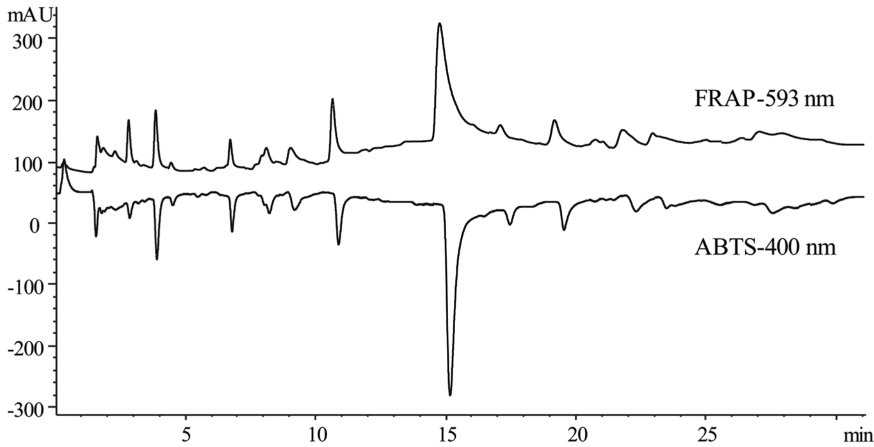

Fig. 3 The chromatograms of the antioxidant detection systems HPLC-FRAP and HPLC-ABTS.

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same as that of the OLE-HPLC system. The conditions of the ESI source were as follows: scanning mode, positive/negative ion mode; drying gas (N_2) flow rate, 11 L min^{-1}; drying gas (N_2) temperature, 350 °C; nebulizer pressure, 40 psi; sheath gas flow rate 10 L min^{-1}; capillary voltage, 4000 V; collision induced dissociation voltage, 120 V; ion scanning range, 100–1500 *m*/z. Collision energy was set at 10 V, 20 V and 40 V, respectively.

2.6 Method validation

As this established approach is a qualitative analysis, only the specificity and repeatability were validated according to the guidelines of *Chinese Pharmacopoeia* (Ch. P).[18] The specificity test was carried out *via* the analysis of standards and samples. In addition, three replicates of the online extraction pools prepared in parallel were analyzed *via* the OLE-HPLC system. Then, recording of the retention time and the area of each common peak was carried out to determine the repeatability of the developed method.

3. Results and discussion

3.1 Optimization of OLE conditions

In general, the sample of the complex mixture contained macropolar and micropolar molecules. The achievement of a complete extraction efficacy in the sample pretreatment process usually required extraction through gradient organic solvents. The established system perfectly solved this problem by the inclusion of a gradient mobile phase. By comparing the results of the OLE-HPLC analysis with those obtained by the traditional ultrasound extraction under different concentrations of methanol (10%, 50%, and 100%), it was found that the developed OLE-HPLC system was able to completely and directly extract the macropolar and micropolar molecules at a higher extraction rate (Fig. S1†), with the advantages of reduced time and reduced use of organic reagents and simplified experimental procedures. Moreover, to investigate the optimum detection wavelength of the compounds contained in the sample, the different detected wavelengths (254, 330, and 360 nm) were compared. The result displayed that the detected wavelength of 254 nm had more chromatographic peaks and a smoother baseline (Fig. S2†).

In addition, in order to obtain a satisfactory extraction effect, different types of dispersants mixed with the samples were investigated. Totally, 5 types of dispersants (acid-washed diatomaceous earth, silica gel, Florisil, neutral alumina, and polyamide) mixed with dried powder of *S. baumii* sample to make the online extraction pool were analyzed *via* the OLE-HPLC system. Fig. S3† indicates that the acid-washed diatomaceous earth when used as a dispersant can better disperse the compounds from the *S. baumii* sample and make it more completely extracted.

3.2 The extraction efficiency of the OLE-HPLC system

To verify the effectiveness of the developed OLE-HPLC method, the pretreated sample (ultrasound extraction) and the OLE sample were analyzed. The results showed that the chromatograms of the OLE sample were almost similar to those of the ultrasound pretreated sample (Fig. 2A and B). Moreover, the same OLE sample was analyzed for the second time to test if the online extraction was complete in one attempt (Fig. 2C). Results showed that the main compounds of *S. baumii* could be completely extracted in one attempt by the OLE-HPLC method, and the extraction efficiency of the OLE-HPLC method was

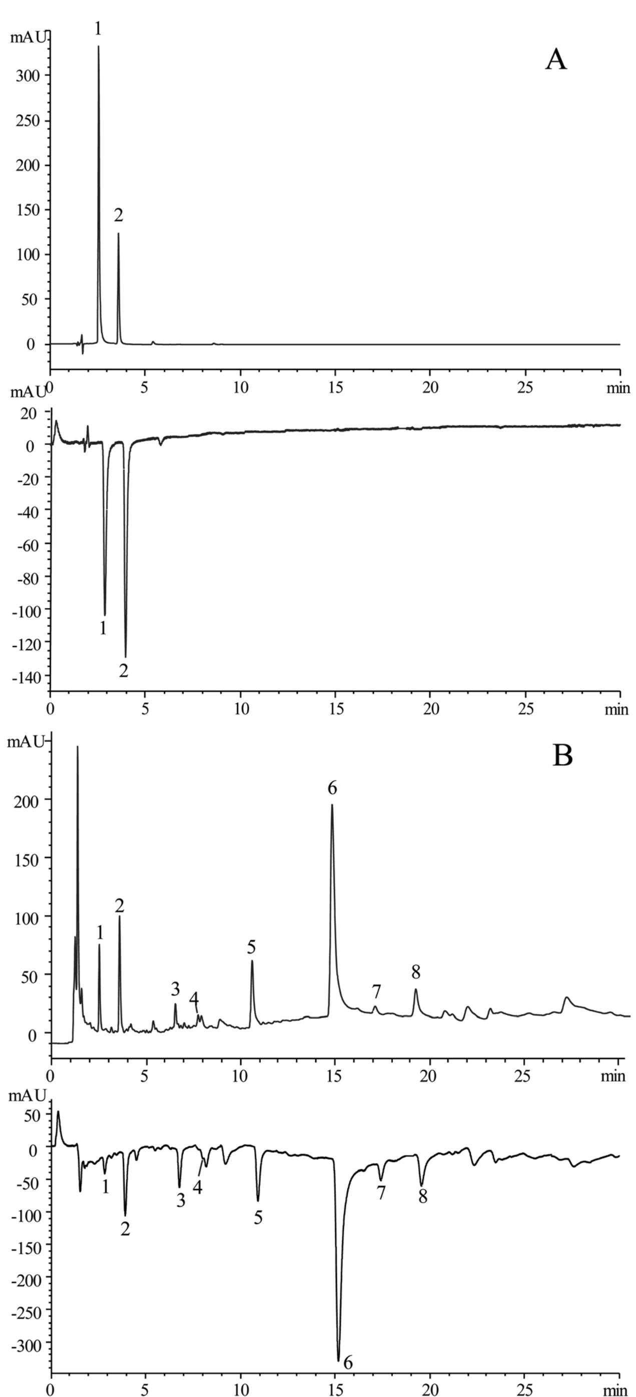

Fig. 4 The chromatograms of the mixed standards (A) and *S. baumii* (B) by OLE-HPLC-ABTS. Protocatechuic acid (1), protocatechualdehyde (2), osmundacetone (3), hispidin (4), davallialactone (5), hypholomine B (6), (4*Z*)-3-(3,4-dihydroxyphenyl)-6-[(*E*)-2-(3,4-dihydroxyphenyl)vinyl]-1-hydroxy-4-(2-oxopropylidene)-3*a*,4-dihydro-3*H*,8*H*-furo[3,4-*c*]oxepin-8-one (7), and inoscavin A (8).

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Table 1 Approaches for screening of the antioxidants in other species of *Sanghuangporus*

		Sample preparation			Sample antioxidant analysis				
No.	Species of *Sanghuangporus*	Extraction methods	Extraction solutions	Time (min)	Method	Time (min)	Total time (min)	Total solvent volume (mL)	Ref.
1	*S. sanghuang* *S. baumii* *S. vaninii*	Dipping extraction	70% ethanol	7200	ABTS, DPPH	20	7220	Unclear	4
2	*S. sanghuang*	Ultrasonic extraction	80% ethanol	40	ABTS, DPPH	20/30	60/70	180	20
3	*Phellinus linteus*	Reflux extraction	Methanol	300	HPLC-DPPH	30	330	3×10^4	21
4	*Phellinus igniarius* *Phellinus linteus*	Heat-assisted extraction	Water	420	FRAP, DPPH	30/20	450/440	Unclear	22
This study	*S. baumii*	Online extraction	—	—	HPLC–ABTS	35	35	42	—

almost identical to the traditional extraction method. The negative sample is shown in Fig. 2D.

3.3 Comparison of HPLC-ABTS and HPLC-FRAP assays

In this study, two common approaches (ABTS and FRAP) were used to evaluate the antioxidant capacities of the samples. Antioxidant effects can work through numerous mechanistic reaction pathways, such as metal chelation, quenching of singlet oxygen and radical scavenging.[19]

The FRAP assay is based on a redox reaction of metal ion assessed through a redox-linked reduction of complex Fe^{3+}–TPTZ to a blue-colored complex (Fe^{2+}–TPTZ). Antioxidants increased the production of Fe^{2+}–TPTZ, and a positive peak was observed at 593 nm. The ABTS radical scavenging assay was based on the oxidization of ABTS free radicals to green free radical cation $ABTS^{\bullet+}$ under the action of appropriate oxidants (potassium persulfate, *etc.*). The antioxidants inhibited the production of $ABTS^{+}$, and a negative peak was observed at 400 nm.

Two samples of the online extraction pools prepared in parallel were analyzed by the two post-column online antioxidant assays of ABTS and FRAP. The wavelength of the HPLC-ABTS assay was set at 400 nm according to the UV scanning result of the ABTS working reagent showing a maximum absorption at this value, and the chromatogram showing the negative peaks was obtained after each run. However, the wavelength of the HPLC-FRAP assay was set at 593 nm, and a chromatogram with positive peaks was recorded. The results (Fig. 3) showed that the chromatographic peak response of the compounds possessing antioxidant activity was maximum, and the baseline noise was smoother in the HPLC-ABTS system. Besides, the chromatogram with a negative peak provided a more intuitive display of antioxidant compounds. Eventually, the HPLC-ABTS post-column assay was used for the online antioxidant analysis.

Table 2 MS data of components in *Sanghuangporus baumii*

Peaks no.	RT (min)	Molecular formula	Molecular ion (*m*/*z*)	Fragment ion (*m*/*z*)	Identification
1	2.530	$C_7H_6O_4$	153.0189	109.0291	Protocatechuic acid
2	3.586	$C_7H_5O_3$	137.0241	108.0211	Protocatechualdehyde
3	6.403	$C_{10}H_{10}O_3$	177.0552	177.0548, 134.0368, 133.0284	Osmundacetone
4	7.530	$C_{13}H_{10}O_5$	245.0456	159.0456, 201.0558	Hispidin
5	10.207	$C_{25}H_{20}O_9$	463.1033	379.0827, 259.0612, 159.0433	Davallialactone
6	14.152	$C_{26}H_{18}O_{10}$	489.0840	445.0935, 403.0829, 241.0506	Hypholomine B
7	16.488	$C_{25}H_{20}O_9$	463.1034	405.0618, 379.0808, 259.0586	(4*Z*)-3-(3,4-Dihydroxyphenyl)-6-[(*E*)-2-(3,4-dihydroxyphenyl)vinyl]-1-hydroxy-4-(2-oxopropylidene)-3*a*,4-dihydro-3*H*,8*H*-furo[3,4-*c*]oxepin-8-one
8	18.381	$C_{25}H_{18}O_9$	461.0877	377.0661, 257.0442, 159.0432	Inoscavin A

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3.4 Method validation

To determine the repeatability of the developed method, three samples of the online extraction pools prepared in parallel were extracted by the online extraction-HPLC-ABTS system. Then, the calculated RSD values of the retention time and peak areas from each common peak were <6%, which indicated that the method was suitable for the intended use and demonstrated great repeatability. Furthermore, the specificity test is verified *via* the analysis of the standards and samples shown in Fig. 4.

3.5 OLE-HPLC-ABTS analysis of *S. baumii*

After the optimization and validation of the method, the OLE-HPLC-ABTS system was successfully applied to the mixed standard stock solution and the OLE sample. The chromatographs with the positive peaks (UV detector at 254 nm) and the antioxidant activity chromatograms with negative peaks (UV detector at 400 nm) are obtained, as shown in Fig. 4. In recent years, numerous approaches for screening of the antioxidant in other species of *Sanghuangporus* were reported.[4,20–22] Comparison with the reported methods showed that the established approach only required 35 min to complete the whole process of antioxidant screening, while the reported methods needed at least 60 min (Table 1). In addition, the OLE-HPLC-ABTS assay only needed less than 50 mL of mobile phase, while the reported methods required above 180 mL of the total solvent. In addition, the reported methods only proved the total antioxidant capacity of *S. Baumii* extraction, while the current approach identified the 8 antioxidant components from *S. Baumii*. Thus, the developed OLE-HPLC-ABTS system for the analysis of the antioxidants in *S. Baumii* was simple, rapid and highly efficient.

3.6 Identification of antioxidants in *S. baumii*

The *S. baumii* sample was further analyzed by the HPLC-Q-TOF-MS system to identify the components with antioxidant activity. Two peaks, protocatechuic acid (1) and protocatechualdehyde (2), were identified by comparing the retention time and the mass spectrometric data of the chromatographic peaks with that of the reference substances. By verifying the mass spectrometry data of the chromatographic peak with the reported data,[23–26] other 6 chromatographic peaks were preliminarily identified, namely osmundacetone (3), hispidin (4), davallialactone (5), hypholomine B (6), (4*Z*)-3-(3,4-dihydroxyphenyl)-6-[(*E*)-2-(3,4-dihydroxyphenyl)vinyl]-1-hydroxy-4-(2-oxopropylidene)-3*a*,4-dihydro-3*H*,8*H*-furo[3,4-*c*]oxepin-8-one (7), and inoscavin A (8). The detailed mass data is provided in Table 2. The results showed that the main antioxidant components in *S. baumii* are flavonoids and polyphenols.

4. Conclusion

In this study, the OLE-HPLC-ABTS technology was used to establish an online screening method for the rapid discovery of the antioxidants in *S. baumii*. By combining of the online extraction and online antioxidant assay, the advantages of extremely lower sample consumption, higher analytical efficiency and less usage of the extraction solvent were observed compared to the previous methods. The results of the antioxidant screening from *S. Baumii* showed that the main antioxidant substances are flavonoids and polyphenols, which could be used as the markers for the quality control of *S. baumii* and its products. In future, the analytical method for the determination of these antioxidants should be developed for the analysis of different *S. baumii* samples. Besides, by virtue of low sample consumption (mg level), the developed method is more suitable for the analysis of the active substances in valuable medicinal materials, such as *Cordyceps sinensis*. To conclude, as a green analytical chemistry method with little pollution, it provided a possible developmental direction for the analysis and quality control of TCM as well as the discovery of bioactive compounds from them.

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Ethical statement

This article does not contain any studies with human participants or animals performed by any of the authors.

Author contributions

Qianhui Shen: investigation, visualization, methodology, and writing (original draft preparation). Qi Huang: visualization, supervision, and writing (review and editing). Juying Xie: conceptualization and writing (review and editing). Kun Wang: resources, project administration, and funding acquisition. Luqi Peng: writing (review and editing). Zhengming Qian: conceptualization, validation, methodology, project administration, funding acquisition, and writing (review and editing). Deqiang Li: conceptualization, project administration, funding acquisition, and writing (review and editing).

Conflicts of interest

All the authors declare that they have no conflicts of interests.

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