Journal of Chromatography B, 870 (2008) 209–215

Contents lists available at ScienceDirect

Journal of Chromatography B

journal homepage: www.elsevier.com/locate/chromb

Determination of 5α-androst-16-en-3α-ol in truffle fermentation broth by solid-phase extraction coupled with gas chromatography–flame ionization detector/electron impact mass spectrometry

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ARTICLE INFO

Article history:
Received 4 April 2008
Accepted 8 June 2008
Available online 13 June 2008

Keywords:
5α-Androst-16-en-3α-ol
Truffle fermentation
Solid-phase extraction
Gas chromatography
Flame ionization detector
Electron impact mass spectrometry

ABSTRACT

A novel method using solid-phase extraction coupled with gas chromatography and flame ionization detector (FID)/electron impact mass spectrometry (EIMS) was developed for the determination of 5α-androst-16-en-3α-ol (androstenol), a steroidal compound belonging to the group of musk odorous 16-androstenes, in truffle fermentation broth. Comparison studies between FID and EIMS indicated two detectors gave similar quantitative results. The highest androstenol concentration of 123.5 ng/mL was detected in *Tuber indicum* fermentation broth, while no androstenol was found in *Tuber aestivum* fermentation broth. For the first time, this work confirmed the existence of androstenol in the truffle fermentation broth, which suggested truffle fermentation is a promising alternative for androstenol production on a large scale.

1. Introduction

Truffle, known as "underground gold", is precious edible fungus widely used in French and Italian cuisines. Truffle culinary and commercial value is mainly due to its organoleptic properties such as aroma [1]. Because of its great demand in market and shortage in wild resource, new approach is eager for truffle production on a large scale. Comparing to the natural field collection and semi-artificial simulation cultivation [2], submerged fermentation of truffle is a promising alternative for the efficient production of mycelia and its metabolites [3], and this idea was firstly demonstrated in our lab by developing the submerged fermentation of Chinese truffle *Tuber sinense* for the production of truffle mycelia and *Tuber* polysaccharides [4–5].

5α-Androst-16-en-3α-ol (androstenol), a steroidal compound belonging to the group of musk odorous 16-androstenes, was firstly isolated from boar testes [6] and always exists in human or animal tissue, saliva, urine, plasma and axillary sweat [7–8]. Recognized as a pheromone [9–10], androstenol could increase the sexual arousal of human female [11]; mediate human menstrual synchrony through regulating the secretion of luteinizing hormone [12]; adjust moods as submissive rather than aggressive in female menstrual cycle [13]; and antagonize anxiety and convulsion by positively modulating the $GABA_A$ receptors [14]. Due to its welcome odor and pharmaceutical function, androstenol has been developed to perfume with high value in market, and it also has potential in drug development for anti-depression.

In 1981, Claus et al. [15] firstly confirmed the presence of androstenol in the fresh fruiting-body of black périgord truffle by the method of thin plate chromatography (TLC) for sample cleaning. Due to its poor isolation capability and recovery, TLC was difficult to get good accuracy and reproducibility, which may be the reason that the method was not chosen for androstenol analysis after its publication. Sample cleaning process is essential for complex mixture analysis, especial the nature products or biological samples. Comparing to TLC, solid-phase extraction (SPE) is widely used in sample extraction and clean up for aqueous samples, such as wine [16], waste water [17], plasma [18], etc. The application of SPE for the determination of androst-16-ene steroids in human testis [19], human urine [20], and boar [21–22] were also reported. The advantage of SPE has been commonly cited including its robustness, potential for automation, capacity for providing clean extracts, selective isolation, even a fractionation of the different sample components. For these reasons, SPE is a powerful preconcentration technique which can be easily adapted for routine analysis, especially for the analyte with low concentration in the complex biologic matrix.

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doi:10.1016/j.jchromb.2008.06.014

Detectors with high sensitivity and selectivity are required for trace amount assay. Gas chromatography (GC) with flame ionization detector (FID) (hereafter referred to as GC-FID) and GC with electron impact mass spectrometry (EIMS) (hereafter referred to as GC-EIMS) [19,20,23,24] have been developed for the analysis of androstenol in testis and urine. Compared to FID, EIMS with lower determination limit is more sensitive. However, the appropriate sample preparation procedure (e.g., SPE) could enrich the analyte and simultaneously removing the impurity, and then satisfy the determination limit of FID. The advantage of FID is sensitive, simple and economical, which is suitable for mass measure.

By developing a novel assay method coupling SPE with GC-FID or EIMS (hereafter referred to as SPE–GC–FID/EIMS) for the determination of androstenol, this work confirmed the presence of androstenol in truffle fermentation broth for the first time. More precisely, this work (1) optimized SPE conditions; (2) studied the method validation of SPE–GC–FID; (3) assayed the concentrations of androstenol in the fermentation broths of *Tuber melanosporum*, *T. sinense*, *Tuber indicum*, and *Tuber aestivum* by SPE–GC–FID, and then confirmed the results by SPE–GC–EIMS. To the best of our knowledge, the determination of androstenol in truffle fermentation broth was firstly reported in this work.

2. Materials and methods

2.1. Chemicals and reagents

Methanol and acetone were purchased from BoDi Chemical Factory (Tianjin, China). Pure water was prepared by Reverse Osmosis Water System, which was produced by PuQing water Industry (Sichuan, China). Cleanert C_{18} cartridges (200 mg/3 mL) were purchased from Agela Technologies Inc. (Beijing, China). 5α-Androst-16-en-3α-ol (androstenol) and 5α-androstan-3β-ol (internal standard, hereafter referred to as I.S.) were purchased from Sigma–Aldrich China Inc. (Beijing, China), their stock solutions were made in acetone at 200 μg/mL, and their working standard solutions ranging from 2 to 200 μg/mL were prepared by diluting 200 μg/ml stock solutions with acetone. These solutions were used for the preparation of spiked fermentation broth sample.

2.2. Truffle fermentation process [6–7]

The strains of *T. melanosporum* Vittad., *T. sinense* and *T. indicum* were kindly provided from Mianyang Institute of Edible Fungi (Sichuan, China). The strain of *T. aestivum* Vittad. was kindly provided by Huazhong Agricultural University (Hubei, China). The strains were maintained on potato–agar–dextrose slants. The slant was inoculated with mycelia and incubated at 25 °C for 5 days, then stored at 4 °C for about 2 weeks. Preculture medium consisted of the following components (g/L): glucose, 35; peptone, 5; yeast extract, 2.5; $KH_2PO_4{\cdot}H_2O$, 1; $MgSO_4{\cdot}7H_2O$, 0.5; Vitamin B_1, 0.05. For the first-stage seed preculture, 40-mL medium with initial pH of 5.0 was prepared in a 250-mL flask, and then 10-mL mycelium suspension from a slant culture was inoculated, and followed by 5-day incubation at 25 °C on a rotary shaker (120 rpm). For the second-stage seed preculture, 180-mL medium was prepared in a 500-mL flask, and inoculated with 20-mL first-stage seed preculture broth (with ca. 400–500 mg dry cell weight (DW)/L), then followed by 2-day incubation at 25 °C on a rotary shaker (120 rpm). The fermentation medium was composed of 65 g/L of sucrose, 5 g/L of peptone, 5 g/L of yeast extract, 0.05 g/L of Vitamin B_1 with 5-mL second-stage seed preculture broth (with ca. 350–450 mg DW of cells per liter) in 50-mL medium in a 250-mL flask. The fermentation was conducted at 25 °C on a rotary shaker at 120 rpm.

For sampling, the fermentation broth on day 5 of culture was filtered with filter paper, and then stored at −20 °C. For the preparation of the blank fermentation broth, the fermentation broth after filtration was subject to boil for 10 min in order to remove the existing androstenol in the fermentation broth.

2.3. Sample preparation

2.3.1. Proposed method

A 10 mL of truffle fermentation broth was spiked with 50 μL of 20.0 μg/mL I.S. and centrifuged in sealed centrifuge tube at 10,000 rpm for 20 min at 4 °C. Then it was loaded into a C_{18} cartridge which had been pretreated with 4 mL of methanol and 4 mL of deionized water. After sample loading, the cartridge was washed by 2 mL of 60% acetone aqueous solution and finally eluted with 1 mL of acetone. The eluate was evaporated to dry under nitrogen stream and then dissolved in 50 μL of acetone.

2.3.2. Optimization of solid-phase extraction (SPE)

Solid-phase extraction (SPE) was optimized by the approach of one-variable-at-a-time, and the blank samples of *T. melanosporum* fermentation broth spiked at 500.5 ng/mL of androstenol and 500.0 ng/mL of I.S. were loaded into C_{18} cartridge for the following optimization. In order to enrich androstenol and I.S. from the matrix as much as possible, the adsorption capability of C_{18} cartridge evaluated by the adsorption percentages of androstenol and I.S. was studied with different loading sample volume of 10, 20, 30, and 40 mL. In order to optimize eluent composition and its rinsing volume, effects of acetone concentration (i.e., 40–100%) in the eluent and the rinsing volume of eluent (i.e., 1, 2 and 3 mL) on the desorption percentages of androstenol and I.S. in C_{18} cartridge was investigated in detail. The concentration of androstenol or I.S in the eluate was analyzed by gas chromatography with flame ionization detector.

2.4. Apparatus and conditions

2.4.1. Gas chromatography with flame ionization detector (GC-FID)

A GC-3900 gas chromatograph (GC) equipped with a split/splitless injector, an auto-sampler and a flame ionization detector (FID) from Varian Technologies Inc. (CA, USA) was used in this study. The separation was carried out on a HP-1 capillary column (30 m × 0.25 mm i.d., 0.20 μm film thickness) from Agilent Technologies Inc. (CA, USA). A volume of 1 μL sample was injected in the split mode at a split ratio of 1:10 and with an injector temperature of 280 °C. GC oven temperature was initially maintained at 100 °C for 2 min, then programmed to 180 °C at a rate 10 °C/min and maintained for 16 min, finally to 300 °C at 40 °C/min and maintained for 2 min. Nitrogen (99.999%) was used as carrier gas at a constant flow rate of 1.0 mL/min. The detector temperature was set at 300 °C.

2.4.2. Comparison between SPE–GC–FID and SPE–GC–EIMS

The analysis of GC equipped with electron impact mass spectrometry (GC-EIMS) was carried out on an Agilent 7890A GC system equipped with a 5975C quadrupole mass spectrometer (MS) detector (CA, USA) for the method comparison. A DB-1-MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) from Agilent Technologies Inc. (CA, USA) was used. The system was operated in the mode of constant flow (1.0 mL/min), using helium as carrier gas, and the sample was injected in the split mode with the split radio of 1:10. The GC temperature program was the same with GC-FID. The injection port, transfer line and ion source temperature were maintained at 200, 250 and 200 °C, respectively. MS was run

in the mode of electron impact (EI) with the electron energy of 70 eV, and the mode of selective ion monitoring (SIM) was applied. The androstenol and I.S. in fermentation broth were identified by comparing their corresponding fragments with the same relative retention time (t_R) occurred in standard solution. Characteristic ions, the m/z of 241 and 274 for androstenol and the m/z of 243 and 276 for I.S., were used for assaying with SIM mode.

2.5. Method validation for SPE–GC–FID

2.5.1. Calibration curve

The blank samples of *T. melanosporum* fermentation broth were spiked with 50 μL various concentrations of androstenol (i.e., 2.0, 5.0, 10.0, 20.0, 50.0 and 100.1 μg/mL) and 50 μL of 5.0 μg/mL I.S. to a final volume of 10 mL to yield 10.0, 25.0, 50.1, 100.1, 250.3, and 500.5 ng/mL androstenol and 25.0 ng/mL I.S., respectively. And then, the samples were treated by the proposed method in Section 2.3.1. The calibration curve was constructed by plotting the peak area ratio of androstenol to I.S. versus the ratio of their corresponding concentrations. The other prepared and analyzed procedures were the same as above.

2.5.2. Recovery

The blank samples of *T. melanosporum* fermentation broth spiked at 10.0, 100.1 and 500.5 ng/mL androstenol were assayed for the determination of recovery. The recovery was evaluated by the percentage of the measured concentration to its spiked concentration. The other prepared and analyzed procedures were the same as above.

2.5.3. Precision and accuracy

For the estimation of the method precision and accuracy, each blank sample of *T. melanosporum* fermentation broth spiked at 10.0, 100.1 and 500.5 ng/mL androstenol was assayed for three times. The intra-day precision was defined as relative standard deviation (R.S.D.) calculated from three independent assay in the same day, and the inter-day precision was the R.S.D. calculated from three independent assays in the separate days. The accuracy was evaluated by the mean deviation between the measured concentration and its spiked concentration.

2.5.4. Sample stability

For the assay of sample (i.e., androstenol) stability, the blank samples of *T. melanosporum* fermentation broth were spiked at 25.0 and 250.3 ng/mL androstenol, respectively. After stored at room temperature for 5 days, at 4 °C for 2 weeks, or at −20 °C for 1 month, the stability of androstenol in the fermentation broth and acetone was defined as a percentage of the mean measured concentration to its spiked value.

2.5.5. Limits of detection and quantitation

For the assay of limit of detection (LOD) and limit of quantitation (LOQ), the blank samples of *T. melanosporum* fermentation broth were spiked at very low amount of androstenol (0.1–10.0 ng/mL). Limit of detection (LOD) was expressed as the concentration of androstenol that generated a response to three times of the signal-to-noise (S/N) ratio, and the limit of quantitation (LOQ) was 10 times of the S/N ratio.

3. Results and discussion

3.1. Optimization of solid-phase extraction

Because of its satisfied recovery and highly selectivity, solid-phase extraction (SPE) was widely reported in the literature for the extraction of androst-16-ene steroids from human testis [19], human urine [20] and boar incubation media [21,22]. Through preliminary test, C_{18} cartridge was identified to be suitable for efficiently removing the coextractives and preconcentrating the analytes. Therefore, C_{18} cartridge was selected as the sorbent for the following SPE experiments.

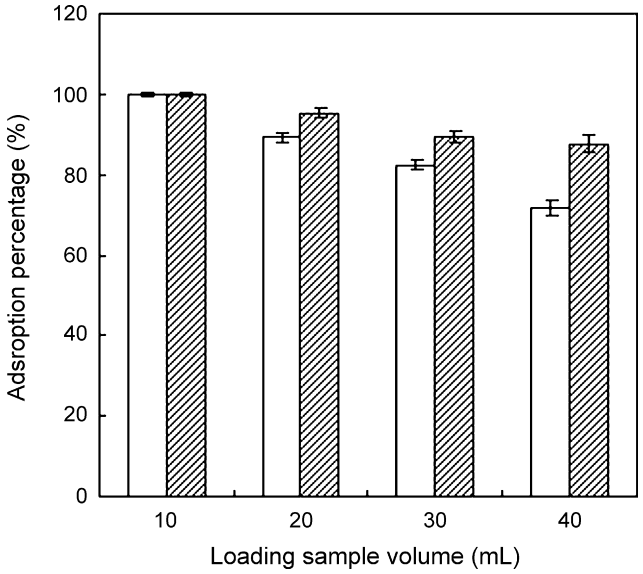

Fig. 1. Effect of loading sample volume on the adsorption percentages of androstenol and internal standard (I.S.) in the C_{18} cartridge. Symbols for the analyte: androstenol (blank bar), and I.S. (hatched bar). The blank samples of *Tuber melanosporum* fermentation broth loaded into the C_{18} cartridge were spiked at 500.5 ng/mL of androstenol and 500.0 ng/mL of I.S., respectively.

Adsorption capability of C_{18} cartridge for analytes is an important factor for SPE. Effect of loading sample volume on the adsorption percentages of androstenol and internal standard (I.S.) in C_{18} cartridge was investigated in order to understand the adsorption capability of C_{18} cartridge. As shown in Fig. 1, androstenol and I.S. had similar retention behavior in the C_{18} cartridge, and their adsorption percentages were decreased with the increase of loading sample volume. When loading sample volume exceeded 10 mL, I.S. had a little bit stronger adsorption capability than androstenol. This indicated the adsorption capability of C_{18} cartridge was varied with the chemical constitution of adsorbate, even the difference was slight. In order to make sure the accuracy of assay, loading sample volume was therefore selected as 10 mL for SPE to ensure all the androstenol and I.S. were completely adsorbed by the sorbent in the C_{18} cartridge.

The desorption capability for the analytes in C_{18} cartridge is another important factor, which is significantly influenced by eluent strength and the rinsing volume of eluent. For SPE, acetone was selected as organic phase in eluent, and its concentration was positively correlated with the eluent strength. Fig. 2 shows the effects of acetone concentration in the eluent and the rinsing volume of eluent on the desorption percentages of androstenol (A) and I.S. (B) in the C_{18} cartridge. Neither androstenol nor I.S. was eluted when acetone concentration was lower than 60%. When acetone concentration increased up to 90%, both androstenol and I.S. were completely eluted with the minimal rinsing volume of 1 mL. This result indicated that eluent strength was the main factor influencing desorption capability. When acetone concentration ranged from 60% to 70%, the eluted androstenol and I.S. increased with the rinsing volume, and the eluted androstenol was a little bit more than the eluted I.S. with the same rinsing volume. This demonstrated that rinsing volume was the second important parameter influencing

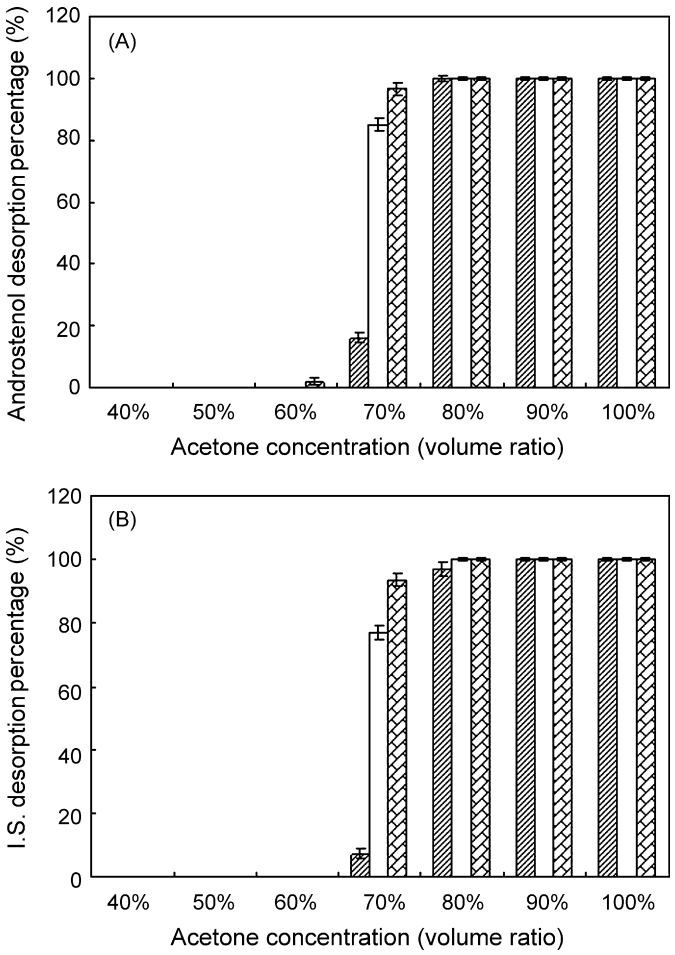

Fig. 2. Effects of acetone concentration in the eluent and the rinsing volume of eluent on the desorption percentages of androstenol (A) and I.S. (B) in the C_{18} cartridge. Symbols for the rinsing volume of eluent: 1 mL (hatched bar), 2 mL (blank bar), and 3 mL (brick bar).

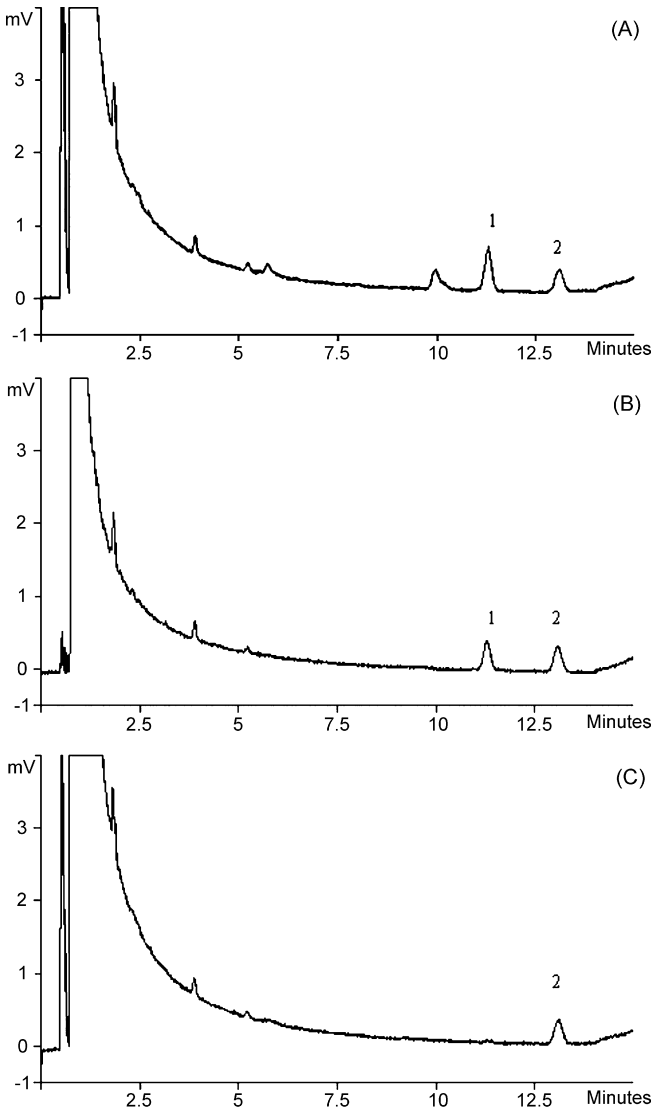

Fig. 3. Typical GC-FID chromatograms of *T. melanosporum* fermentation broth spiked at 25.0 ng/mL I.S. (A); of the standard control (25.0 ng/mL of androstenol and I.S.) (B); and of *T. melanosporum* fermentation broth blank sample spiked at 25.0 ng/mL I.S. (C). Compound: 1, androstenol; 2, I.S. All of the samples were treated by the proposed method of SPE.

desorption process. In order to remove the impurities with polarity and middle-polarity as much as possible but not affect the analytes, 2 mL of 60% acetone was chosen as washing solution. And then 1 mL of acetone was chosen as eluting solvent, as it is much easier to dry than acetone aqueous solution.

Since androstenol concentration was very low in truffle fermentation broth, the sample should be concentrated as much as possible to reach the quantitation limit. So the elution solvent was evaporated to dry under nitrogen stream and finally dissolved in 50 μL acetone.

3.2. Method validation of SPE–GC–FID

3.2.1. Specificity, linearity, recovery, precision, accuracy and limits of detection/and quantitation

Fig. 3 shows the typical GC-FID chromatograms of *T. melanosporum* fermentation broth, which indicated the existence of androstenol in *T. melanosporum* fermentation broth. A baseline separation of androstenol and I.S. was obtained under the specified chromatographic conditions, and no interfering peaks were detected.

Further specificity experiment was carried out with the analysis of GC-EIMS. Fig. 4 shows the EIMS chromatogram of androstenol and I.S. in *T. melanosporum* fermentation broth spiked with 50 μL of 20.0 μg/mL I.S. The characteristic ion fragments were the *m*/*z* of 274 and 241 for androstenol (Fig. 4A), and the *m*/*z* of 276 and 243 for I.S. (Fig. 4B), which were the same with the standard control. According

Table 1
The method recovery of SPE–GC–FID calculated from its assay results of the blank samples of *T. melanosporum* fermentation broth spiked at 10.0, 100.1 and 500.5 ng/mL androstenol ($n = 3$)

Compound	Spiked concentration (ng/mL)	Recovery (%)	R.S.D. (%)	Average (%)
Androstenol	10.0	92.9 ± 2.6	2.8	95.1
	100.1	100.8 ± 3.8	3.8	
	500.5	91.7 ± 2.8	3.1	

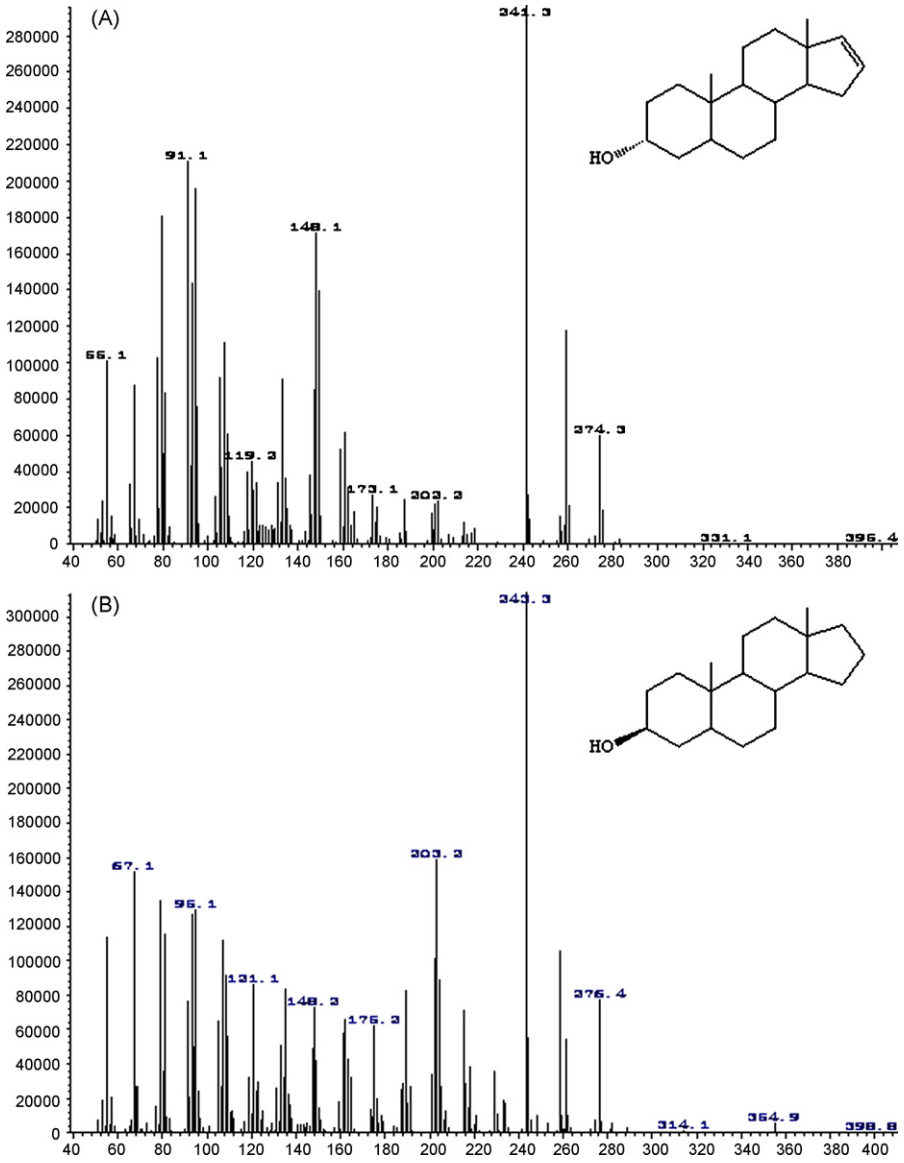

Fig. 4. EIMS chromatograms of androstenol (A) and I.S. (B) in *T. melanosporum* fermentation broth spiked at 50 μL of 20.0 μg/mL I.S.

to the evidence of ion spectrum, the existence of androstenol was further confirmed in *T. melanosporum* fermentation broth.

Under the optimal conditions for GC-FID, the calibration curves showed a dynamic linear range from 10.0 to 500.5 ng/mL. The relationship between the concentration ratio of androstenol to I.S. (y) and their corresponding ratio of peak area (x) was shown with regression equation as follows: $y = 0.849x - 0.059$, $R^2 = 0.999$.

As shown in Table 1, the recovery of androstenol ranged from 91.7% to 100.8%, which indicates the method of SPE–GC–FID were suitable for the determination of androstenol.

As indicated in Table 2, the R.S.D. was not higher than 6.7%, and the highest analytical error was 8.2%. The satisfied precision and accuracy indicated the good performance of SPE–GC–FID for the quantitative analysis of androstenol in truffle fermentation broth.

The limit of detection (LOD) and limit of quantitation (LOQ) for the method of SPE–GC–FID were 3.2 and 10.1 ng/mL, respectively, and their corresponding values were 0.1 and 0.5 ng/mL for the method of SPE–GC–EIMS with SIM mode.

Table 2
Intra-day and inter-day precision and accuracy for the method of SPE–GC–FID calculating from its assay results of the blank samples of *T. melanosporum* fermentation broth spiked at 10.0, 50.1 and 500.1 ng/mL androstenol ($n = 3$)

	Spiked concentration (ng/mL)	Intra-day assay			Inter-day assay		
		Measured concentration (ng/mL)	Error (%)	R.S.D. (%)	Measured concentration (ng/mL)	Error (%)	R.S.D. (%)
Androstenol	10.0	9.2 ± 0.6	7.7	6.7	9.2 ± 0.4	8.2	4.6
	50.1	49.1 ± 0.7	1.8	1.5	48.4 ± 0.9	3.3	1.8
	500.5	481.8 ± 8.3	3.7	1.7	487.2 ± 9.0	2.6	1.8

Table 3
Stability of sample (i.e., androstenol) in *T. melanosporum* fermentation broth and acetone under various store conditions ($n = 3$)

Concentration (ng/mL)	Store condition	Percentage of measured concentration to spiked value (%)	
		In fermentation broth	In acetone
25.0	Stored at room temperature for 5 days	48.0 ± 3.4	89.0 ± 2.5
	Stored at 4 °C for 2 weeks	90.0 ± 1.6	97.0 ± 1.0
	Stored at −20 °C for 1 month	93.1 ± 1.6	97.4 ± 1.8
250.3	Stored at room temperature for 5 days	43.8 ± 3.5	89.6 ± 2.6
	Stored at 4 °C for 2 weeks	89.5 ± 2.6	96.5 ± 1.7
	Stored at −20 °C for 1 month	92.7 ± 2.7	97.1 ± 1.4

3.2.2. Sample stability

The stability of sample (i.e., androstenol) under various store conditions is compared in Table 3. Regardless of various store conditions, androstenol dissolved in acetone was relatively stable because at least 89.0% of androstenol was detected after various treatments. Stored at 4 °C for 2 weeks or −20 °C for 1 month, androstenol in the fermentation broth remained stable regardless its concentrations. However, stored at room temperature for 5 days, androstenol in the fermentation broth was unstable, maybe owe to the volatility of androstenol in aqueous solution at room temperature. So the fermentation broth sample should be treated as soon as possible.

3.3. Comparison between SPE–GC–FID and SPE–GC–EIMS

The performance of SPE–GC–FID was examined by comparing its detected androstenol concentration in the four kinds of truffle fermentation broths (*T. melanosporum, T. sinense, T. indicum* and *T. aestivum*) with the analysis result of SPE-GS-EIMS. Fig. 5 shows the typical SIM chromatograms of standard control (A) and

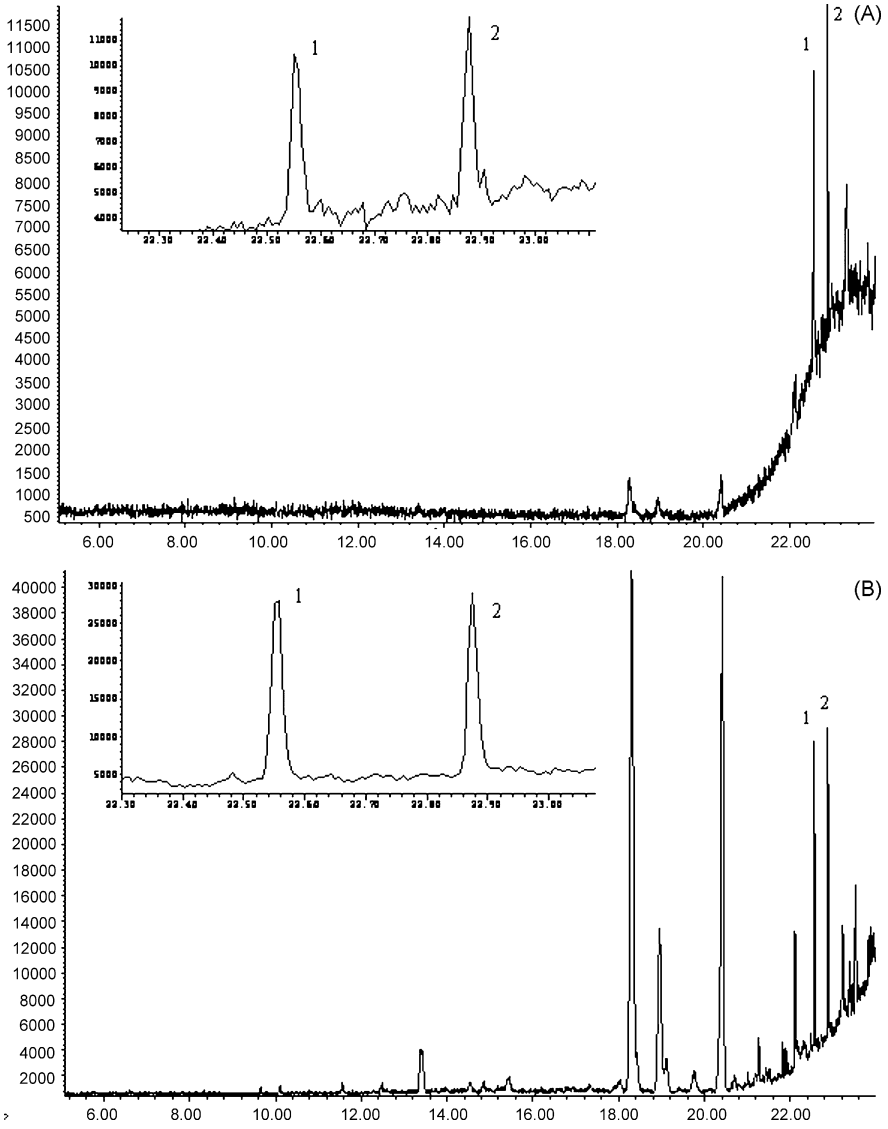

Fig. 5. SIM chromatograms of the standard control (5.0 ng/mL of androstenol and I.S.) (A); and of *T. melanosporum* fermentation broth sample (B). Compound: 1, androstenol; 2, I.S. All of the samples were treated by the proposed method of SPE.

Table 4
Quantitative analysis of androstenol in various truffle fermentation broths by SPE–GC–FID compared with SPE–GC–EIMS ($n = 3$)

	Androstenol concentration (ng/mL)	
	Analyzed by SPE–GC–FID	Analyzed by SPE–GC–EIMS
T. melanosporum	47.8 ± 3.5	48.1 ± 3.4
T. sinense	24.4 ± 4.1	24.6 ± 3.9
Tuber indicum	123.5 ± 1.1	124.7 ± 1.2
T. aestivum	n.d.	n.d.

n.d.: not detected.

T. melanosporum fermentation broth (B). As compared in Table 4, no significant difference was observed for androstenol concentrations analyzed by SPE–GC–FID and SPE–GC–EIMS. This shows the developed method of SPE–GC–FID allows the sensitive and selective detection for androstenol even at trace level in truffle fermentation broth. Androstenol concentrations in four kinds of truffle fermentation broths were obviously different, and its highest concentration of 123.5 ng/mL was detected in *T. indicum* fermentation broth, while no androstenol was detected in *T. aestivum* fermentation broth. The results confirmed the presence of androstenol in truffle submerged fermentation system for the first time, which suggested that truffle fermentation is a promising alternative for androstenol production on a large scale.

4. Conclusion

A novel method using solid-phase extraction (SPE) coupled with gas chromatography (GC) and flame ionization detector (FID)/electron impact mass spectrometry (EIMS) for the quantitative determination of 5α-androst-16-en-3α-ol (androstenol) in truffle fermentation broth was developed in this work. Through the optimized SPE procedure, androstenol and internal standard (I.S.) were selectively enriched to reach FID quantitation limit. The specificity of SPE–GC–FID was verified by SPE–GC–EIMS analysis. The method of SPE–GC–FID provided good accuracy and reproducibility (recovery >91.7%; R.S.D. <6.7%; and analytical error <8.2%) for spiked truffle fermentation broth. The calibration curve was linear in the range of 10.0–500.5 ng/mL ($R^2 = 0.999$), and the limits of detection and quantitation were 3.2 and 10.1 ng/mL, respectively. Comparison studies between FID and EIMS indicated two detectors gave similar quantitative results, although FID detection limit was not as low as mass spectrometry with selected ion monitor mode. The novel method of SPE–GC–FID was demonstrated to be a simple, sensitive, accurate and reproducible method. Furthermore, FID is much simple, economical and convenient, which is more suitable for mass measure to monitor the kinetics of androstenol production by truffle fermentation.

By using the novel method, androstenol concentration in truffle fermentation broth was assayed and reported for the first time, which confirmed the presence of androstenol in truffle submerged fermentation system. The results obtained in this study suggested that truffle fermentation is a promising alternative for androstenol production on a large scale.

Acknowledgements

The financial supports from the National Natural Science Foundation of China (NSFC, Project No. 20706012), National High Technology Research and Development Key Program of China (Project No. 2007AA021506), the Scientific Research Foundation for the Returned Overseas Chinese Scholars (Ministry of Personnel, State Education Ministry and Hubei Provincial Department of Personnel), Hubei Provincial Innovative Research Team in University (Project No. T200608), Hubei Provincial Science Foundation for Distinguished Young Scholars (Project No. 2006ABB034), Hubei Provincial International Cooperation Foundation for Scientific Research (Project No.2007CA012), the Science and Technology Commission of Wuhan Municipality "Chenguang Jihua" (Project No. 20065004116-31), the Scientific Research Key Foundation from Hubei University of Technology (Project No. 306.18002), and the Open Project Program of the State Key Laboratory of Bioreactor Engineering (ECUST) are gratefully acknowledged. Ya-Jie Tang also thanks the Chutian Scholar Program from Hubei Provincial Department of Education, China (2006).

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