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# Determination of fatty acids from mushrooms using high performance liquid chromatography with fluorescence detection and online mass spectrometry

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ABSTRACT

Owing to the growing consumer interest in low-fat foods, it is necessary to provide our customers with the information about the fatty acid composition of mushrooms. In this study, a selective and sensitive method based on pre-column derivatization using 2-(12-oxobenzo[*b*]-acridin-5(12*H*)-yl)-ethyl-4-toluenesulfonate (BAETS) as the labeling reagent has been optimized by high-performance liquid chromatography with fluorescence detection and online mass spectrometry identification (HPLC–FLD–MS/MS). Fatty acids (FA) were derivatized by BAETS and separated on a reversed-phase Hypersil BDS C8 column with a gradient elution. Eighteen FA investigated were found to give excellent linear responses with correlation coefficients of >0.9996. Limits of detection and quantification (LOD and LOQ) were in the range of 0.46 to 1.02 ng $mL^{-1}$ and 1.43 to 3.48 ng $mL^{-1}$, respectively. This method was applied to the quantitative analysis of FA from a wild and four cultivated mushroom species. The wild mushroom named *Armillaria luteo-virens* contained higher unsaturated fatty acids (UFA) when compared to the cultivated species (*Flammulina velutiper*, *Pleurotus eryngii*, *Copyinds comatus* and *Agrocybe aegerita*). Ratio of UFA:SFA (SFA, saturated fatty acids) for *A. luteo-virens* was >5 whereas the values for the cultivated species were <4.09. Therefore, BAETS derivatization allowed the development of a highly sensitive and specific method for the determination of FA in mushroom samples.


## 1. Introduction

Lipids are essential nutrients that play an important role in the human body. In many cases, biologically active lipids act like hormones or their precursors to regulate body functions. They can help the digestion process; serve as the source of energy for muscles; work as structural and functional components of biomembranes, even as constituents of myelin sheath and as thermal insulators (Ribeiro, Guedes de Pinho, Andrade, Baptista, & Valentão, 2009). Therefore, lipids are indispensable components of human body composition and necessary for our daily food intake. However, when a high dietary lipid is consumed, there might be an increasing risk of some chronic diseases such as diabetes, hypertension, atherosclerosis and cardiovascular dysfunctions (Berenson & Srinivasan, 2001; Eyre, Kahn, & Robertson, 2004; Gidding, Leibel, Daniels, & Rosenbaum, 1996; Yusuf, Reddy, Ounpuu, & Anand, 2001). Today, people around the world are becoming more aware of the relationship between diet and health. This concept has stimulated the interest in the consumption of low-fat foods. Edible mushrooms are increasingly consumed in many countries. Their low fat content, especially, the high proportion of UFA is considered a significant contributor to the rise in consumption (Kurtzman, 1997; Lakhanpal & Rana, 2005; Mattila, Salo-Väänänen, Könkö, Aro, & Jalava, 2002; Sanmee, Dell, Lumyong, Izumori, & Lumyong, 2003).

Edible mushrooms, even the most cultivated species, contain a high content of UFA (Kavishree, Hemavathy, Lokesh, Shashirekha, & Rajarathnam, 2008; Pedneault, Angers, Gosselin, & Tweddell, 2006; Ribeiro et al., 2009; Yilmaz, Solmaz, Turkekul, & Elmastas, 2006). UFA, especially the omega-3 and -6 series, are necessary in human health due to their effects on normal health and chronic diseases, such as the regulation of cholesterol levels, reduction of the risk of cardiovascular diseases, promotion of neuronal development and immune functions (Benatti, Peluso, Nicolai, & Calvani, 2004). In this respect, compositional analysis of various FA in edible mushroom is of great interest. *Armillaria luteo-virens*, distributed at an altitude of around 3000–4300 m, is a well-known valuable edible and medicinal fungus in Qing-Tibet Plateau (Diao, 1997). Over the years, *A. luteo-virens* has been traditionally consumed by local residents because of its excellent flavor and texture (Li, 2005). Recently, more attentions have been paid to this wild mushroom including isolation of lectin (Feng et al., 2006) and polysaccharides (Liu, Yu, Mao, Meng, & Liu, 2007), cultivation of mycelium (Jiao, Chen, Zhou, Zhang, & Chen, 2008; Xu, Fu, Chen, & Liu, 2010) and investigation of antioxidative and antitumor activities (Li,

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Chen, & Bi, 2004). Furthermore, eighteen amino acids, three vitamins (Diao, 1997) and ten minerals (Li & Diao, 2008) have been found in *A. luteo-virens*. Herein, FA profile of *A. luteo-virens* was conducted in order to improve the knowledge of its nutritional value.

Determination of FA from mushrooms is often done by GC coupled with derivatization. The most commonly used derivatization procedure is transesterification of both conjugated and free FA with methanol to their methyl esters based on catalytic reaction after preliminary hydrolysis and lipid extraction (Barros et al., 2007; Heleno, Barros, Sousa, Martins, & Ferreira, 2009; Kavishree et al., 2008; Pedneault et al., 2006; Ribeiro et al., 2009; Yilmaz et al., 2006). Although this method is well developed and fairly effective, there are some problems associated with volatility of polar compounds, stability of derivatives and their thermal decomposition during analysis (Aluyor, Ozigagu, Oboh, & Aluyor, 2009; Perret, Gentili, Marchese, Sergi, & Caporossi, 2004; Toyo'oka, 2002; Wood & Lee, 1983). Compared with GC, HPLC can provide several advantages: (1) HPLC is the method that allows the nonvolatile thermally labile compounds to be separated with mild operating conditions; (2) detectors used in HPLC are nondestructive for the analytes, which makes it possible to collect the fraction for further investigation; (3) HPLC method allows FA to be converted to a large number of different derivatives. In addition, with the availability of various solvents, column packing materials and strong ultraviolet-absorbing or fluorescent tagging probes, a highly selective and sensitive HPLC analysis is possible (Perret et al., 2004; Puttmann, Krug, Von Ochsenstein, & Kattermann, 1993; Shukla, 1988). Commonly, chemical derivatization with fluorescence detection has been widely adopted in order to reduce detection limits. In this work, a selective and sensitive method for the determination of FA using 2-(12-oxobenzo[*b*]acridin-5(12*H*)-yl)-ethyl-4-toluenesulfonate (BAETS) as the labeling reagent with fluorescence detection coupled with post-column MS identification was developed. At the same time, a comparative study on the FA composition from the extracts of *A. luteo-virens* and four cultivated mushroom species, including *Flammulina velutiper*, *Pleurotus eryngii*, *Copyinds comatus* and *Agrocybe aegerita*, was evaluated.

## 2. Materials and methods

### 2.1. Equipments

HPLC analysis was carried out using an Agilent HP 1100 series high performance liquid chromatography. This LC apparatus was composed of an online vacuum degasser (model G1322A), a quaternary pump (model G1311A), an autosampler (model G1329A), a thermostated column compartment (model G1316A) and a fluorescence detector (FLD) (model G1321A). For identification of FA, Aglient 1100 Series LC–MSD Trap-SL (ion trap) mass spectrometer from Bruker Daltonics (Bremen, Germany) was used. This mass spectrometer was equipped with electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) ion sources.

### 2.2. Chemicals and reagents

Standards of eighteen FA were purchased from Sigma Reagent Co. (St. Louis, MO, USA). HPLC-grade acetonitrile was obtained from Yuwang Chemical Reagent Co. (Jining, Shandong, China). Water was purified on a Milli-Q system (Millipore, Bedford, MA, USA). All other reagents used were of analytical grade unless otherwise stated. The fluorescent regent BAETS was synthesized as described previously (You, Fu, Sun, & Suo, 2010).

### 2.3. Preparation of standards and samples

FA standards at individual concentrations were prepared by the dilution of the corresponding stock solutions ($1.0\times10^{-2}$ mol $L^{-1}$) with N,N′-dimethylformamide (DMF). BAETS solution ($1.0\times10^{-2}$ mol $L^{-1}$) was prepared by dissolving 44.3 mg in 10 mL DMF. The fruit body of *A. luteo-virens* was collected from Qilian Mountain in Qinghai Province of China. Fruit bodies of *F. velutiper*, *P. eryngii*, *C. comatus* and *A. aegerita* were bought from a local supermarket. After collection, the mushrooms were immediately transferred to the laboratory and washed with pure water to remove dust particles. The materials were then cooled, dried, ground and sieved to obtain granular products of 20–40 mesh. Crude fat from each mushroom species was extracted by a Soxhlet apparatus for 8 h with petroleum ether as the extraction solvent. Subsequently, the extract was concentrated to near dryness using a rotary evaporator at 60 °C. The residue was further evaporated to dryness with a stream of nitrogen gas. Crude fat used to evaluate total fatty acids (TFA) was saponified according to the method of Ma (Ma, Wierzbicki, Field, & Clandinin, 1999), while fat used to evaluate free fatty acids (FFA) was directly dissolved in DMF.

### 2.4. Optimum derivatization

Taguchi approach (Mandal, Mohan, & Hemalatha, 2008) was used for optimization of the derivatization reaction between BAETS and FA. With DMF as the derivatization co-solvent (Wang et al., 2007; Zhao et al., 2006), the optimization was evaluated by varying four parameters with three levels of each (Table 1). To complete the procedure, nine tests were performed and each test was repeated three times (Table 2). Software Mintab 16 was dedicated to treat the experimental data.

### 2.5. Derivatization procedures

To 20 mg anhydrous $K_2CO_3$ in a 2-mL vial, 35 μL of mixed standard FA solution, 145 μL DMF, 120 μL BAETS solution were consecutively added. The vial was sealed and allowed to react in a water-bath for 40 min at 95 °C. After the reaction was completed, the mixture was taken to cool at room temperature. Then the derivatization solution was diluted with DMF and injected directly for analysis. Derivatization for the saponified or unsaponified samples was similar as described above.

### 2.6. HPLC separation

HPLC separation of eighteen FA was performed on a reversed-phase Hypersil BDS C8 column (200 × 4.6 mm i.d., 5 μm) by a gradient elution. Eluent A was 30% acetonitrile containing 10 mM ammonium formate buffer (ACN/$H_2O$ = 30:70, *v*/*v*); B was 100% acetonitrile. The elution conditions were as follows: 50–90% B from 0 to 40 min; 90–100% B from 40 to 45 min; 100% B from 45 to 50 min. The flow rate was constant at 1.0 mL $min^{-1}$ and the column temperature was kept at 30 °C. The fluorescence excitation and emission wavelengths were set at 272 nm and 505 nm, respectively.

### 2.7. MS conditions

Two kinds of ion sources (ESI and APCI) were evaluated for identification of FA derivatives. APCI conditions: nebulizer pressure, 413 kPa; dry gas temperature, 350 °C; dry gas flow, 5.0 L $min^{-1}$; APCI Vap

**Table 1**
Derivatization parameters and levels for the Taguchi orthogonal design.

| Levels | Derivatization temperature (°C) | Derivatization time (min) | Reagent concentration (fold molar) | Amount of $K_2CO_3$ (mg) |
|---|---|---|---|---|
| 1 | 75 | 20 | 2 | 20 |
| 2 | 85 | 30 | 5 | 40 |
| 3 | 95 | 40 | 8 | 60 |

**Table 2**
Taguchi orthogonal design and results for optimizing derivatization.

| Tests | A[a] | B | C | D | Derivatization yield (peak area)[b] | S/N[c] for derivatization yield |
|---|---|---|---|---|---|---|
| 1 | 1 | 1 | 1 | 1 | 697.43 | 56.87 |
| 2 | 1 | 2 | 2 | 2 | 1235.73 | 61.69 |
| 3 | 1 | 3 | 3 | 3 | 2032.74 | 66.13 |
| 4 | 2 | 1 | 2 | 3 | 1344.84 | 62.56 |
| 5 | 2 | 2 | 3 | 1 | 3884.54 | 71.78 |
| 6 | 2 | 3 | 1 | 2 | 1506.16 | 63.49 |
| 7 | 3 | 1 | 3 | 2 | 2891.20 | 69.20 |
| 8 | 3 | 2 | 1 | 3 | 1147.75 | 61.17 |
| 9 | 3 | 3 | 2 | 1 | 3413.75 | 70.66 |

[a] A, derivatization temperature (°C); B, derivatization time (min); C, reagent concentration (fold molar); D, amount of $K_2CO_3$ (mg).
[b] Average of three triplicates.
[c] Signal-to-noise ratio.

temperature, 450 °C; corona current (nA), 4000 (pos); capillary voltage, 3500 V. ESI conditions: spray pressure, 413 kPa; dry gas temperature, 350 °C; dry gas flow, 5.0 L $min^{-1}$; corona current (nA), 4000 (pos); capillary voltage, 3500 V. The positive-ion detection mode was selected for both APCI and ESI ion sources.

### 2.8. Validation parameters

The method was evaluated by linearity, LOD and LOQ, precision and accuracy. Standard solutions containing eighteen FA at seven different concentrations between 7.29 and 0.11 μmol $L^{-1}$ were determined (n = 3) to construct calibration curves. LOD and LOQ were determined based on a signal-to-noise ratio of 3 (S/N = 3) and 10 (S/N = 10), respectively. Both the instrument and method precision were estimated. The instrument precision was investigated by injection of the mixed FA standards with five replicates; at the same time, precision of the chromatographic method was checked by applying the whole procedure, including saponification, derivatization, and injection with *A. luteo-virens* sample (n = 3). Accuracy of the method was checked by spiking known amount of FA standards in *A. luteo-virens* sample. Overall process included saponification, derivatization and determination.

### 2.9. Statistical analysis

Three samples were used for each of the mushroom species and all the assays were performed three times. Results obtained were expressed as mean values and standard deviation (SD). Software SPSS 16.0 was used.

## 3. Results and discussion

### 3.1. Optimum derivatization

Optimization of derivatization reaction (Fig. 1) was a key step to ensure the sufficient label of analytes. Taguchi approach was used as the statistical design method for optimizing the selected derivatization parameters in terms of maximizing the derivatization yields (Section 2.4). Results (Table 2, Fig.2) offered by this method showed that the optimized parameters were 8-fold molar excess of BAETS reagent over total molar FA, 20 mg catalyst ($K_2CO_3$), reaction time 40 min and derivatization temperature 95 °C. With these parameters presented above, derivatization yield (expressed as peak area, n = 3) was 4315.66 whereas the predicted value was 4351.80. When a statistical analysis of variance (ANOVA) was performed, $p$ values were all <0.01, indicating the four parameters with the selected levels significantly affected the derivatization yields. Obviously, Taguchi method was a powerful modeling tool that allowed optimization with a small number of experiments (Mandal et al., 2008; Nagarjun, Rao, Rajesham, & Rao, 2005; Zhao et al., 2010). With other three optimized parameters proposed above, a single factor test for BAETS concentration was evaluated. Results showed that further increasing the excess of reagent (>8-fold molar) did not significantly alter the derivatization yields. Therefore, quantitative conversion of FA to their BAETS derivatives was usually guaranteed by using, at least, an 8-fold molar excess of BAETS to total FA.

### 3.2. MS characterization of the BAETS derivatives

Ionization of FA was easily achieved in negative mode due to the presence of carboxyl group. Unfortunately, best chromatographic resolution with reversed-phase columns was usually achieved at acidic

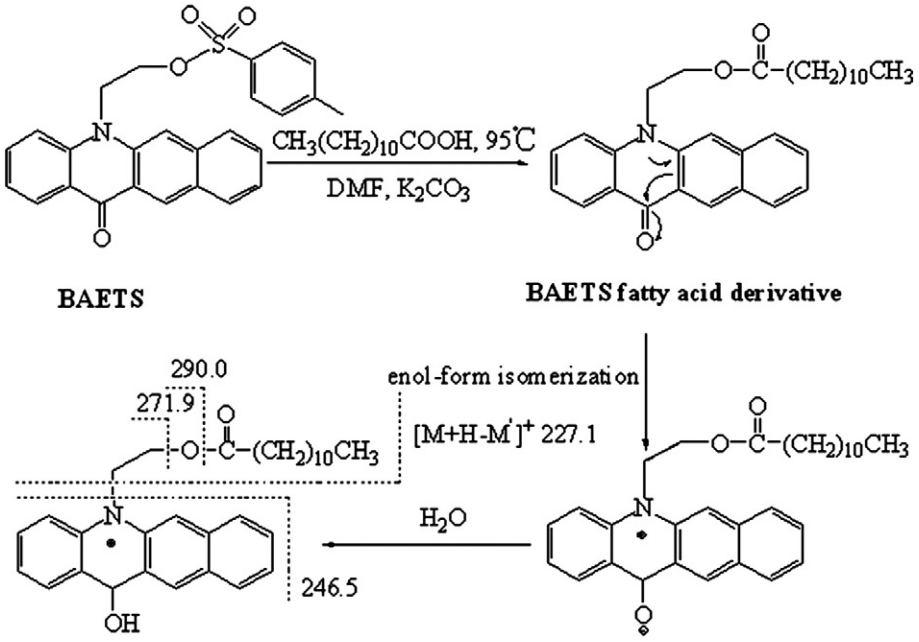


**Fig. 1.** Derivatization scheme of 2-(12-oxobenzo[*b*]-acridin-5(12*H*)-yl)-ethyl-4-toluenesulfonate (BAETS) with representative lauric acid (C12:0) and MS cleavage mode of the core structure of BAETS (M′, corresponding to the protonated vinyl ester of laurate moiety).

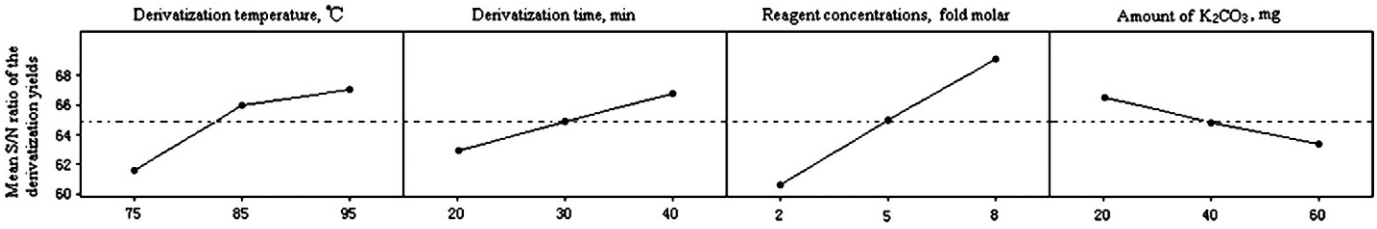


**Fig. 2.** Effects plot for signal-to-noise ratio (S/N) of the Taguchi method.

pH where the ionization of carboxyl group in MS ion chamber would be suppressed and resulted in low mass sensitivity (Yang, Adamec, & Regnier, 2007). This problem could be easily resolved by derivatization (You et al., 2005, 2007), which converted FA to a positively charged target molecule by the reaction of carboxylic group with the labeling reagent. In this work, BAETS was used and it could easily convert FA to a nitrogen-contained target molecule.

Owing to the existence of the nitrogen atom in the molecular core, BAETS could easily transform to its isomer (You et al., 2010). The isomerized molecule with a phenoxide ion could easily accept a $[H]^+$ proton to form a quaternary ammonium ion (Fig. 1), and resulted in the significant gains in sensitivity for ESI and APCI in positive-ion detection mode. Compared data for MS analysis of BAETS-FA derivatives using ESI and APCI in positive-ion detection mode were summarized in Table 3. Results showed that two ion sources gave similar fragmentation, but the obtained ionization ability for molecular ion peaks with APCI–MS was higher relative to that of ESI–MS (see Fig. 3). Therefore, APCI–MS was selected for all subsequent experiments.

As expected, all FA derivatives including SFA and UFA exhibited intense molecular ion peaks at $m/z$ $[M+H]^+$. The positive-ion mass spectra, corresponding to SFA, were relatively simple (Fig. 4); the collision-induced dissociation spectra for representative lauric acid (C12:0) derivative produced the specific fragment ions at $m/z$ 246.5, $m/z$ 271.9, $m/z$ 290.0 and $m/z$ $[M+H\text{-}M']^+$ (M′: corresponding to the protonated vinyl ester of laurate moiety, $m/z$ 227.1). The characteristic ions at $m/z$ 246.5, $m/z$ 271.9 and $m/z$ 290.0 were common to all FA derivatives. These ions were mainly produced by the cleavage of the molecular core of BAETS. The specific fragment ion at $m/z$ 246.5 was from the cleavage of N – C bond of the N-linked side chain, corresponding to the protonated molecular core of benzoacridine. The specific fragment ions at $m/z$ 271.9 and $m/z$ 290.0 were, respectively, from the cleavage of C – O bond and O – CO ester bond of the N-linked side chain, corresponding to the protonated vinylbenzoacridine and ethoxidebenzoacridine moieties, respectively (Fig. 1).

In most cases, the collision-induced dissociation spectra for UFA produced an intense fragment ion by losing one $H_2O$ molecule at $m/z$ $[M+H-H_2O]^+$. With all SFA, ions at $m/z$ $[M+H-H_2O]^+$ were not observed. Fragment ions were particularly rich for polyunsaturated fatty acid (PUFA) derivatives. This should be attributed to the cleavage of poly-double-bonds in PUFA molecules. The collision-induced dissociation spectra for the representative linoleic acid (C18:2n6c) produced the specific fragment ions at $m/z$ 246.0, $m/z$ 272.2, $m/z$ 289.8, $m/z$ 306.7

**Table 3**
MS and MS/MS analysis for the BAETS-fatty acid derivatives using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) ion sources.

| Fatty acid derivatives | | Molecular ion $[M+H]^+$ | | $[M+H-H_2O]^+$ | | MS/MS | |
|---|---|---|---|---|---|---|---|
| | | ESI | APCI | ESI | APCI | ESI | APCI |
| Saturated fatty acids | C10:0 | 444.1 | 444.0 | nd[a] | nd | 199.2, 246.3, 272.2, 290.3 | 198.9, 246.1, 272.2, 290.0 |
| | C11:0 | 458.1 | 457.9 | nd | nd | 213.1, 246.1, 272.3, 290.0 | 213.1, 246.3, 272.0, 290.1 |
| | C12:0 | 472.1 | 471.9 | nd | nd | 227.3, 246.4, 272.4, 290.1 | 227.1, 246.5, 271.9, 290.0 |
| | C13:0 | 486.0 | 485.9 | nd | nd | 241.2, 246.1, 272.3, 290.2 | 241.0, 245.8, 271.8, 290.0 |
| | C14:0 | 500.2 | 499.8 | nd | nd | 246.1, 255.3, 272.1, 290.3 | 245.2, 255.2, 272.0, 290.0 |
| | C15:0 | 514.1 | 513.8 | nd | nd | 245.5, 269.2, 272.3, 290.3 | 245.8, 268.9, 272.1, 289.8 |
| | C16:0 | 528.2 | 527.8 | nd | nd | 246.3, 272.0, 283.3, 290.1 | 246.0, 271.9, 282.9, 289.8 |
| | C17:0 | 542.1 | 541.9 | nd | nd | 245.9, 272.3, 290.3, 297.2 | 245.7, 272.0, 289.9, 297.0 |
| | C18:0 | 556.2 | 555.8 | nd | nd | 246.2, 272.1, 290.3, 311.2 | 245.7, 271.9, 289.8, 310.7 |
| | C19:0 | 570.1 | 569.9 | nd | nd | 246.2, 272.4, 290.2, 325.3 | 244.7, 271.9, 289.7, 324.8 |
| | C20:0 | 584.2 | 583.9 | nd | nd | 246.4, 272.3, 290.2, 339.3 | 245.2, 271.8, 289.9, 338.8 |
| | C21:0 | 598.2 | 597.9 | nd | nd | 245.6, 272.4, 290.4, 353.2 | 246.0, 272.8, 289.6, 352.9 |
| | C22:0 | 612.1 | 611.8 | nd | nd | 246.2, 272.0, 290.3, 367.2 | 245.8, 271.9, 289.9, 366.9 |
| Unsaturated fatty acids | C18:1n9c | 554.1 | 553.8 | 536.3 | nd | 245.4, 272.0, 290.2, 309.2, 400.8, 428.0, 455.9, 456.7, 469.7 | 245.7, 271.8, 289.9, 308.8, 400.5, 413.6, 428.8, 456.6, 469.5 |
| | C18:2n6c | 552.2 | 551.8 | 533.7 | 534.0 | 245.4, 272.1, 290.2, 307.0, 385.7, 400.4, 414.9, 427.1, 440.1, 455.4, 470.4, 495.9 | 246.0, 272.2, 289.8, 306.7, 386.4, 399.5, 413.4, 425.6, 441.2, 454.2, 467.8, 495.8 |
| | C18:3n3c | 550.1 | 549.9 | 532.3 | 533.2 | 246.5, 272.0, 290.3, 305.4, 400.0, 413.9, 427.6, 441.9, 456.0, 466.2, 479.9, 494.2, 507.6 | 245.8, 271.7, 289.8, 305.2, 400.1, 413.7, 427.7, 441.8, 455.3, 466.0, 480.2, 493.7, 507.6 |
| | C20:4n6c | 576.1 | 575.8 | 558.0 | 557.9 | 245.0, 272.4, 290.1, 331.2, 372.5, 386.0, 400.2, 413.5, 426.0, 440.1, 453.1, 466.0, 478.0, 491.8, 506.1, 519.8, | 245.2, 272.2, 289.9, 330.9, 386.1, 399.9, 411.2, 424.0, 437.7, 451.8, 465.6, 479.2, 493.8, 506.0, 519.9 |
| | C22:6n3c | 600.1 | 599.7 | 581.9 | 582.0 | 246.3, 272.2, 290.1, 355.2, 383.7, 398.0, 412.5, 424.0, 438.2, 451.7, 463.8, 478.0, 491.3, 506.1, 520.0, 529.9, 557.6, 572.1 | 245.3, 272.0, 290.1, 354.9, 385.7, 397.9, 411.8, 425.2, 437.9, 451.6, 465.6, 479.9, 491.5, 505.6, 517.6, 529.8, 543.9, 558.3, 571.6 |

[a] No detection or no observed.

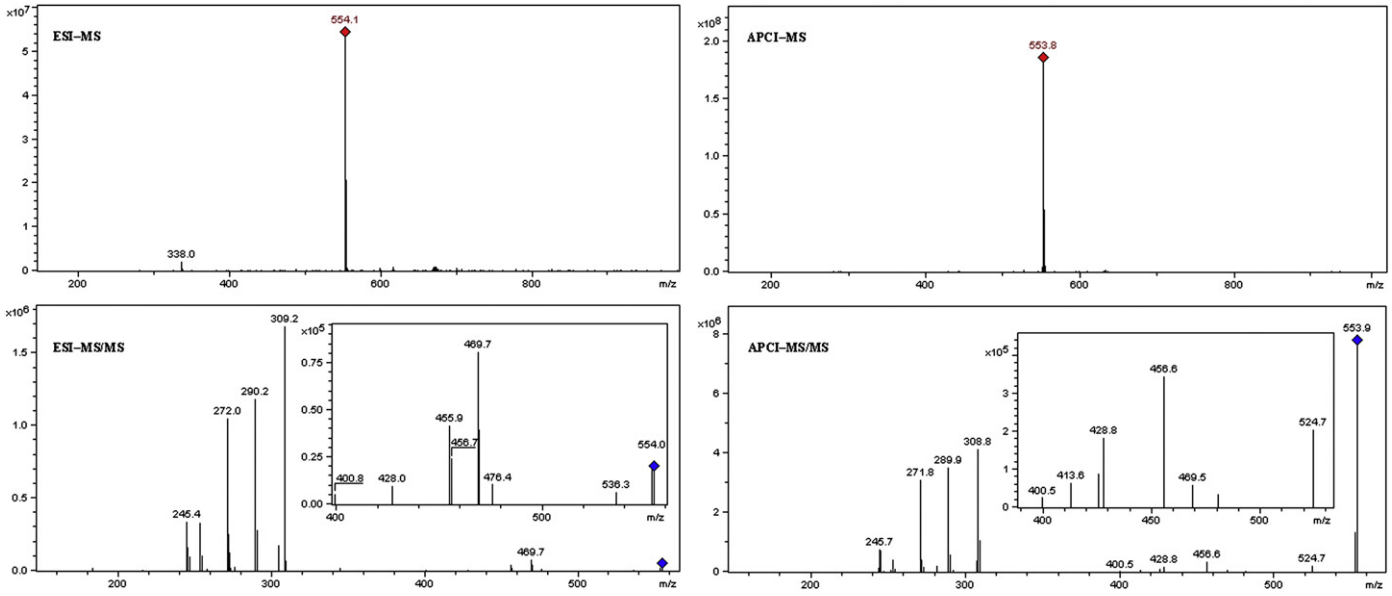


**Fig. 3.** The comparison of ionization abilities for the representative oleic acid (C18:1n9c) between electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) ion sources.

$[M+H-M']^+$, m/z 386.4, *m/z* 399.5, *m/z* 413.4, *m/z* 425.6, *m/z* 441.2, *m/z* 454.2, *m/z* 467.8, *m/z* 495.8 and *m/z* 534.0 $[M+H-H_2O]^+$ (Fig. 5). Fragment ion at *m/z* 399.5 was produced by means of McLafferty rearrangement with a cleavage of C7 – C8 bond by losing of neutral molecule of undeca-1,5-diene. Similarly, fragment ion at *m/z* 495.8 also occurred by means of McLafferty rearrangement with C14 – C15 bond cleavage by losing of neutral molecule of but-1-ene and further elimination of $CH_2{=}CH_2$ to produce a fragment ion at *m/z* 467.8. Fragment ion at *m/z* 454.2 was formed by C11 – C12 bond cleavage by losing of neutral molecule of hept-1-ene and further elimination of neutral $CH_2{=}CH_2$ to produce the fragment ion at *m/z* 425.6. The fragment ion at *m/z* 386.4 was obtained by means of McLafferty rearrangement by losing of neutral molecule of penta-1,3,-diene. Fragment ion at *m/z* 441.2 occurred by C10 – C11 bond cleavage by losing of neutral molecule of octa-1,3-diene and further elimination of neutral fragment $CH_2{=}CH_2$ to produce the fragment ion at *m/z* 413.4 (Fig. 6). The fragmentation patterns were basically similar to the previous description (Hsu & Turk, 2008; Kerwin, Wiens, & Ericsson, 1996; Yang et al., 2007).

### 3.3. Validation of the method for determination of FA derivatives

Calibration curves were established using the regression of the peak area versus concentration of each FA standard. All FA derivatives showed excellent linear responses with coefficients > 0.9996 (Table 4). LOD and LOQ ranged from 0.46 to1.02 ng $mL^{-1}$ and 1.43 to 3.48 ng $mL^{-1}$, respectively (Table 4). Such low limits were mainly contributed to the highly conjugated π–π system in the molecular core of the labeling reagent (Bagheri & Mohammadi, 2003). The relative standard deviations (R.S. D %) of the inter- and intra-day validation for the instrument precision were less than 0.88 and 1.32, respectively (Table 4), whereas the RSD values for the method precision were less than 4.55 and 5.71, respectively (Table 4). These data showed that the developed HPLC–FLD method

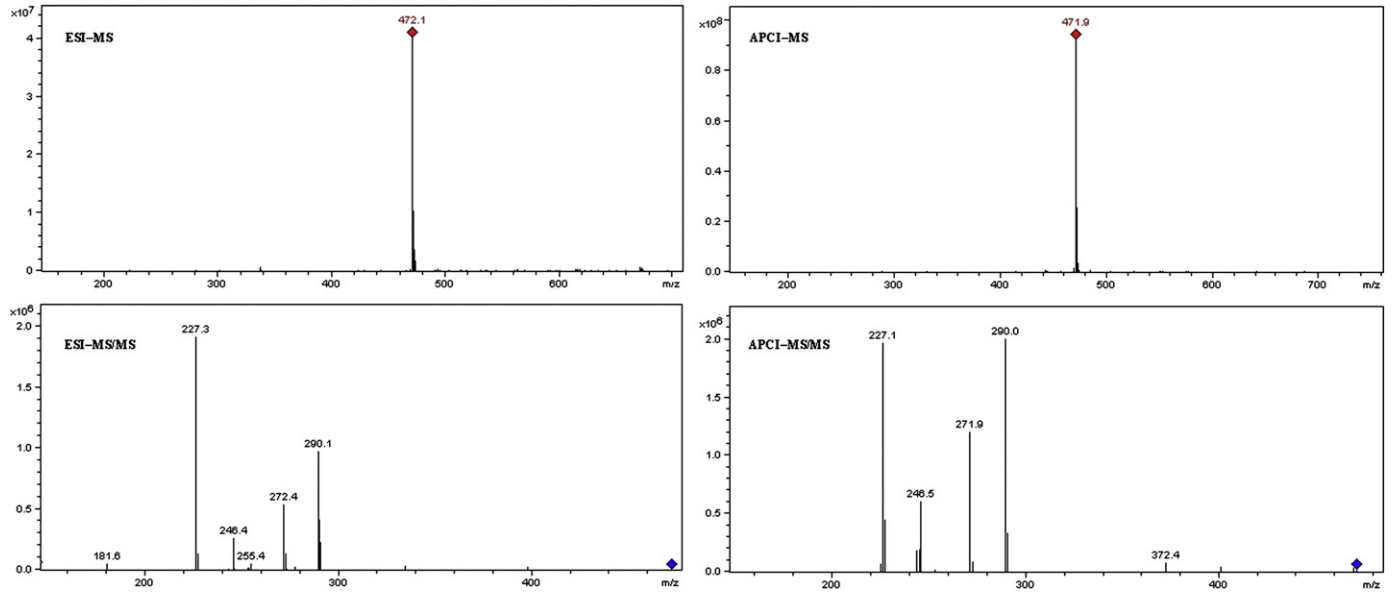


**Fig. 4.** The mass spectra for the representative lauric acid (C12:0) using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) ion sources.

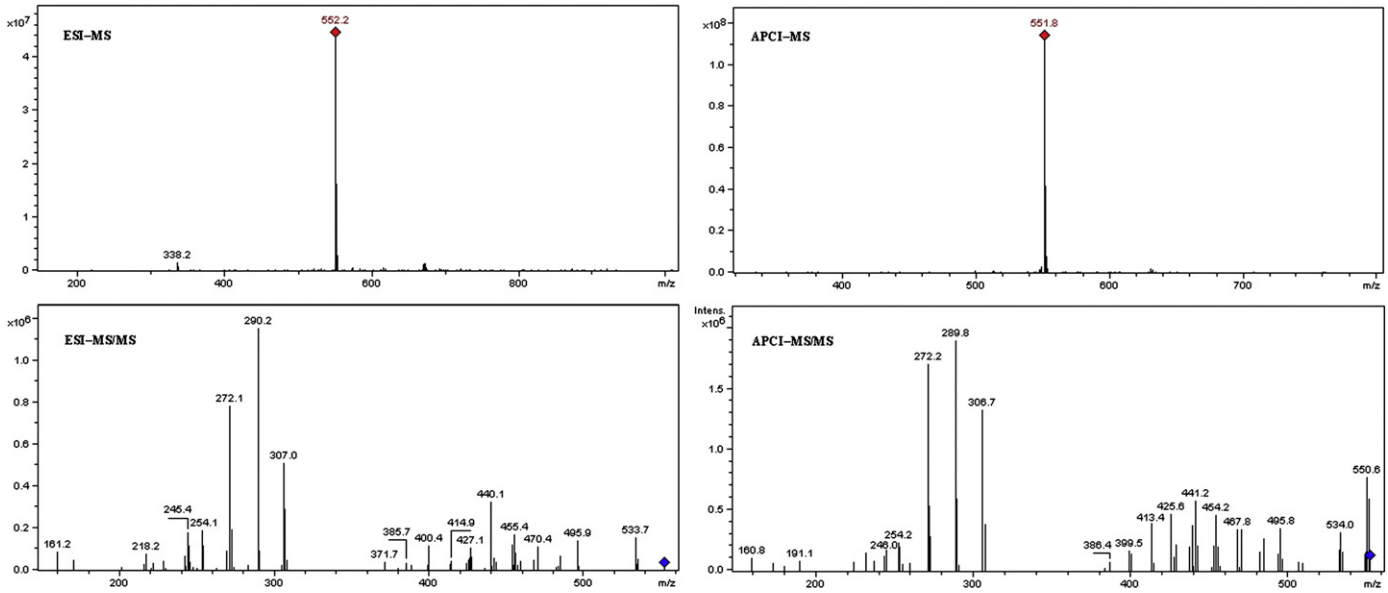


**Fig. 5.** The mass spectra for the representative linoleic acid (C18:2n6c) using electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) ion sources.

was feasible to determine FA from mushroom samples. Recoveries ranged from 93.21 to 104.62% and RSD values were less than 2.51% (Table 4). This information further demonstrated that this method was precise and practical for determination of FA from mushrooms.

### 3.4. Analysis of FA from mushrooms

Both the compositions of TFA and FFA from mushrooms were investigated (Table 5), and the typical chromatogram was shown in Fig. 7. Total TFA values for *A. luteo-virens*, *F. velutiper*, *P. eryngii*, *A. aegerita* and *C. comatus* (on a dry weight basis) were 83.08, 80.11, 42.60, 48.95 and 79. 21 mg 10 $g^{-1}$, respectively. *A. luteo-virens*, even the four cultivated species, contained a high proportion of UFA. Similar results have been observed in many mushroom species (Kavishree et al., 2008; Pedneault et al., 2006). UFA accounted for an average of 78.37% of total TFA. *A. luteo-virens* presented the highest value (5.14) whereas the ratios for the cultivated species were <3.97; UFA:SFA ratios for *F. velutiper*, *P. eryngii*, *A. aegerita* and *C. comatus* were 3.97, 3.23, 3.29 and 3.03, respectively. Linoleic (C18:2n6c) and oleic (C18:1n9c) acids were considered to be the main contributors to the high ratio of UFA. Content of linoleic acid (C18:2n6c) varied from 27.17 to 49.34 mg 10 $g^{-1}$, while oleic acid (C18:1n9c) content ranged from

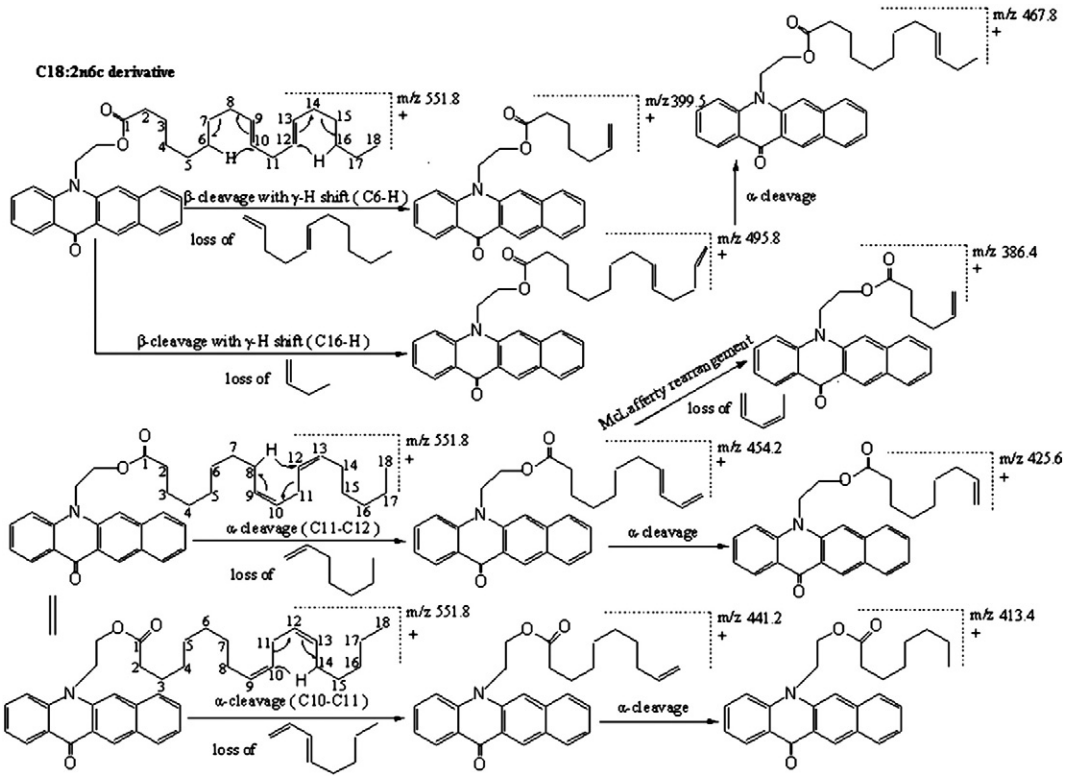


**Fig. 6.** The cleavage mode for the representative linoleic acid (C18:2n6c).

**Table 4**
Calibration curves, correlation coefficient, precision, accuracy, limits of detection and quantification (LOD and LOQ).

| Fatty acid | Y = aX + b X: injected amount (pmol) (n = 3) Y: peak area | Correlation coefficient $r^2$ | LOD (ng $mL^{-1}$) | LOQ (ng $mL^{-1}$) | Precision (RSD, %) | | | | Accuracy (n = 3) | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Instrument precision (n = 5) | | Method precision (n = 3) | | Recovery (%) | RSD (%) |
| | | | | | Inter-day | Intra-day | Inter-day | Intra-day | | |
| C10:0 | Y = 12.81X + 6.06 | 0.9998 | 0.46 | 1.43 | 0.76 | 1.11 | 2.35 | 3.64 | 98.46 | 1.94 |
| C11:0 | Y = 13.50 X − 13.67 | 0.9998 | 0.54 | 1.78 | 0.56 | 1.30 | 3.68 | 5.40 | 93.96 | 1.43 |
| C12:0 | Y = 13.01X + 5.58 | 0.9996 | 0.52 | 1.63 | 0.75 | 0.98 | 2.10 | 3.34 | 94.37 | 2.03 |
| C13:0 | Y = 15.80X − 11.60 | 0.9997 | 0.64 | 2.16 | 0.66 | 1.14 | 2.43 | 3.53 | 100.05 | 2.31 |
| C18:3n3c | Y = 23.58X − 10.87 | 0.9999 | 0.59 | 1.76 | 0.19 | 1.32 | 1.68 | 3.68 | 93.21 | 1.54 |
| C22:6n3c | Y = 15.91X − 9.15 | 0.9998 | 0.92 | 3.02 | 0.69 | 0.78 | 2.53 | 3.72 | 104.62 | 1.65 |
| C14:0 | Y = 14.11X + 1.65 | 0.9997 | 0.70 | 2.13 | 0.78 | 1.13 | 1.83 | 4.89 | 103.30 | 0.91 |
| C20:4n6c | Y = 18.57X − 8.37 | 0.9996 | 0.90 | 2.76 | 0.68 | 1.24 | 3.35 | 5.62 | 99.34 | 1.76 |
| C18:2n6c | Y = 24.32X − 6.68 | 0.9999 | 0.62 | 1.97 | 0.82 | 1.02 | 2.77 | 5.21 | 96.28 | 2.24 |
| C15:0 | Y = 15.52X + 0.29 | 0.9999 | 0.66 | 2.20 | 0.71 | 1.07 | 2.26 | 4.46 | 98.18 | 2.12 |
| C16:0 | Y = 23.69X + 4.74 | 0.9998 | 0.56 | 1.75 | 0.80 | 0.92 | 4.12 | 5.71 | 102.46 | 1.85 |
| C18:1n9c | Y = 21.29X + 4.70 | 0.9999 | 0.67 | 2.03 | 0.58 | 1.17 | 1.90 | 3.77 | 94.57 | 2.37 |
| C17:0 | Y = 27.38X − 9.72 | 0.9998 | 0.58 | 1.84 | 0.72 | 1.04 | 2.61 | 4.66 | 97.04 | 1.72 |
| C18:0 | Y = 16.22X − 3.93 | 0.9999 | 0.75 | 2.29 | 0.79 | 1.23 | 4.55 | 5.14 | 94.34 | 1.88 |
| C19:0 | Y = 15.13X − 2.61 | 0.9996 | 0.92 | 3.06 | 0.88 | 1.19 | 2.11 | 4.01 | 102.53 | 2.51 |
| C20:0 | Y = 15.43X − 11.57 | 0.9997 | 0.91 | 3.12 | 0.74 | 1.20 | 2.25 | 4.56 | 95.03 | 2.03 |
| C21:0 | Y = 18.05X − 6.25 | 0.9997 | 0.78 | 2.75 | 0.87 | 1.06 | 2.80 | 4.98 | 102.17 | 2.30 |
| C22:0 | Y = 11.88X − 0.24 | 0.9998 | 1.02 | 3.48 | 0.52 | 1.08 | 3.01 | 4.87 | 94.20 | 1.74 |

4.08 to 22.15 mg 10 $g^{-1}$. With respect to SFA, abundant palmitic (C16:0) and stearic (C18:0) acids were found in all the species investigated. Content of palmitic acid (C16:0) varied from 6.49 to 14.88 mg 10 $g^{-1}$ while stearic acid (C18:0) content ranged from 1.15 to 3.00 mg 10 $g^{-1}$. Although linoleic (C18:2n6c), oleic (C18:1n9c), palmitic (C16:0) and stearic (C18:0) acids as the major FA presented in all mushroom samples, there were differences in the levels. *A. luteo-virens* had a significantly higher content of oleic acid (C18:1n9c) than the cultivated species. Higher contents of linoleic acid (C18:2n6c) were observed in *A. luteo-virens* and *C. comatus* when compared to *F. velutiper*, *P. eryngii*, and *A. aegerita*. The highest contents of palmitic acid (C16:0) and stearic (C18:0) acid were found in *C. comatus* and *A. luteo-virens*, respectively. In addition, significantly higher content of linolenic (C18:3n3c) acid (18.02 mg 10 $g^{-1}$) existed in *F. velutiper*, and lower amounts were obtained in the remaining species. Considering *P. eryngii* and *F. velutiper* species, relative content of the major FA, namely, palmitic (C16:0), stearic (C18:0), oleic (C18:1n9c), linoleic (C18:2n6c) and linolenic (C18:3n3c) acids, were 15.24%, 2.69%, 12.13%, 63.77%, 0.48%, and 12.09%, 2.69%, 7.15%, 50.23%, and 22.50%, respectively. As reported in the literature (Reis, Barros, Martins, & Ferreira, 2012), contents of FA presented above for *P. eryngii* were 12.8%, 1.7%, 12.3%, 68.8% and 0.3%, respectively, while the relative contents for *F. velutiper* were 11.0%, 2.0%, 5.7%, 45.4%, and 28.8%, respectively. With species of *A. aegerita* and *C. comatus*, the main FA found were linoleic acid

**Table 5**
Fatty acid compositions (on a dry weight basis) from five mushroom species (mg 10 $g^{-1}$, mean ± SD).

| Fatty acids | TFA[a] | | | | | FFA[b] | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | *Armillaria luteo-virens* | *Flammulina velutiper* | *Pleurotus eryngii* | *Agrocybe aegerita* | *Copyinds comatus* | *Armillaria luteo-virens* | *Flammulina velutiper* | *Pleurotus eryngii* | *Agrocybe aegerita* | *Copyinds comatus* |
| C10:0 | 0.12 ± 0.00 | 0.06 ± 0.02 | 0.71 ± 0.08 | 0.39 ± 0.05 | 0.29 ± 0.09 | 0.07 ± 0.01 | 0.04 ± 0.00 | 0.46 ± 0.05 | 0.29 ± 0.02 | 0.10 ± 0.00 |
| C11:0 | nd [c] | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| C12:0 | 0.15 ± 0.03 | 0.57 ± 0.21 | 0.09 ± 0.01 | 0.09 ± 0.00 | 0.24 ± 0.05 | 0.04 ± 0.00 | 0.23 ± 0.07 | 0.05 ± 0.02 | 0.01 ± 0.00 | 0.11 ± 0.02 |
| C13:0 | 0.10 ± 0.02 | 0.22 ± 0.06 | 0.10 ± 0.03 | 0.08 ± 0.01 | 0.27 ± 0.05 | 0.06 ± 0.00 | 0.10 ± 0.03 | 0.07 ± 0.01 | 0.04 ± 0.01 | 0.19 ± 0.08 |
| C18:3n3c | 0.26 ± 0.03 | 18.02 ± 0.26 | 0.20 ± 0.00 | 0.16 ± 0.02 | 0.77 ± 0.10 | 0.09 ± 0.01 | 9.23 ± 0.34 | 0.08 ± 0.02 | 0.09 ± 0.00 | 0.51 ± 0.12 |
| C22:6n3c | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| C14:0 | 0.69 ± 0.17 | 2.01 ± 0.16 | 0.73 ± 0.05 | 0.49 ± 0.02 | 0.97 ± 0.07 | 0.19 ± 0.01 | 0.85 ± 0.21 | 0.39 ± 0.07 | 0.12 ± 0.04 | 0.52 ± 0.18 |
| C20:4n6c | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| C18:2n6c | 47.14 ± 0.63 | 40.23 ± 0.87 | 27.17 ± 0.15 | 33.31 ± 0.13 | 49.34 ± 0.80 | 30.05 ± 0.60 | 19.04 ± 0.23 | 17.64 ± 0.47 | 23.14 ± 0.09 | 32.02 ± 0.30 |
| C15:0 | 0.31 ± 0.05 | 0.31 ± 0.01 | 0.30 ± 0.00 | 0.41 ± 0.07 | 0.34 ± 0.02 | 0.05 ± 0.00 | 0.14 ± 0.07 | 0.19 ± 0.03 | 0.27 ± 0.07 | 0.07 ± 0.01 |
| C16:0 | 8.29 ± 0.16 | 9.69 ± 0.10 | 6.49 ± 0.18 | 6.93 ± 0.20 | 14.88 ± 0.32 | 5.69 ± 0.21 | 4.63 ± 0.10 | 4.90 ± 0.08 | 4.32 ± 0.20 | 8.86 ± 0.25 |
| C18:1n9c | 22.15 ± 0.35 | 5.73 ± 0.10 | 5.17 ± 0.12 | 4.08 ± 0.08 | 9.41 ± 0.24 | 15.53 ± 0.34 | 2.42 ± 0.05 | 3.13 ± 0.08 | 3.00 ± 0.02 | 6.57 ± 0.12 |
| C17:0 | 0.12 ± 0.04 | 0.10 ± 0.01 | 0.11 ± 0.00 | 0.13 ± 0.05 | 0.14 ± 0.02 | 0.03 ± 0.00 | 0.04 ± 0.01 | 0.09 ± 0.00 | 0.08 ± 0.03 | 0.10 ± 0.01 |
| C18:0 | 3.00 ± 0.08 | 2.16 ± 0.12 | 1.15 ± 0.06 | 2.20 ± 0.13 | 1.68 ± 0.09 | 1.76 ± 0.13 | 1.00 ± 0.05 | 0.75 ± 0.05 | 1.29 ± 0.10 | 0.86 ± 0.10 |
| C19:0 | nd | nd | nd | nd | nd | nd | nd | nd | nd | nd |
| C20:0 | 0.25 ± 0.02 | 0.14 ± 0.00 | 0.12 ± 0.02 | 0.17 ± 0.05 | 0.24 ± 0.08 | 0.14 ± 0.03 | 0.10 ± 0.01 | 0.04 ± 0.00 | 0.11 ± 0.01 | 0.10 ± 0.02 |
| C21:0 | nd | 0.18 ± 0.05 | 0.05 ± 0.01 | 0.03 ± 0.00 | nd | nd | 0.12 ± 0.02 | 0.01 ± 0.00 | 0.02 ± 0.00 | nd |
| C22:0 | 0.50 ± 0.08 | 0.66 ± 0.05 | 0.22 ± 0.05 | 0.50 ± 0.03 | 0.64 ± 0.09 | 0.36 ± 0.10 | 0.24 ± 0.10 | 0.14 ± 0.01 | 0.35 ± 0.13 | 0.18 ± 0.02 |
| Total content | 83.08 ± 1.60 | 80.11 ± 2.15 | 42.60 ± 0.80 | 48.95 ± 0.92 | 79.21 ± 2.00 | 54.06 ± 1.30 | 38.19 ± 1.13 | 27.94 ± 0.90 | 33.13 ± 0.85 | 50.18 ± 1.12 |
| UFA:SFA[d] | 5.14 | 3.97 | 3.23 | 3.29 | 3.03 | 5.44 | 4.09 | 2.94 | 3.80 | 3.52 |

[a] Total fatty acids, sum of nonesterified and esterified fatty acids.
[b] Free fatty acids, named the nonesterified fatty acids.
[c] No detection or below the LOQ.
[d] UFA:SFA, ratio between unsaturated fatty acids and saturated fatty acids.

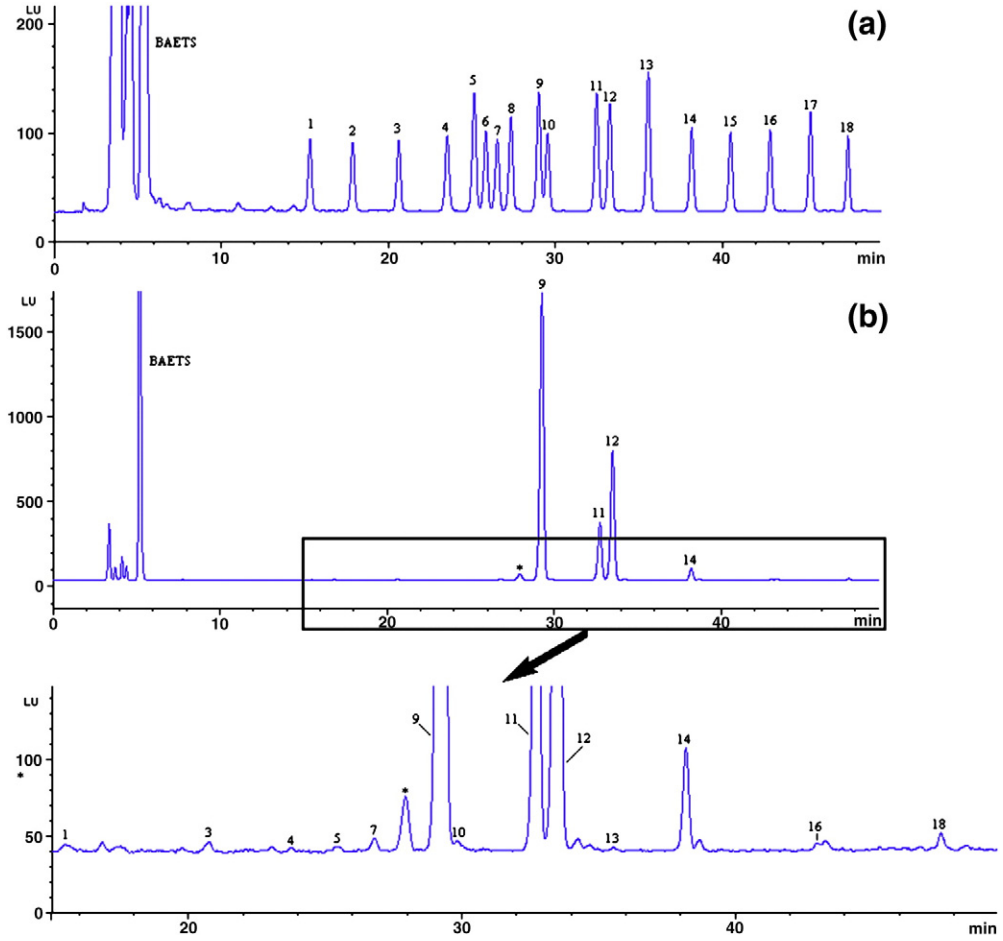


**Fig. 7.** (a) Chromatogram for fatty acid standards; (b) typical chromatogram of fatty acid derivatives from *Armillaria luteo-virens*. (1) Capric acid (C10:0); (2) undecanoic acid (C11:0); (3) lauric acid (C12:0); (4) tridecanoic acid (C13:0); (5) linolenic acid(C18:3n3c); (6) docosahexaenoic acid (C22:6n3c); (7) myristic acid (C14:0); (8) arachidonic acid (C20:4n6c); (9) linoleic acid (C18:2n6c); (10) pentadecanoic acid (C15:0); (11) palmitic acid (C16:0); (12) oleic acid (C18:1); (13) heptadecanoic acid (C17:0); (14) stearic acid (C18:0); (15) nonadecanoic acid (C19:0); (16) arachidic acid (C20:0); (17) heneicosanoic acid (C21:0); (18) behenic acid (C22:0). * identified by mass spectra, palmitoleic acid (C16:1n9c).

(C18:2n6c) > palmitic acid (C16:0) > oleic (18:1n9c) acid, which were consistent with previous studies (Lei, Jiang, Weng, Tang, & Su, 2007; Wang, 2004; Wang, Chen, Yang, & Tian, 2010).

Total FFA values for *A. luteo-virens*, *F. velutiper*, *P. eryngii*, *A. aegerita* and *C. comatus* were 54.06, 38.19, 27.94, 33.13, and 50.18 mg 10 $g^{-1}$, respectively (Table 5). The monitoring results showed that SFA and UFA were simultaneously present in the mushroom species. High content of UFA was also found in the mushroom species as the same as the profile of TFA. The highest ratio of UFA:SFA was observed in *A. luteo-virens* with the value of 5.44. Lower values were obtained in the cultivated species. UFA:SFA ratios of *F. velutiper*, *P. eryngii*, *A. aegerita* and *C. comatus* were 4.09, 2.94, 3.80 and 3.52, respectively. Three FA including linoleic (C18:2n6c), oleic (C18:1n9c) and palmitic (C16:0) acids were principal in the mushrooms (Table 5). High content of linolenic (C18:3n3c) acid was found in *F. velutiper*. Linoleic (C18:2n6c) acid was the most abundant one, varying from 17. 64 to 32.02 mg 10 $g^{-1}$.

## 4. Conclusions

The HPLC–FLD–MS/MS method based on pre-column derivatization developed in this work proved to be reproducible (good sensitivity, linearity and precision) and accurate allowing the quantification of FA in *A. luteo-virens* as well as four cultivated mushroom species (*F. velutiper*, *P. eryngii*, *A. aegerita* and *C. comatus*).

Linoleic (C18:2n6c), oleic (C18:1n9c) and palmitic (C16:0) acids were found to be the major FA in all the mushrooms investigated. Furthermore, all the mushroom species contained high content of UFA. The highest value of UFA:SFA was obtained in *A. luteo-virens*. Such high content of UFA is very significant from a nutritional standpoint.

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