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Rapid determination of total solanesol in tobacco leaf by ultrasound-assisted extraction with RP-HPLC and ESI-TOF/MS

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Abstract

A reliable and rapid method based on high-performance liquid chromatography (HPLC-UV) and positive ion electrospray-time of flight mass spectrometry (ESI-TOF/MS) has been developed for the characterization and quantification of solanesol in extracts of tobacco leaves from different sources. The solanesol was extracted from tobacco leaf via saponification and ultrasonic-assist extraction, and the extraction conditions were optimized. The HPLC conditions are as following: Hypersil C_4 (4.6 mm × 150 mm, 5 μm) column, acetonitrile and water as mobile phase, flow-rate is 0.8 ml/min, detection length of UV is 202 nm, injection volume is 10 μl. The results indicated that the developed HPLC method is simple, sensitive and reliable for the determination of solanesol in tobacco leaves with a linear dynamic range of 3.65–4672 ng, a detection limit of 1.83 ng, and an average recovery of 98.7%. The method has been applied to analyze and compare different tobacco samples. The results show that the solanesol content in samples of different geographic locations varies widely from 0.20 to 1.50%. When different parts of the tobacco plant are compared, the top parts of the leaves are more abundant in solanesol content than those of lower parts.

Keywords: Tobacco leaves; Solanesol; Saponification; Ultrasound-assisted extraction; HPLC; ESI-TOF/MS

1. Introduction

Solanesol ($C_{45}H_{74}O$) is a trisesquiterpenoid alcohol (polyisoprenoid alcohols) which was first isolated from flue-cured tobacco by Rowland et al [1]. The chemical structure of solanesol is illustrated in Fig. 1. Solanesol has diverse applications as cardiac stimulant, lipid antioxidant and antibiotic, etc. [2]. Besides being used as a medicine itself, the chemical is also used widely as an intermediate for the synthesis of coenzyme Q_{10} or vitamin K_2. In these processes, the solanesol molecule is incorporated as a side-chain component in the product molecules. Solanesol as a chemical is generally isolated from natural plant species rather than synthesized chemically. Among these plant species, the notable ones include tobacco leaves, mulberry leaves and silkworm feces. In both mulberry leaves and silkworm feces, the contents of solanesol are very low. As was reported by Rowland et al. [1], solanesol is present in comparatively larger quantities only in tobacco leaves, making it the major commercial source of solanesol. The solanesol content of tobacco leaves depends upon a number of factors including the type of tobacco, the stalk position, and the growing time of the plant. Several studies have been reported pertaining to these subjects [3–6]. The reported content of solanesol in tobacco leaves range from 0.3 to 3% of the dry weight of tobacco leaves [7–9]. A substantial portion of the solanesol in tobacco is as esters of fatty acids rather than free molecules. To extract the solanesol component, these "bound"(esterified) solanesol must first be liberated by alkaline hydrolysis [3].

Many analytical techniques including, thin-layer chromatography [10,11], gas chromatography [4,6,12], high-performance liquid chromatography [13–18], etc. have been developed to

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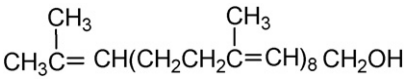

Fig. 1. The structure of solanesol.

assay solanesol in tobacco leaves and tobacco smoke. The thin-layer chromatographic densitometric method as reported by woolen and Jones is problematic because of considerable solanesol decomposition on the plates [11]. Gas chromatography method is also considered undesirable because it involves lengthy extraction and derivatization procedures. HPLC is a popular method, and various detectors including UV, RID, ELSD have been used to determine solanesol [13–18]. Reports on mass spectrometric analysis of solanesol has been scarce although one paper has been published recently on the determination of solanesol in tobacco smoke by HPLC-ES–MS [16].

In the present paper, a rapid and sensitive RP-HPLC-UV method has been developed for the quantitative determination of solanesol in the crude extract of tobacco leaf. Consideration has been given especially on the selection of HPLC conditions with optimum separation efficiency and better detection sensitivity. ESI-TOF/MS coupled with HPLC has also been applied to the analysis of tobacco leaf extracts and the identification of solanesol. In addition, a new extraction process involving saponification followed by ultrasound-assisted extraction was optimized to maximize solanesol extraction yield. The developed method has been applied to compare solanesol yield from tobacco leaves of different breeds.

2. Experimental

2.1. Materials and reagents

Twenty-one samples of tobacco leaves were obtained from different regions in Fujian province of China, and were each labeled according to their source (Table 3). Standard of solanesol (>98%) was obtained from National Institute for the Control of Pharmaceutical and Biological Products of China. Acetonitrile were of HPLC grade from Fisher Chemicals (USA). Other chemicals, such as *n*-hexane, ethanol, KOH were all of analytical grade from Shanghai Chemical Factory. Water was purified using a Milli-Q water purification system (Milipore, USA).

2.2. Sample preparation

Dried ground powder of tobacco leaves (about 2.0 g) was weighed into a 100-ml flask, and then 20 ml ethanol and 0.4 g KOH were added. The flask was sealed with cap and the mixture was heated at 60 °C with a water bath for 4 h, to insure complete hydrolysis of the bound solanesol. After cooling, the mixture was filtered through a filter paper. The filtrate was diluted to 35 ml volume with water, and then extracted with *n*-hexane for four times (*n*-hexane volume: 20, 20, 15, and 15 ml). The extraction was conducted in a funnel, which was vigorously shaken to effect solvent partitioning. The residue from filtration was ultrasonically (SY3200, Shanghai Shengyuan, China) extracted with 20 ml *n*-hexane for 20 min and then filtered. This ultrasonic extraction was repeated three additional times. It is has been found that three ultrasonic extractions (20 min each) were sufficient to completely extract solanesol from tobacco leaf residues. The filtrate was extracted with *n*-hexane (liquid–liquid extraction) for four times, and the solanesol in filtrate could be extract completely by *n*-hexane. The combined extracts were evaporated to dryness by a stream of nitrogen at 40 °C using a TurboVap II concentration workstation (Caliper Life Science). The residue was then dissolved in 50 ml of acetonitrile and filtered through a 0.45 μm nylon filter membrane. The solutions were then diluted for HPLC analysis.

2.3. HPLC and ESI-TOF/MS conditions

The HPLC analyzes were performed with a HPLC instrument (Agilent1100, USA) equipped with a quaternary solvent delivery system, an autosampler, a column oven and a diode array UV–vis detector. The column (Hypersil C_4, 15 mm × 4.6 mm, 5 μm, Dalian Elite Analytical Instruments Co. Ltd.) was eluted isocratically with a binary mixture of acetonitrile and water (volume ratio 9:1) at a total flow-rate of 0.8 ml/min. Elution was monitored at 202 nm on the diode array detector.

A G1969A TOF mass spectrometer (Agilent, USA) with an electrospray ion source coupled to the HPLC system described above was used to analysis the solanesol in tobacco leaf. Data were processed using Analyst QS software equipped in Agilent TOF/MS. The chromatographic conditions were the same as those described above, except an extra 0.3% formic acid was added into the mobile phase. The outlet of flow cell was connected to a split valve in order to divert a flow of 0.4 ml/min to the electrospray ion source via a short length of fused silica tubing. The mass spectrometer conditions were optimized for solanesol detection as follows. The lens voltages were in positive ion mode by turning on the respective ions of interest. A spray voltage of 4.5 kV was employed and the velocity of drying gas was 10 l/min. The MS data acquisition process was composed of three scans, each with different collision induced dissociation (CID) voltage of 100, 200, and 300 V, respectively. The temperature of the heated transfer capillary was 300 °C. The mass spectrometer was scanned from *m/z* 100 to 1000 in full scan mode. Reference solution was used and two *m/z*, 121.050873 and 922.009798 were selected for solanesol detection.

2.4. HPLC method validation

The calibration curve was constructed by running six standards of different concentrations in triplicate. Consequently, calibration curves were constructed. The correlation coefficient was determined using a linear regression model. The limit of detection was defined as three times the noise level obtained from running matrix blank samples.

The precision of solanesol and HPLC retention time (repeatability) measurements were established by the observed relative standard deviations of the respective measurements from six repeated runs. The reproducibility of the method was also evaluated by running six replicate samples (no. 1) prepared

independently in a single day. Sample stability was tested by periodically analyzing sample solution which was kept at room temperature at a frequency of every 12 h within 3 days. The relative standard deviation was taken as the indicator for stability.

The recovery of the solanesol was determined by standard addition method. Known quantities of solanesol standard was added into the known amounts of tobacco leaf samples. Six runs were performed at three different concentration levels, and with duplicate runs performed at each level. The spiked samples were extracted and analyzed with the established HPLC method. The percentage recoveries were calculated based on a comparison between results from the spiked and the unspiked samples.

3. Results and discussion

3.1. Extraction method development

Since substantial amount of the solanesol in tobacco leaves is bound as esters, an effective extraction method capable of measuring the total solanesol in both free and bound forms is needed. This requires first the hydrolysis of the ground tobacco in a saponification flask, followed by solvent extraction of the dissociated solanesol [4,12]. An ultrasound-assisted extraction procedure was utilized in the post-hydrolysis extraction step.

The saponification conditions are the main parameters that affect the extraction efficiency. Based on literature reports described previously [4,6,12], ethanolic KOH was selected as the hydrolysis solvent while *n*-hexane was selected as the extraction solvent. Saponification time is a critical factor affecting extraction efficiency. Prolonged saponification time should be avoided because of potential analytical artifact problems of solanesol degradation in the hydrolysis process. The time for saponification varied from 1 to 5 h in this study. The results in Fig. 2A show that the saponification efficiency of solanesol increases with time from 1 to 4 h. Further increase in reaction time results in significant reduction of solanesol yield, and 4 h saponification time by 20 mg/ml KOH in ethanol at 60 °C provides the best yield.

The amount of KOH required was investigated by analyzing the amount of solanesol saponified with the concentrations of KOH in ethanol (hydrolysis solvent) varied from 2.5, 5, 10, 20 to 30 mg/ml. The results (Fig. 2B) suggest that the highest yield of solanesol was obtained by using 20 mg/ml KOH in ethanol.

The yields of solanesol extracted under different saponification conditions are summarized in Table 1. The highest yield of solanesol was obtained under the conditions of 4-h time, 60 °C temperature and 20 mg/ml KOH in ethanol. The yield at room temperature under otherwise identical conditions was significantly lower than those at 60 °C.

Table 1
Comparison of the solanesol extraction ratio from tobacco leaves under different saponification conditions

Saponification conditions	Relative extraction ratio
2 h, at 60 °C, 20 mg/ml KOH in ethanol	0.73
4 h, at 60 °C, 20 mg/ml KOH	1.00
6 h, at 60 °C, 20 mg/ml KOH	0.78
24 h, at room temperature, 10 mg/ml KOH	0.38
24 h, at room temperature, 20 mg/ml KOH	0.39

3.2. Optimization and validation of HPLC method

The chromatographic conditions were developed and optimized using both solanesol standards and real tobacco leaf sample. Reversed phase HPLC was used instead of the normal-phase procedures reported in the literature to improve separation and detection sensitivity [13–14,17]. Because the absorptions of the solanesol lies in the vacuum UV range with λ max of 200 nm (Fig. 3), 202 nm was therefore chosen as the detection wavelength. The use of such short wavelength UV dictates the use of acetonitrile instead of methanol in water as the mobile phase in order to minimize detector baseline noise. Compared with results presented in previous reports [14,15,18] with the use of refractive index detector, UV 211 nm

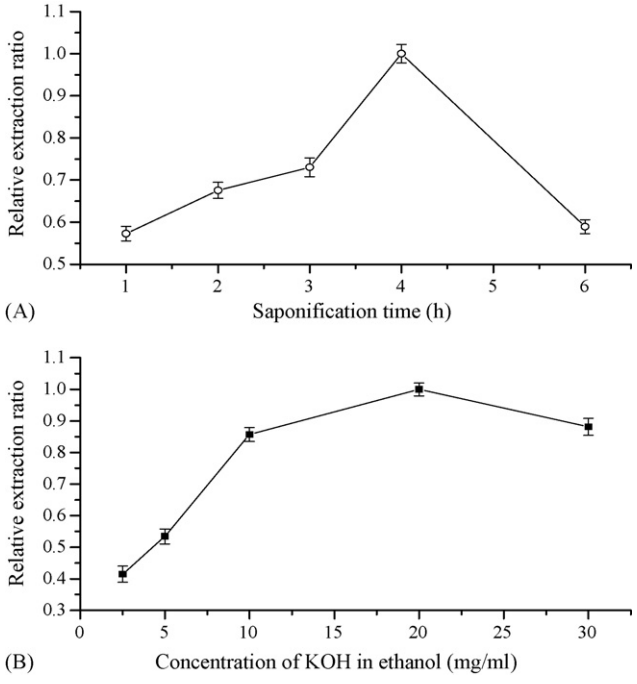

Fig. 2. The effect of saponification time (A) and concentration of KOH in ethanol (B) on solanesol extraction yield.

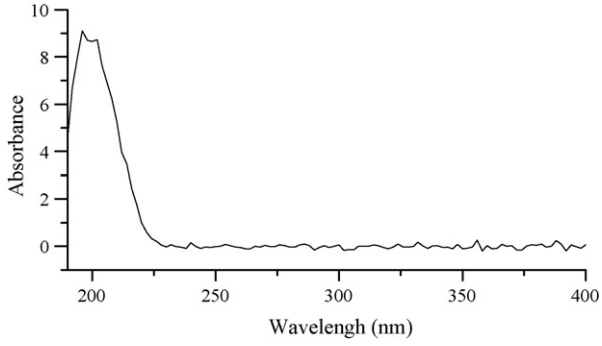

Fig. 3. The UV spectrum of the solanesol peak isolated in HPLC.

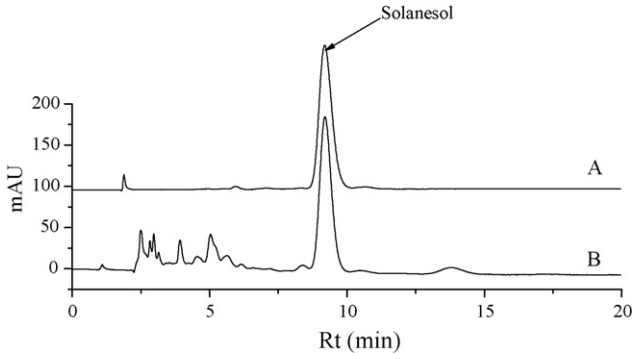

Fig. 4. HPLC chromatograms of solanesol standard (A), and extracts of tobacco leaf sample (B).

and evaporative light scattering detectors, our results show significant improvement in detection sensitivity for solanesol.

The performance of four columns: C_{18} (250 mm), C_{18} (150 mm), C_8 (250 mm) and C_4 (150 mm) were evaluated. It was found that the two C_{18} and C_8 columns are too retentive towards solanesol, while C_4 column provided good separation. Under the developed HPLC conditions, solanesol peak can be well separated from all the other component peaks in tobacco leaves as illustrated in Fig. 4.

Under the chromatographic conditions used in this study, calibration curves exhibited good linearity from regression analysis ($r^2 > 0.999$). The regression equation obtained is $Y = 0.1317X - 5.4596$. The calibration curve for the solanesol is linear within the range of 3.65–4672 ng. The linearity of the calibration curve was verified by the correlation coefficient as well as visual inspection.

The detection limit for solanesol, defined as a signal-to-noise ratio of 3, was 1.8 ng. The value is about 100 times more sensitive than those reported in the literature using UV [15] or evaporative light scattering detection [18].

The results of precision, reproducibility and stability test showed that the relative standard deviation (R.S.D.) of retention time and peak area for precision found were 0.07 and 0.21%, for reproducibility were 0.49 and 3.72%, for stability were 0.39 and 3.67%, respectively. All the results demonstrate that the developed analytical method is reproducible with good precision and is stable within 3 days.

The recovery results are summarized in Table 2. The recoveries were within the range of 95.6–105.1% with a R.S.D. of 3.40%, demonstrating superior performance of the method in both recovery and accuracy.

3.3. Identification of solanesol by LC-ESI-TOF/MS

A typical HPLC chromatogram of an extract sample and the UV spectra of solanesol peak are shown in Figs. 4B and 3, respectively. With a diode-array detector and the corresponding computer software, the evaluation of peak purity allows checking the singularity of the peak component. Using this peak purity check routine, the solanesol peak in Fig. 4B has high peak purity according to the normalized match factor value (>990) (with 1000 being 100% pure).

The samples were also run by LC-ESI-TOF/MS to identify unequivocally the target species. Our preliminary direct infusion studies with solanesol confirmed that positive ion mode was more sensitive. The mass spectrometer conditions were optimized for solanesol standard in order to achieve maximum sensitivity. The results showed that the spray voltage of 4.5 kV could give a best sensitivity. The CID voltage of 200 V could produce the best signals and the lowest limits. When the CID voltage was more than 300 V, the $[M + NH_4]^+$ ion was disappeared. The effects of pH of the mobile phase on the ionization efficiency of solanesol were also evaluated. The use of 0.3–1.0% acetic acid in mobile phase was found to give satisfactory sensitivity towards solanesol detection by ESI-TOF/MS. In fact, no solanesol signal was observed in ESI-TOF/MS when the amount of acetic acid in the mobile phase was below 0.3% able (Fig. 5).

The extracts of the tobacco leaf sample was analyzed by LC-ESI-TOF/MS with positive ion mode under the optimized mass spectrometer conditions. Solanesol, which was detected at a retention time of 9.02 on HPLC, was unambiguously identified by TOF/MS from both exact mass measurement and by comparing with authentic standard. Within the mass range scanned, two major fragment ions were observed at *m/z* 613.5705 and 648.6072. A much less abundant (2–6% of the base peak) protonate $[M + H]^+$ ions (observed at *m/z* 631.5811) was also occasionally detected (Fig. 6A). The exact masses of these ions ($([M - H_2O + H]^+$, $[M + H]^+$ and $[M + NH_4]^+$) were consistent with the elemental compositions of $C_{45}H_{73}$, $C_{45}H_{75}O$ and $C_{45}H_{78}NO$ with mass errors under 2 ppm, respectively. The molecular mass of solanesol was calculated to be 630.57 which was confirmed by the observation of an $[M + H]^+$ ion of 631.5811 using positive ion ESI-TOF mass spectrometry. The exact mass measurement of the base peak at *m/z* 613.5705 suggests a molecular composition o f $C_{45}H_{73,}$ derived from the loss of a H_2O moiety and the addition of a H^+ onto the parent ions of solanesol [M-H_2O]. The loss of water was expected since solanesol is a polyisoprenoid alcohol containing a hydroxyl group, from which

Table 2
Results for recovery of solanesol determined by standard addition method

Numbers	Original (mg)	Added (mg)	Detected (mg)	Recovery (%)	Average recovery (%)	R.S.D. (%)
1	13.00	3.31	16.48	105.1	98.7	3.40
2	13.00	3.35	16.24	96.8		
3	6.50	3.34	9.75	97.4		
4	6.50	3.30	9.65	95.6		
5	3.30	3.32	6.59	99.1		
6	3.30	3.31	6.55	98.3		

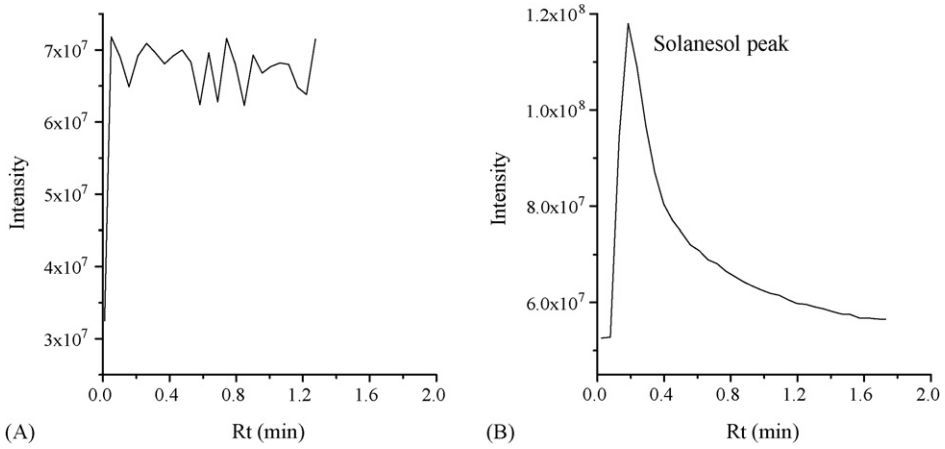

Fig. 5. Total ion current (TIC) obtained from ESI-TOF/MS analysis of solanesol standard solution. The standard solution was injected into ESI-TOF/MS directly. Solanesol was dissolved in HPLC mobile phase of acetonitrile and water with the addition of: (A) 0.1% formic acid and (B) 0.3% formic acid.

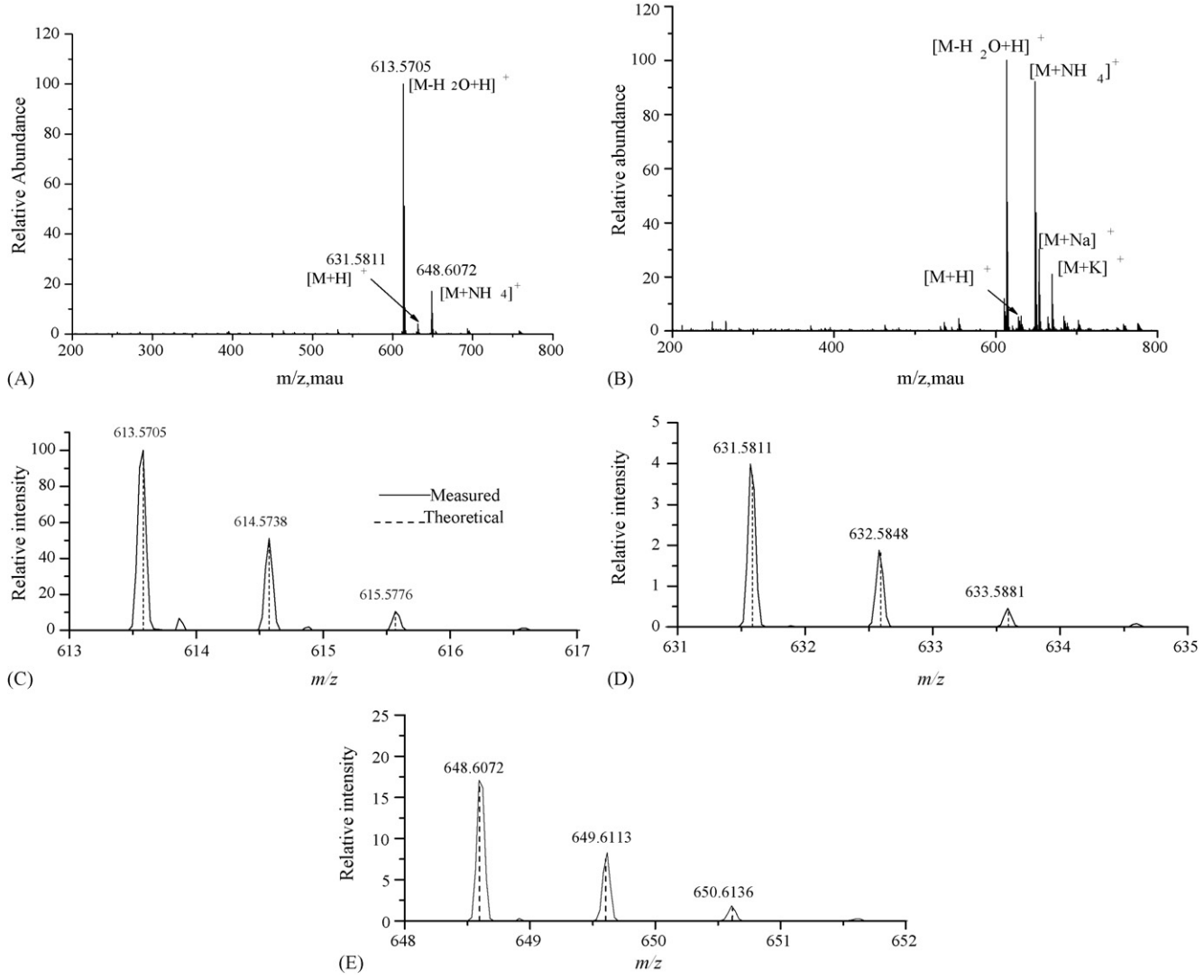

Fig. 6. (A) MS spectra of solanesol obtained using acetonitrile, water and formic acid (volume ratio 90:10:0.3) as mobile phase in the positive mode; (B) MS spectra of solanesol obtained using a solvent mixture of acetonitrile, water and ammonium hydroxide (volume ratio 90:10:0.5) as the mobile phase in the positive mode; (C) observed mass ($[M-H_2O+H]^+$) of solanesol and its isotopics compared with calculated isotopics; (D) observed mass ($[M+H]^+$) of solanesol and its isotopics compared with calculated isotopics; (E) observed mass ($[M+NH_4]^+$) of solanesol and its isotopics compared with calculated isotopics.

the water moiety could be lost readily [19]. The exact mass measurement of *m/z* 648.6072 suggests a molecular composition of $C_{45}H_{78}NO$, in which an ammonium ion had been introduced into the solanesol molecules. Fig. 6C–E show that measured isotopics of the three formulas match perfectly with the theoretical values (including positions and intensities).

Tucker and Pretty [16] have identified solanesol in tobacco smoke by ES–MS in the positive ion mode using a mobile phase consisting of methanol/0.50% ammonium hydroxide. In the present study, 0.50% ammonium hydroxide also added to the mobile phase for MS analysis in the positive ion mode. The $[M+NH_4]^+$ ion abundance in Fig. 6B was relative higher than in Fig. 6A and the $[M+Na]^+$, $[M+K]^+$ ions were also observed, which can be seen by comparing Fig. 6A and B. However, the detection sensitivity of *m/z* 613.5705 with 0.50% ammonium hydroxide as additives in mobile phase decreased obviously comparing with acetic acid as additives.

In our TOF/MS analysis, an isotope ratio matching technique was used in the compound identification process using the Analyst QS software available in the instrument. In the exercise, once the exact mass of the peak was decided, one or several possible elemental compositions will then be proposed. The software can then give the theoretical isotopic ratios (shown with broken lines) of all the possible structures. The exact masses and the intensities of these theoretical isotopic peaks are then compared with those of the actual examples for compound identification. In this study, the excellent agreement obtained between the proposed and the actual isotopic ratios of the peaks associated with solanesol can be seen in Fig. 6C–E.

3.4. Quantification of solanesol in the samples

The developed method has subsequently been applied to determine the solanesol contents in different tobacco samples. Representative chromatograms of the extracts of these tobacco leaf samples are shown in Fig. 4B, and the observed yields of solanesol are summarized in Table 3.

The results in Table 3 illustrate that the contents of solanesol in tobacco leaves obtained from different geographic origins vary over a wide range from 0.20 to 1.50%, consistent with those of previous reports [1]. It is found that the contents of solanesol in 21 tobacco leaves cultivated in Fujian province of China correlate with product grade but not plant origins or breeds. The commercial tobacco products are classified as B, C and X grades, representing tobacco leaves being picked, respectively from the top, middle or bottom parts of the tobacco plant. Our results show that five of the 21 samples contain solanesol yield over 1.00%, and all these five samples belong to the B grade. Our data, although limited in sample size, thus suggest that the leaves grown in the top part of the tobacco plant are relatively more abundant in solanesol content.

Table 3
Contents of solanesol in the tobacco leaf samples from different growing regions of Fujian province of China (with different breeds and grades) ($n=3$)

Sample numbers	Origins	Breed	Grade	Contents (%)	R.S.D. (%)
1	Shaowu	K326	B	1.27	2.64
2	Shaowu	K326	C	0.66	3.12
3	Shaowu	K326	C	0.41	3.45
4	Shaowu	K326	C	0.54	2.83
5	Shaowu	K326	X	0.43	3.59
6	Shaowu	K326	X	0.27	3.28
7	Shaowu	Yunyan85	B	1.21	2.57
8	Shaowu	Yunyan85	B	1.50	2.71
9	Shaowu	Yunyan85	C	0.63	3.82
10	Shaowu	Yunyan85	C	0.64	3.39
11	Shaowu	Yunyan85	X	0.20	4.10
12	Shaowu	Yunyan85	X	0.34	3.52
13	Yongan	K326	B	1.17	2.46
14	Yongan	K326	C	0.50	3.24
15	Yongan	K326	X	0.41	3.66
16	Ninghua	CB-1	B	1.06	2.93
17	Ninghua	CB-1	C	0.33	3.88
18	Ninghua	CB-1	X	0.43	3.94
19	Longyan	Yunyan85	C	0.34	4.05
20	Longyan	K326	X	0.53	3.81
21	Longyan	K326	C	0.93	3.19

4. Conclusion

The HPLC method developed in this work is well suited for rapid routine quantification of solanesol in tobacco leaf samples. The combination of saponification with ultrasound-assisted extraction provides an efficient and reliable method for the quantitative recovery of solanesol from tobacco leaves. With a detection limit of 1.8 ng per injection, the sensitivity (detection limit 1.8 ng on the column) of this method was improved by more than 100 times over those reported previously using UV–vis detector. The coupling of high resolution HPLC separation with accurate mass measurement capability of ESI-TOF/MS provides an attractive tool for the identification of "unknowns" compounds in tobacco leaves.

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