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Preparative isolation and structural characterization of sucrose ester isomers from oriental tobacco

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ABSTRACT

To date, the structures of the sucrose tetraester (STE) isomers, a main kind of sucrose esters (SEs) in *Solanum*, have not been conclusively assigned. In this study, three groups of STE isomers with the molecular weight 650, 664 and 678 (designated as STE I, STE II and STE III, respectively) have been isolated and purified from the oriental tobacco-*Komotini Basma* using a semi-preparative RP-HPLC method. The full characterization of the isomers in the three groups of STE were investigated for the first time by MS (HRMS, MS^2) and NMR (^1H, ^{13}C, HSQC) spectroscopy combined with alkaline hydrolysis and STE derivation experiments. The STE III (a single compound) was confirmed as a known sucrose tetraester. Furthermore, the STE II was found to contain three isomers and the structures were first unambiguously established as 6-*O*-acetyl (2,3 or 2,4 or 3,4)-di-*O*-3-methylvaleryl-(4 or 3 or 2)-*O*-2-methylbutyryl-α-D-glucopyranosyl-β-D-fructofuranoside. Finally, the STE I was discovered to contain seven isomers and the structures were elucidated as 6-*O*-acetyl (2 or 3 or 4)-*O*-3-methylvaleryl-(3,4 or 2,4 or 2,3)-di-*O*-2-methylbutyryl-α-D-glucopyranosyl-β-D-fructofuranoside, 6-*O*-acetyl (2 or 3 or 4)-*O*-3-methylvaleryl-(3,4 or 2,4 or 2,3)-di-*O*-isovaleryl-α-D-glucopyranosyl-β-D-fructofuranoside and 6-*O*-acetyl (2,3 or 2,4 or 3,4)-di-*O*-3-methylvaleryl-(4 or 3 or 2)-*O*-isobutyryl-α-D-glucopyranosyl-β-D-fructofuranoside (one of the 3 isomers).

1. Introduction

Sucrose esters (SEs) are the second major class of tobacco surface chemicals, which are biosynthesized exclusively in trichome glands from sucrose and activated acids derived from branched-chain amino acid metabolism.[1–4] The most abundant tobacco leaf SEs were identified as sucrose tetraesters (STEs).[1,5,6] SEs isolated from the leaf surface extracts are recognized as precursors of important oriental tobacco smoke flavour components. It has been proved that SEs readily release fatty acids such as isobutyric acid, methylbutyric acid, and methylvaleric acid on thermolysis during tobacco combustion.[7,8] SEs also have been shown to possess insecticidal,[9–12] antibacterial and plant growth regulating activities.[13–17] Studies have shown that some other related *Solanum* (e.g., potato, tomato, petunia) can also produce sucrose esters.[18–23]

There are already several reports on the separation and structural characterisation of the different types of SE homologs from tobaccos by silica gel column or HPTLC combining with spectroscopic methods,[13,14,24] LC–MS,[25,26] and LC–MS^n,[27] in previous studies. However, to the best of our knowledge, few attempts have been made to report the full characterization of the SE isomers with the same molecular weight.[5,28]

In this paper, three groups of STE isomers with the molecular weight 650, 664 and 678 were isolated and purified from oriental tobacco-*Komotini Basma* by semi-preparative RP-HPLC. By direct NMR and MS spectra along with alkaline hydrolysis and STE derivation experiments, the structures of the three isomers in the STE group of MW 664 were established unequivocally and the structures of the seven isomers in the STE group of MW 650 were elucidated for the first time.

2. Experimental

2.1. Material and reagents

N,*O*-Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) (98% purity), acetic acid, isobutyric acid, 2-methylbutyric acid (99% purity) were purchased from Acros Organics (USA). Isovaleric acid and 3-methylvaleric acid (99% purity) were from Tokyo Kasei Kogyo Co., Ltd (Tokyo, Japan). Acetonitrile and methanol (HPLC grade) purchased from Siyou Fine Chemical Co. Ltd (Tianjing, China). HPLC grade water (18 mΩ) was prepared using a Millipore Milli-Q purification system (Millipore Corp., USA). The oriental tobacco-*Komotini Basma* leaves sample was kindly provided from the R&D center of

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Hongta Tobacco (Group) Co., Ltd (Yuxi, China). Tobacco sample was dried at 45 °C and ground to pass through a 40-mesh sieve for further analysis.

2.2. Extraction and isolation of sucrose tetraesters

A sample of tobacco leaves (50.0 g) was extracted by ultrasonic assistant extraction with dichloromethane (1000 mL) at 25 °C for 20 min. After filtration, the residue was washed with dichloromethane (3 × 50 mL) and the resulting solution were combined and concentrated under reduced pressure to give a residue which was dissolved in CH_3OH/H_2O (80:20, v/v) (100 mL). This solution was extracted with *n*-hexane (3 × 100 mL), and the CH_3OH/H_2O phase was collected and extracted with $CHCl_3/H_2O$/saturated aq KCl (4:2:1, v/v) (3 × 70 mL). The $CHCl_3$ phase was collected, washed with water (10 mL), dried over anhydrous sodium sulfate (15 g) and then concentrated to a volume of 10 mL. This solution was then subjected to column chromatography (column size 4 cm × 60 cm) on silica gel (100–200 mesh), Initial elution was with *n*-hexane/acetone (1:1, v/v) at a rate of 4 mL/min to afford fractions 1–7 (40 mL/fraction), and then with *n*-hexane/acetone (2:3, v/v) to give fractions 8–25. For the routine TLC screening test of STEs, each fraction was concentrated to 5 mL before being applied to the plate.

2.3. TLC of sucrose tetraesters

TLC Silica Gel 60 F254 plates, 6.5 cm × 2.5 cm, thickness 0.5 mm (Qingdao Haiyang Chemical Group Co. Ltd, China) were developed in a twin trough unsaturated chamber using PhMe/MeCOEt/MeOH/H_2O (10:5:4.5:0.2, v/v/v/v). Plates were developed to a distance run by the solvent of 4 cm at room temperature and relative humidity of 40%. The developed plates were dried in a stream of cold air for 3 min and then immersed for 20 min into a color reagent of $CO(NH_2)_2/H_3PO_4$/*n*-BuOH (1 g of $CO(NH_2)_2$ and 4.5 mL 85% (v/v) H_3PO_4 dissolved in 48 mL *n*-BuOH saturated by water), by means of the Camag chromatogram immersion device II. Heating at 70 °C for 20 min furnished coloured bands for separated compounds.[29]

2.4. Analytical and semi-preparative HPLC separation procedure

The combined fractions containing STEs was concentrated to 20 mL and filtered through a membrane filter (0.45 μm, Millipore). Two milliliters of the resulting filtrate (named F_{STE}) was injected into the semi-preparative HPLC. The HPLC system equipped with a Waters 600 controller, a Waters Delta 600 pump, a differential refractive index detector (RID, Waters 2414), and a sunFire™ prep C18 column (250 mm × 10 mm i.d.,10 μm, Waters Corp., USA). The

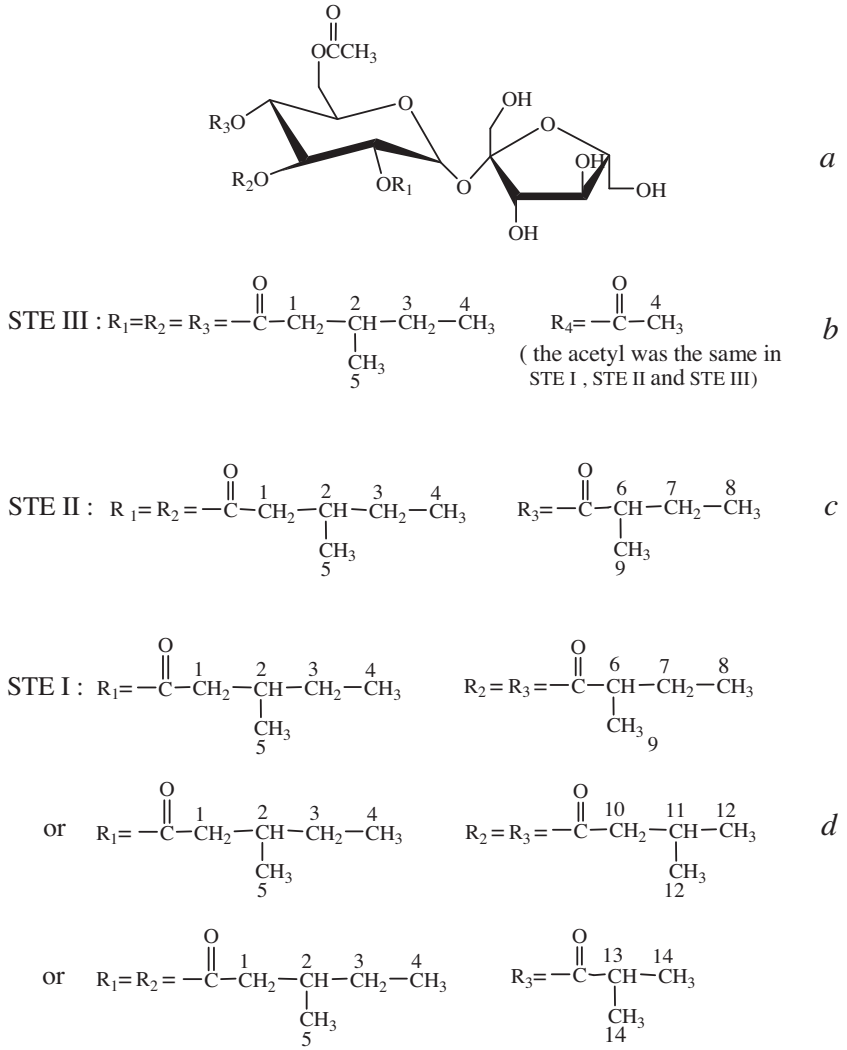

Figure 1. Structures and numbering systems of the isolated STEs, (a) general structural formula of the STEs homologs; (b) structures of the acyl groups in STE III; (c) structures of the acyl groups at the positions of O-2, O-3 and O-4 of the glucose residue in STE II; (d) structures and combination modes of the acyl groups at the positions of O-2, O-3 and O-4 of the glucose residue in STE I.

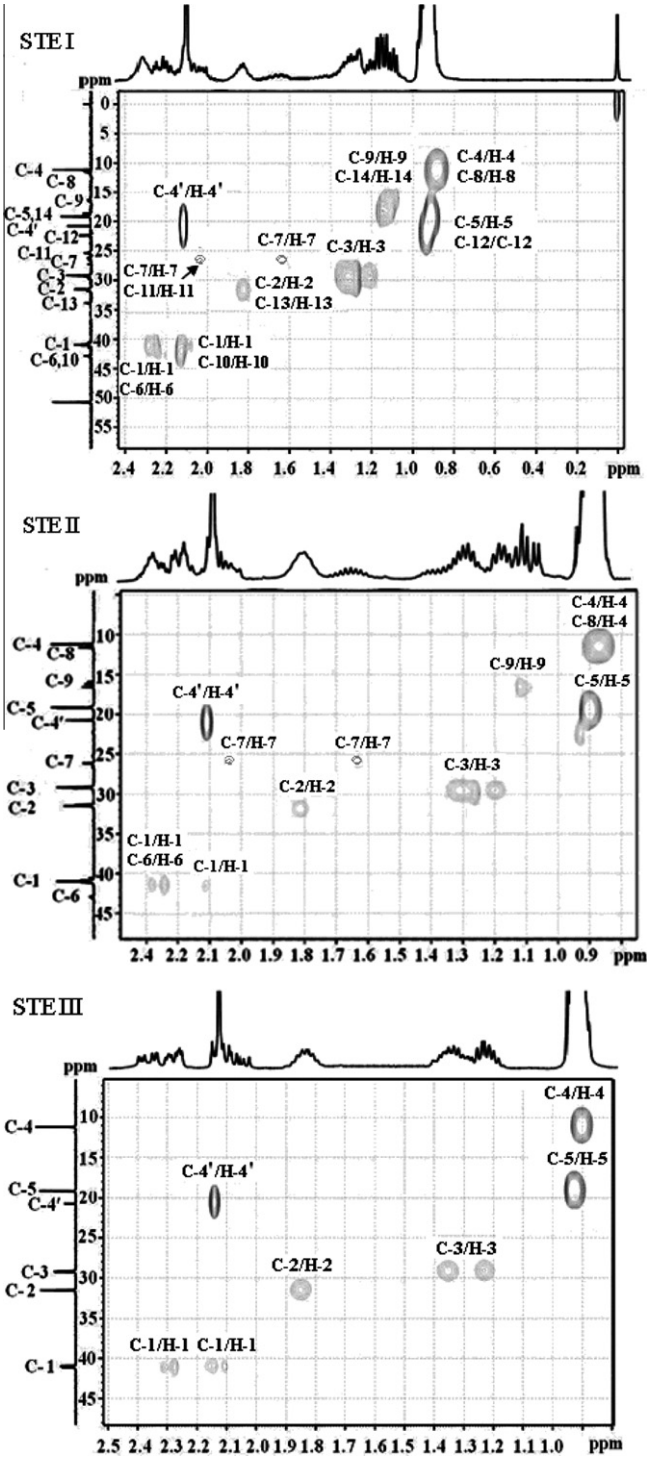

Figure 2. HSQC spectra of the acyl region of STE I–STE III. The numbering of carbons and the attached protons in HSQC spectra are the same as that shown in Figure 1b, c and d.

mixed solvent CH_3CN/H_2O (70:30, v/v) was used as eluent at a flow rate of 4.0 mL/min.

HPLC analyses of the F_{STE} and the purified STE fractions were performed on a Waters 1525 (Waters Corp., USA), using a sunFire™ C18 column (250 mm × 4.6 mm i.d. 5 μm, Waters). CH_3CN/H_2O (70:30, v/v) was used as eluent at a flow rate of 1.0 mL/min. STEs were detected by evaporation light scattering detector (ELSD, Waters 2424). The purity of the STE fractions was expressed on an area percentage basis.

2.5. Structural identification of STE isomers

All MS analyses (HRMS, MS^2) were performed on a Waters Q-TOF Micro mass spectrograph (Waters Corp., USA) by full scan ESI-MS and collision induced dissociation (CID) ESI-MS/MS. The analytical parameters were spray voltage: 3.5 kV, cone voltage: 35 V, collision voltage: 26.0 V. Data were acquired in the positive-ion MS and MS^2 modes. The sample was injected using a peristaltic pump.

1D and 2D NMR spectra were acquired on a Bruker-DPX-400 (400 MHz) NMR spectrometer, with samples (3–10 mg) dissolved in 0.4–0.6 mL $CDCl_3$, using tetramethylsilane (TMS) as internal standard. The ^1H, ^{13}C, H, H-COSY, HSQC experiments were standard Bruker implementations.

2.6. Alkaline hydrolysis experiments

Each group of purified STE isomers (5 mg) was dissolved in 2 mL $KOH/CH_3OH/H_2O$ solution (1 mol KOH dissolved in 1 L of CH_3OH/H_2O (80:20, v/v)), and the solution was heated at 60 °C for 30 min, after which time concentrated hydrochloric acid (0.3 mL) was added, and the mixture then centrifuged. The fatty acids in the supernatant layer were analysed by GC–MS. GC–MS analyses were performed on a GC 6890-MS 5973 N Gas chromatography–mass spectrometry (equipped with an automatic sampler, G1701CA MSD chemical workstation and NIST 05 mass spectral data base, Agilent Corp., USA), GC conditions were as follows: HP-INNOWax capillary column (30 m × 250 μm × 0.25 μm), with an initial column temperature of 80 °C, increasing at 5 °C/min to 160 °C, which was then held for 3 min. Injector temperature was 250 °C. The carrier gas was helium at a flow rate of 1.0 mL/min. Injection volume was 1 μL, with a split ratio of 5:1. MS were measured with electron impact (EI) ionization energy of 70 eV. Interface temperature was 250 °C, and the quadrupole temperature was 150 °C; the electron multiplier was voltage 1.34 kV. The MS measurements were made in the scan method with a mass scan range of 35–500 *m*/*z*. Standard acid solutions (acetic acid, isobutyric acid, 2-methylbutyric acid, isovaleric acid and 3-methylvaleric acid) of different concentrations (1 μg/mL, 20 μg/mL and 40 μg/mL) were also analyzed under the above conditions.

3. Results and discussion

3.1. TLC analyses

TLC revealed the existences of sucrose tetraesters from the 16th to the 22nd of the 25 fractions from the tobacco extracts. The developing agent $CO(NH_2)_2/H_3PO_4/n$-BuOH proved to be a sensitive reagent for STEs giving bluish grey spots. R_f values on TLC for the 20nd fraction (F_{20}) was 0.69. No material was observed at this R_f in the 12th fraction (F_{12}) indicated indicating the absence of STEs.

3.2. Analytical and semi-preparative HPLC separation

The crude sample (F_{STE}) was analyzed as described in Section 2.4 and three groups of STE isomers designated as STE I, STE II and STE III were observed with retention times of 6.04 min, 7.43 min, and 9.46 min, respectively.

Semi-preparative HPLC separation of the F_{STE} gave the three different purified STE fractions as viscous oils. As a result, from 50.0 g of a tobacco leaves sample, 14.8 mg of STE I, 30.4 mg of STE II and 25.2 mg of STE III were obtained, and the purity of STE I, STE II and STE III was 99.6%, 99.2% and 99.8%, respectively, in a single run.

3.3. Structural identification of the isomers in the three groups of STEs

The HRMS analysis results further confirmed that STE I, STE II and STE III were three groups of STE isomers since only one major peak was observed in every case. The molecular formulas of STE I, STE II and STE III were established as $C_{30}H_{50}O_{15}$, $C_{31}H_{52}O_{15}$ and $C_{32}H_{54}O_{15}$ by HRMS analysis (*m*/*z* 673.3051 $[M_{STE\ I}+Na]^+$, calcd for $C_{30}H_{50}O_{15}Na$, 673.3047), *m*/*z* 687.3206 $[M_{STE\ II}+Na]^+$ calcd for $C_{31}H_{52}O_{15}Na$, 687.3204, and *m*/*z* 701.3363 $[M_{STE\ III}+Na]^+$, calcd for $C_{32}H_{54}O_{15}Na$, 701.3360, respectively. On the basis of the molecular formulae and literature data,[4,5,27] these STEs probably possessed a general structure formula which is shown in Figure 1a. In the MS^2 spectra, a major peak at *m*/*z* 511.2 $[M_{STE\ I}+Na-162]$, *m*/*z* 525.3 $[M_{STE\ II}+Na-162]$ and *m*/*z* 539.3 $[M_{STE\ III}+Na-162]$, corresponding to the loss of a fructose unit, were observed for STE I, STE II and STE III, respectively. This further confirmed that these STEs belong to the same structural type showed in Figure 1a. It contains an acetyl group on O-6 of the glucose ring, three acyl groups of 4 to 6 carbons on O-2, O-3 and O-4 of the glucose ring, and four free hydroxyl groups on the fructose ring. Furthermore, on the basis of literature data,[4,5,27] the combination of fatty acids bound to the glucose ring can be $C_2C_5C_5C_6$ or $C_2C_4C_6C_6$ in STE I, $C_2C_5C_6C_6$ in STE II and $C_2C_6C_6C_6$ in STE III where the subscript denotes the number of carbons in the acid. Except for STE III, each group contains several isomers. In this study, the structures of the four acyl groups on O-6, O-2, O-3, and O-4 were further investigated by 1D and 2D NMR spectra along with the alkaline hydrolysis and derivation experiments of STEs, and the structures of the isomers in STE I and STE II were confirmed or elucidated. Except for the ^{13}C NMR data of STE III,[5] the NMR and HRMS spectra from the authentic samples of STE I, STE II and STE III have been obtained for the first time.

The HSQC spectra of the acyl region (Fig. 2) made possible unambiguous assignments of the chemical shifts of carbon and the attached protons for all four acyls in STE III and STE II, and allow most of the chemical shifts of acyls region to be assigned in STE I. The ^1H NMR and ^{13}C NMR data are summarised in Table 1.

In Table 1 (STE III), the data of the acyl region indicated the presence of three 3-methylvaleryls and an acetyl in STE III by comparison with the data obtained by measurements on standards of these fatty acids. In addition, the NMR data were confirmed by the experiment result of alkaline hydrolysis. The hydrolyzates after acidification were analyzed by GC–MS. By searching with NIST 05 MS mass spectral data base and comparison with the mass spectra of standard acids, the fatty acid compositions of the STEs could be achieved. The molar response value of the acids could be calculated according to the GC–MS analysis results of the standard acid solutions (Section 2.6). The results showed that the alkaline hydrolysis experiment of STE III gave acetic acid and 3-methylvaleric acid with the molar ratio being approximately 1:3. Based on the NMR data and alkaline hydrolysis experiment, it is clear that the four acyl groups on O-6, O-2, O-3 and O-4 positions of STE III were one acetyl and three 3-methylvaleryls (Fig. 1b). Therefore, STE III was essentially a single compound, and the structure was established as 6-*O*-acetyl 2,3,4-tri-*O*-3-methylvaleryl-α-D-glucopyranosyl-β-D-fructofuranoside. It is the only STE that has to date been characterised in this homologous series.[5]

In Table 1 (STE II), the data of the acyl region indicated the presence of two 3-methylvaleryls, one 2-methylbutyryl and one acetyl in STE II by comparison with the data from standards of the fatty acids. Moreover, the analysis of hydrolyzate after acidification by GC–MS gave acetic acid, 2-methylbutyric acid and 3-methylvaleric acid with the molar ratio being approximately 1:1:2. Therefore, the three acyls at O-2, O-3 and O-4 in STE II were two 3-methylvaleryls and one 2-methylbutyryl (Fig. 1c) and three arrangements of these acyl groups located at the O-2, O-3 and O-4 positions of the glucose molecule are possible. Thus three isomers of STE II may exist, which was confirmed by silylation with BSTFA/DMF (1:1, v/v) and examination of the product by GC–MS, when three peaks were observed (data not shown). Therefore the structures can be assigned as 6-*O*-acetyl (2,3 or 2,4 or 3,4)-di-*O*-3-methylvaleryl-(4 or 3 or 2)-*O*-2-methylbutyryl-α-D-glucopyranosyl-β-D-fructofuranoside.

In Table 1 (STE I), the data of the acyl region also indicated the presence of 3-methylvaleryls, 2-methyl butyryl and one acetyl in STE I by comparison with the data from STE II. The complete assignments of carbons and protons in the acyls of STE I were difficult due to some overlap of the resonance signals in ^1H NMR, ^{13}C NMR and HSQC spectra. This problem would be resolved with the assistance of the alkaline hydrolysis experiment. The analysis of hydrolyzate of STE I after acidification by GC–MS gave acetic acid, isobutyric acid, 2-methylbutyric acid, isovaleric acid and 3-methylvaleric acid with the approximate molar ratio of acetic acid/isobutyric acid/methylbutyric acid/3-methylvaleric acid = 7:1:12:8. According to the fatty acid compositions, the rest of the NMR data of STE I (Table 1) was assigned to isovaleryl and isobutyryl groups, respectively. Overall, the above results suggested that the structures and combinations of the three acyls at O-2, O-3 and O-4 positions of glucose in STE I were as shown in Figure 1d. According to the three different structural arrangements in Figure 1d and the approximate molar ratio of the fatty acids mentioned above, it is deduced that STE I may consist of seven isomers, and the structures are 6-*O*-acetyl (2 or 3 or 4)-*O*-3-methylvaleryl-(3,4 or 2,4 or 2,3)-di-*O*-2-methylbuty-

Table 1
^{13}C NMR and ^1H NMR date (δ ppm) of the acyl region of STE I-STE III

No*	STE I			STE II			STE III		
	δ_C	δ_H		δ_C	δ_H		δ_C	δ_H	
1	41.1	2.09-2.35 (m)		41.1	2.07-2.37(4H,m)		41.1	2.07-2.37 (6H,m)	
2	31.4	1.85 (m)		31.4	1.85 (2H,m)		31.5	1.85 (3H,m)	
3	29.2	1.16-1.30 (m)	3-methylvaleryl	29.2	1.10-1.32(4H,m)	3-methylvaleryl	29.2	1.06-1.32 (6H,m)	3-methylvaleryl
4	11.2	0.84-0.94 (m)		11.2	0.84-0.95(6H,m)		11.2	0.87-0.94 (9H,m)	
5	19.2	0.84-0.94 (m)		19.2	0.84-0.95 (6H,m)		19.2	0.87-0.94 (9H,m)	
6	42.8	2.09-2.35 (m)		42.8	2.07-2.37 (1H,m)		20.7		
7	26.2	1.65-2.13 (m)	2-methylbutyryl	26.2	1.65-2.13 (2H,m)	2-methylbutyryl			
8	11.0	0.84-0.94 (m)		11.0	0.84-0.95 (3H,m)				
9	16.3	1.06-1.16 (m)		16.3	1.06-1.10 (3H,m)				
10	43.0	2.09-2.35(m)		20.7					
11	25.6	1.65-2.13 (m)	isovaleryl						
12	22.4	0.84-0.94 (m)							
13	34.0	1.85 (m)	isobutyryl						
14	18.8	1.06-1.16 (m)							
4′	20.7	2.11 (3H,s)	— acetyl		2.11 (3H,s)	— acetyl		2.11 (3H,s)	— acetyl

*The serial numbers of carbons and attached protons are those for the 3-methylvaleryl (1–5), 2-methylbutyryl (6–9), isovaleryl (10–12), isobutyryl (13 and 14) and acetyl (4′) groups as shown in Fig. 1.

ryl-α-D-glucopyranosyl-β-D-fructofuranoside, 6-*O*-acetyl (2 or 3 or 4)-*O*-3-methylvaleryl-(3,4 or 2,4 or 2,3)-di-*O*-isovaleryl-α-D-glucopyranosyl-β-D-fructofuranoside and 6-*O*-acetyl (2,3 or 2,4 or 3,4)-di-*O*-3-methylvaleryl-(4 or 3 or 2)-*O*-isobutyryl-α-D-glucopyranosyl-β-D-fructofuranoside (one of the 3 isomers). The molar ratio of acetyl, isobutyryl, methyl butyryl and 3-methylvaleryl in these seven isomers is 7:1:12:8, which is coincident with the result of hydrolysis experiment.

4. Conclusions

In summary, three groups of STE isomers with the molecular weight 650, 664 and 678 have been isolated and purified from the oriental tobacco-*Komotini Basma* with a purity of 99.6%, 99.2% and 99.8%, respectively, using a semi-preparative RP-HPLC method for the first time. The structures of all isomers in the three groups of STEs were identified by MS (HRMS, MS^2) and NMR (^1H, ^{13}C, HSQC) spectra along with alkaline hydrolysis and STE derivation experiments. As a result, the structures of the three isomers in STE II were identified conclusively. The structures of the seven isomers in STE I were elucidated. The provision of detailed NMR and mass spectrometry data will be useful for the future identification of other STE compounds and, additionally, using our isolation method, the groups of STE isomers can be isolated from tobaccos or other plants on a large scale and may be applied for the bioactivity studies.

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