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Isolation of Tetra-acyl Sucrose Esters from Turkish Tobacco Using Supercritical Fluid CO_2 and Comparison with Conventional Solvent Extraction

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Pure supercritical CO_2 at various pressures and temperatures was used to effect the fractionation of tetra-acyl sucrose esters (SE) from dried, ground Turkish tobacco without any further pretreatment of the matrix. It was determined that SE cannot be extracted using low density CO_2 (150 atm, 60 °C, and 0.62 gm/mL or 200 atm, 100 °C, and 0.49 gm/mL), whereas other analytes, which strongly interfere with the conventional solvent extraction of SE, can be easily removed under the same conditions. At the higher temperature (100 °C), these same analytes that interfere with the conventional solvent extraction of SE are even more readily removed, while the very poor extractability of SE is not affected. It was demonstrated, however, that SE can be removed from the pre-extracted tobacco with supercritical CO_2 if the density is greater than (or equal to) 0.73 gm/mL. The supercritical fluid extraction method has been compared with other previous extraction methods that employ conventional solvents. This study provides one of the clearest examples of how the variable density property of a supercritical fluid can be utilized to effect the fractionation of a complex mixture.

INTRODUCTION

It has been established that the leaf surface chemistry of tobacco plays an important role in the plant's biology, as well as its flavor and aroma characteristics. Various groups have characterized the leaf surface chemistry of different types of tobacco. It has been established that the sucrose esters (SE) in the cuticular waxes of green tobacco are the major components of Turkish tobacco. These compounds are antimicrobial agents, and they appear to be precursors to tobacco flavor and aroma compounds (*1*, *2*). Severson et al. (*3*) used NMR to determine that the sucrose displayed a fully esterified glucose with an acetate group at the C6 position, while the fructose portion showed four free hydroxyl groups (**Figure 1**). They were able to isolate a series of SE in which each glucose hydroxyl moiety was esterified with a mixture of C_3-C_8 fatty acids. It has been determined that SE containing isobutyric, isovaleric, and β-methylvaleric acids were the three most dominant precursors of Turkish tobacco smoke flavor (*4*).

Different methods have been used to extract and isolate SE from green tobacco leaves. Severson et al. (*5*) developed a gel filtration (GF)−partition chromatography method using Sephadex LH-20 to isolate individual groups of SE. In this method, tobacco leaves were washed with CH_2Cl_2 followed by a series of evaporation and extraction steps using different solvents. The final extract in $CHCl_3$ was fractionated first on a LH-20 column with an i.d. of 2.54 cm and bed length of 58 cm. The SE gel fractions (GF #75−140) were evaporated to dryness and redissolved in 3 mL of $CHCl_3$. This fraction was separated once again on a 1.37 cm i.d. column (bed length, 110 cm). Elution with $CHCl_3$ produced another fraction (GF #45−65, 95+% SE). This fraction was then used with another GF column to isolate each SE. Severson et al. were able to isolate six groupings of ester isomers, differing by 14 amu. Later, Danehower (*6*) used a silica gel solid phase cartridge to clean the CH_2Cl_2 extract of the leaf surface. First, the extract was loaded into the solid phase extraction (SPE) cartridge and washed with CH_2Cl_2. It was demonstrated that most of the polar compounds including SE were retained on the column and were not washed out with

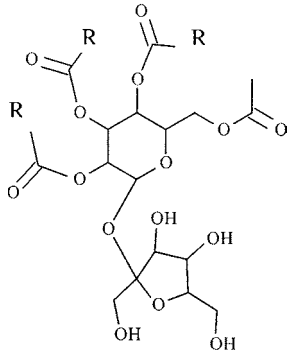

Figure 1. Structure of tetra-acyl SE (R = C_3 – C_8, n = 2–7).

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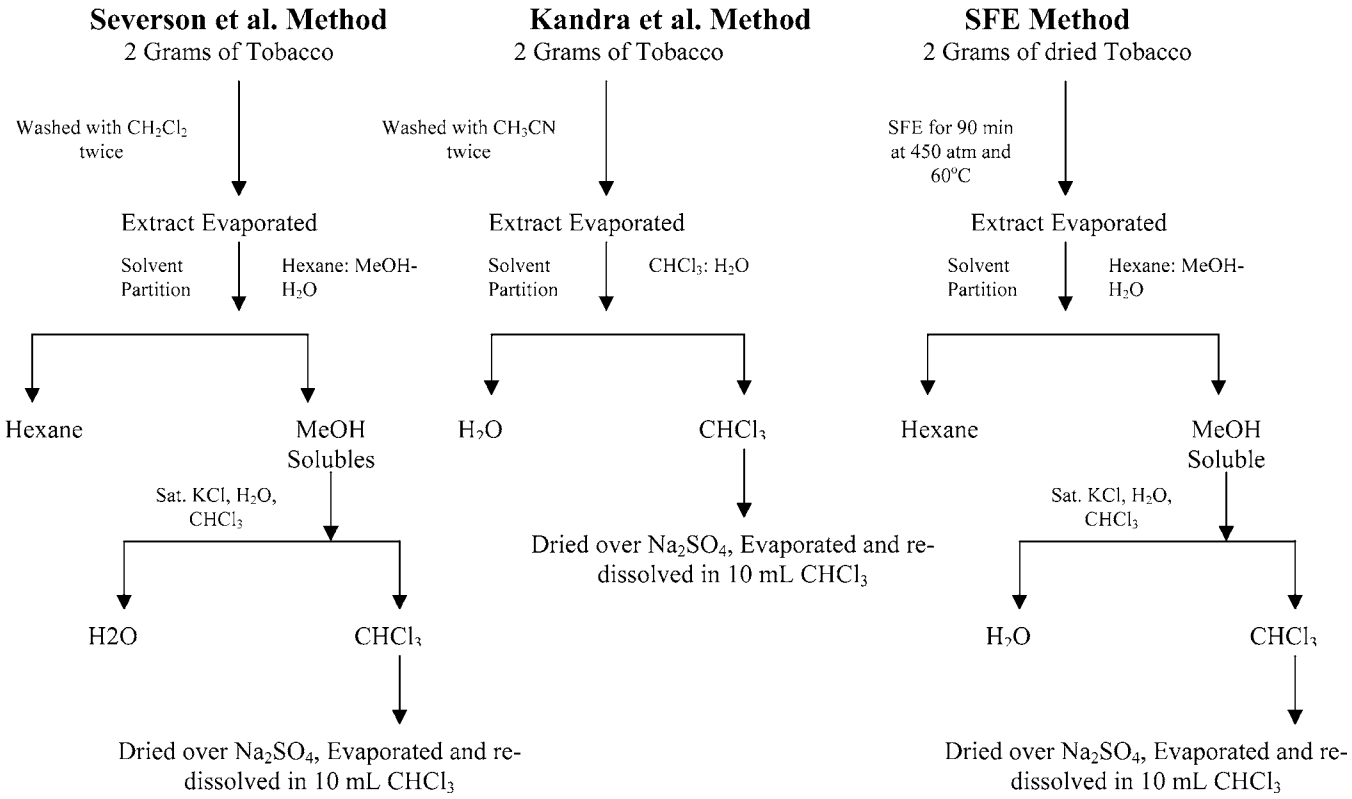

Figure 2. Schematic for isolation of SE from Turkish tobacco using various extraction methods.

CH_2Cl_2. Next, methanol was used to quantitatively remove the SE from the silica column. It was shown by gas chromatography (GC) that 50% of the total material from the leaf surface was cleaned up using this technique, while 99% of the SE was retained on the SPE column. In a later study by Kandra et al. (*7*), CH_3CN was used as an extraction solvent in order to wash the leaf surface. They did not use the more common CH_2Cl_2 solvent, to avoid extraction of cuticular hydrocarbons, which would have interfered with the SE separation. After CH_3CN extraction, the SE-enriched sample was extracted with $CHCl_3$/H_2O (2/1, v/v) to remove H_2O soluble materials. The $CHCl_3$ phase was dried and later was separated by high-performance liquid chromatography (HPLC) using a Cyano column. The workers were able to isolate four fractions from the HPLC separation with the last fraction being SE.

In the past decade, supercritical fluids have been used for extraction of different components from tobacco and tobacco byproducts. For example, methanol-modified carbon dioxide has been used to remove nicotine from tobacco (*8*). The same fluid was also used to quantitatively remove N-nitrosamines from both smokeless tobacco and cigarettes (*9*−*11*). The object of our research was first to use supercritical fluid CO_2 for both cleanup and extraction of SE from processed commercially available Turkish tobacco. Also, we wished to compare supercritical fluid extraction (SFE) with other traditional techniques, which have been used previously for extraction of SE from green tobacco matrices.

EXPERIMENTAL PROCEDURES

All air-dried ground Turkish tobacco samples were obtained from R. J. Reynolds Tobacco Co. (Winston-Salem, NC). The air-dried tobacco samples were ground mechanically such that the ground material passed through a 20 mesh stainless steel screen. Solvents were EM Science (Gibbstown, NJ) HPLC grade and were used as received. Dimethylformamide (DMF), sodium acetate, and sodium sulfate were obtained from Sigma-Aldrich (Milwaukee, WI). N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) (Alltech Associate, Deerfield, IL) was silylation grade.

Tobacco extracts and SE were analyzed as their trimethylsilyl ether derivatives using an Agilent 5890 gas chromatograph (Wilmington, DE) equipped with a 5972 mass selective detector. All separations were obtained on a DB-5 MS capillary column (15 m × 0.25 mm i.d., and 0.25 μm d_f). All SFEs were performed on a Isco-Suprex AP-44 extractor (Lincoln, NE) using 5 mL extraction vessels. All HPLC separations were conducted using an Agilent series 1050 HPLC equipped with a multiwavelength UV detector and 3396 integrator. All HPLC separations were obtained using analytical or semipreparative Supelco (Bellefonte, PA) CN column (250 mm × 4.6 mm and 250 mm × 10 mm, 5 μm d_p).

Extraction Procedures. Different extraction procedures were used to extract and isolate SE from dried tobacco. In the first extraction method, Severson's cleanup procedure was used minus the GF cleanup procedure (**Figure 2**). For each extraction, 2 g of tobacco was transferred into a 100 mL bottle fitted with a Teflon-coated cap. Then, 20 mL of CH_2Cl_2 was added to the bottle and the sample was manually shaken for 3−5 min. The solution was filtered using a type 1 filter paper (Whatman Co., Maidstone, United Kingdom). Next, the residual tobacco and filter paper were transferred into the bottle where tobacco was re-extracted using an additional 20 mL of fresh CH_2Cl_2. Next, the combined CH_2Cl_2 extracts were evaporated to dryness using a nitrogen stream. The residue was then partitioned between 20 mL each of hexane and 80/20% MeOH−H_2O (**Figure 2**). The MeOH−H_2O solution was re-extracted a second time using an additional 20 mL of hexane. Next, 10 mL of saturated KCl solution was added to the MeOH−H_2O solution followed by 15 mL of $CHCl_3$. The $CHCl_3$ solubles were removed, and the aqueous phase was extracted with another 15 mL of $CHCl_3$. The combined $CHCl_3$ fractions were then washed with H_2O and filtered through a bed of anhydrous Na_2SO_4. After the solvent was removed, the extract was quantitatively redissolved in 10 mL of $CHCl_3$.

In the second extraction method (Kandra et al.), 2 g of tobacco was transferred into a 100 mL bottle equipped with a Teflon-coated cap (**Figure 2**). Then, 20 mL of CH_3CN was added to the bottle and the sample was manually shaken for 3−5 min. The solution was filtered using a type 1 filter paper. Next, tobacco and filter paper were transferred into the 100 mL bottle and the tobacco was re-extracted

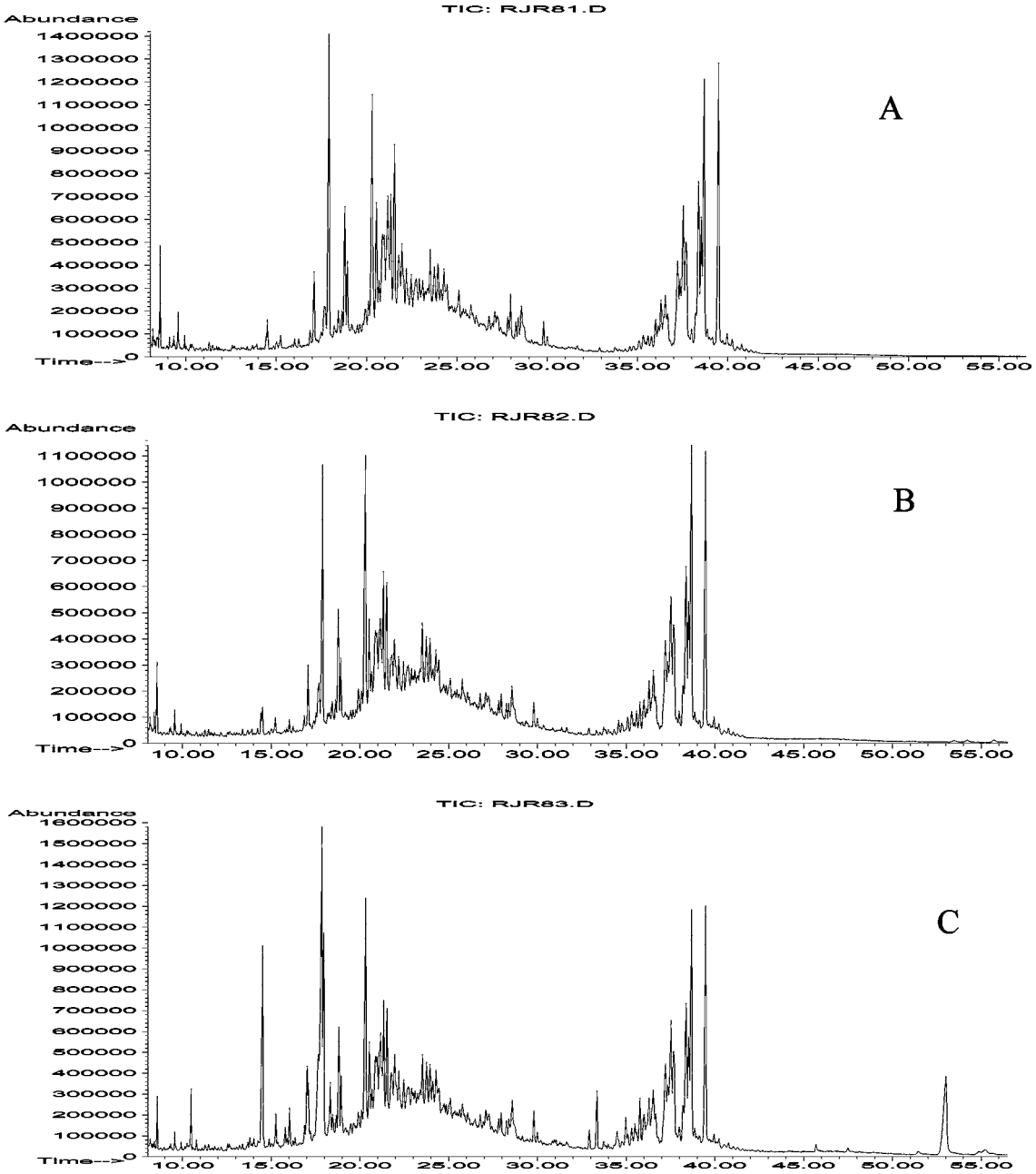

Figure 3. GC/MS separation of SE extract from Turkish tobacco using different extraction methods. (**A**) SFE. Refer to **Figure 2** and text for details of extraction conditions. (**B**) Severson et al. extraction and (**C**) Kandra et al. extraction.

using an additional 20 mL of fresh CH_3CN. Next, the combined CH_3CN extracts were evaporated to dryness using a nitrogen stream. The residue was partitioned between 20 mL of $CHCl_3$ and 10 mL of H_2O (**Figure 2**) in order to remove H_2O soluble materials. The $CHCl_3$ phase was dried with anhydrous Na_2SO_4 and evaporated to dryness using a stream of N_2. The extract residue was then quantitatively redissolved in 10 mL of $CHCl_3$.

In the third extraction method, a 5 mL extraction vessel was filled with 2 g of tobacco (**Figure 2**). Next, samples were extracted using supercritical CO_2 at different pressures and temperatures. Extracts were collected in a trap packed with stainless steel balls. After completion of each extraction step, the trap was rinsed with 5 mL of 50/50% $MeOH/CH_2Cl_2$ into a 12 mL vial. The trap rinse solvent was evaporated using a nitrogen gas stream. The resulting residue was next partitioned between 20 mL each of hexane and 80/20% MeOH−H_2O (**Figure 2**) and cleaned like the Severson et al. method. Surprisingly, all extraction methods provided a similar extraction recovery of SE from the tobacco. It is important to note here that the Kandra and Severson methods extracted much more polar analytes than supercritical fluid CO_2, which no doubt accounted for the fact that the color of the solvent extracts was darker than the supercritical fluid extracts even after application of the cleanup procedure.

Table 1. Recovery of SE from Turkish Tobacco as a Function of Molecular Mass Using Various Extraction Methods

		concn (μg/g)		
molecular mass	extracted ion from GC/MS	Severson method	Kandra method	SFE method
622	443	13.8	11.5	16.4
636	457	57.4	62.0	58.9
650	471	152.9	163.6	162.9
664	485	184.4	192.4	192.9
678	499	77.8	80.5	83.9
total concn		472.5	498.5	498.5

GC/MS Analysis. After completion of each extraction method, 1.0 mL of $CHCl_3$ solution from each extract was quantitatively transferred

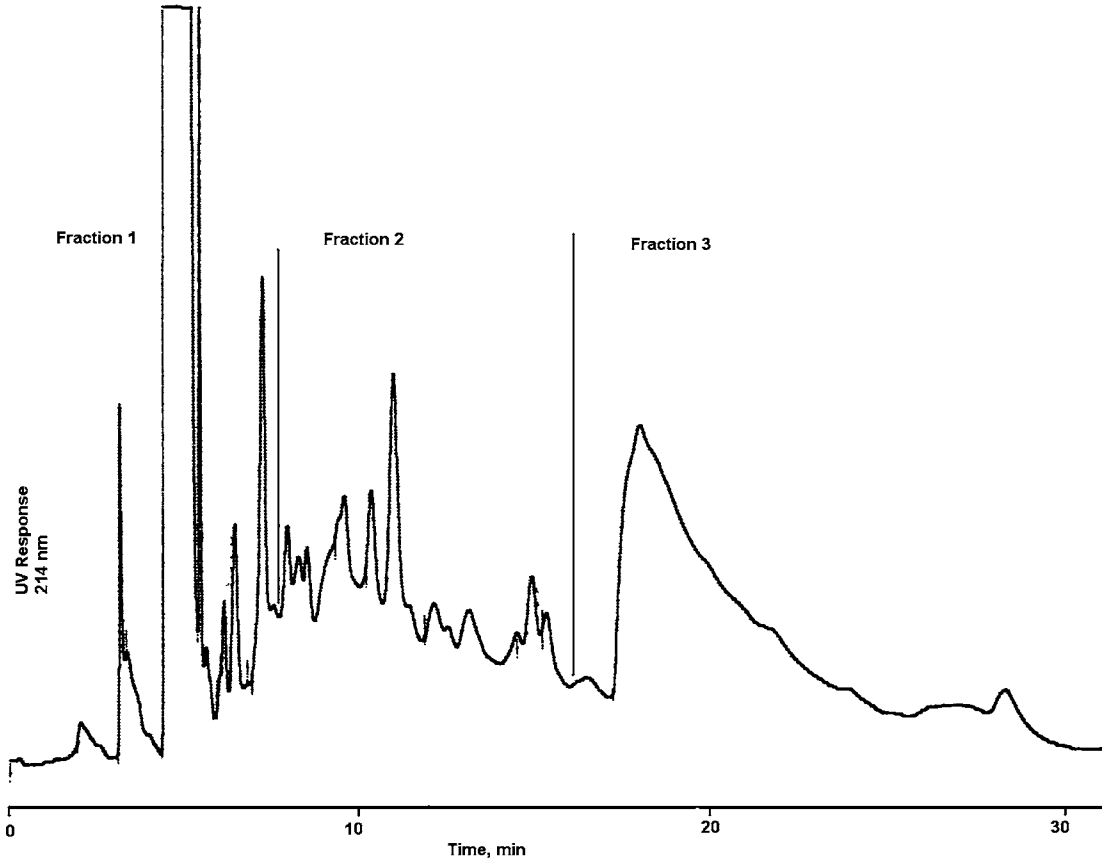

Figure 4. Normal phase HPLC/UV separation of tobacco extract using the Severson et al. extraction technique. HPLC conditions: 85/15% isooctane/ethanol; 0.8 mL/min; cyano column (250 mm × 4.6 mm, 5 μm dp); detection, 214 nm; and injection volume, 40 μL.

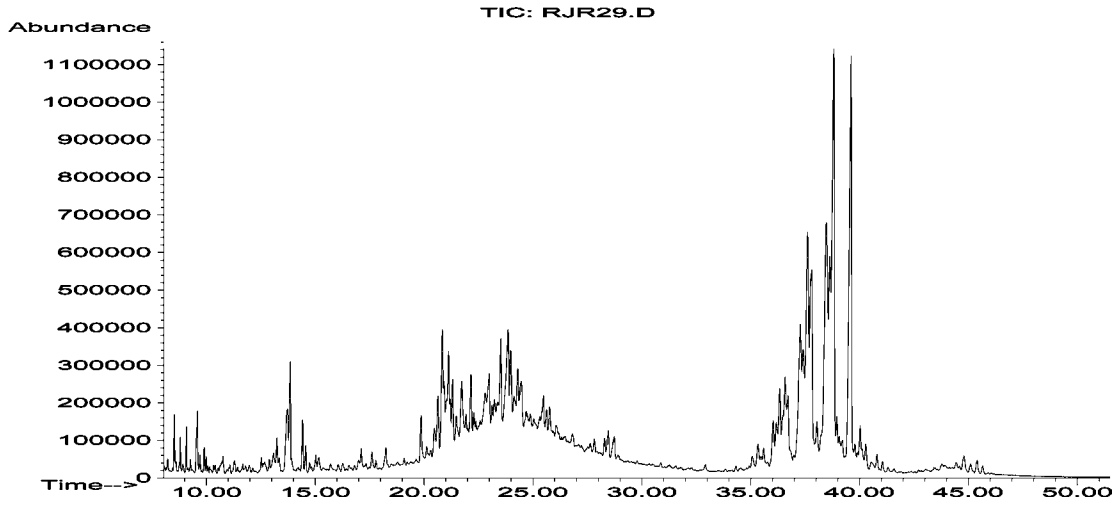

Figure 5. GC/MS profile of derivatized SE (Severson extract) isolated via semipreparative HPLC/UV prior to derivatization.

into a GC vial for derivatization. The solvent was evaporated to dryness using N_2 at room temperature. A 500 μL portion of 1:1 BSTFA−DMF was added to each vial for the purpose of forming silyl ethers of the four hydroxyl groups on each SE molecule (**Figure 1**). Each vial was purged with N_2 and capped with a Teflon-lined cap and heated at 70 °C for 30 min. After it was cooled, the sample was placed inside the 7673 Agilent autosampler for GC analysis. All GC runs were obtained with a DB-5 MS capillary column (15 m × 0.25 mm i.d.) as described earlier using the following temperature program: initial temperature 80 °C, hold for 2 min, ramp to 140 °C at a rate of 10 °C/min and then ramp to 290 °C at a rate of 4 °C/min, hold at 290 °C for 10 min.

HPLC Analysis. To obtain pure fractions of SE, HPLC was applied to each of the extracts employing a cyano-bonded phase as described earlier. Separation was achieved via isocratic elution using isooctane: ethanol (85/15%) at room temperature flowing at 0.8 mL/min. UV detection was set at 214 nm.

RESULTS AND DISCUSSION

In the first part of this research, three methods of extraction were compared (e.g., liquid−solid extraction (LSE) with CH_2Cl_2, LSE with CH_3CN, and nonoptimized SFE with CO_2).

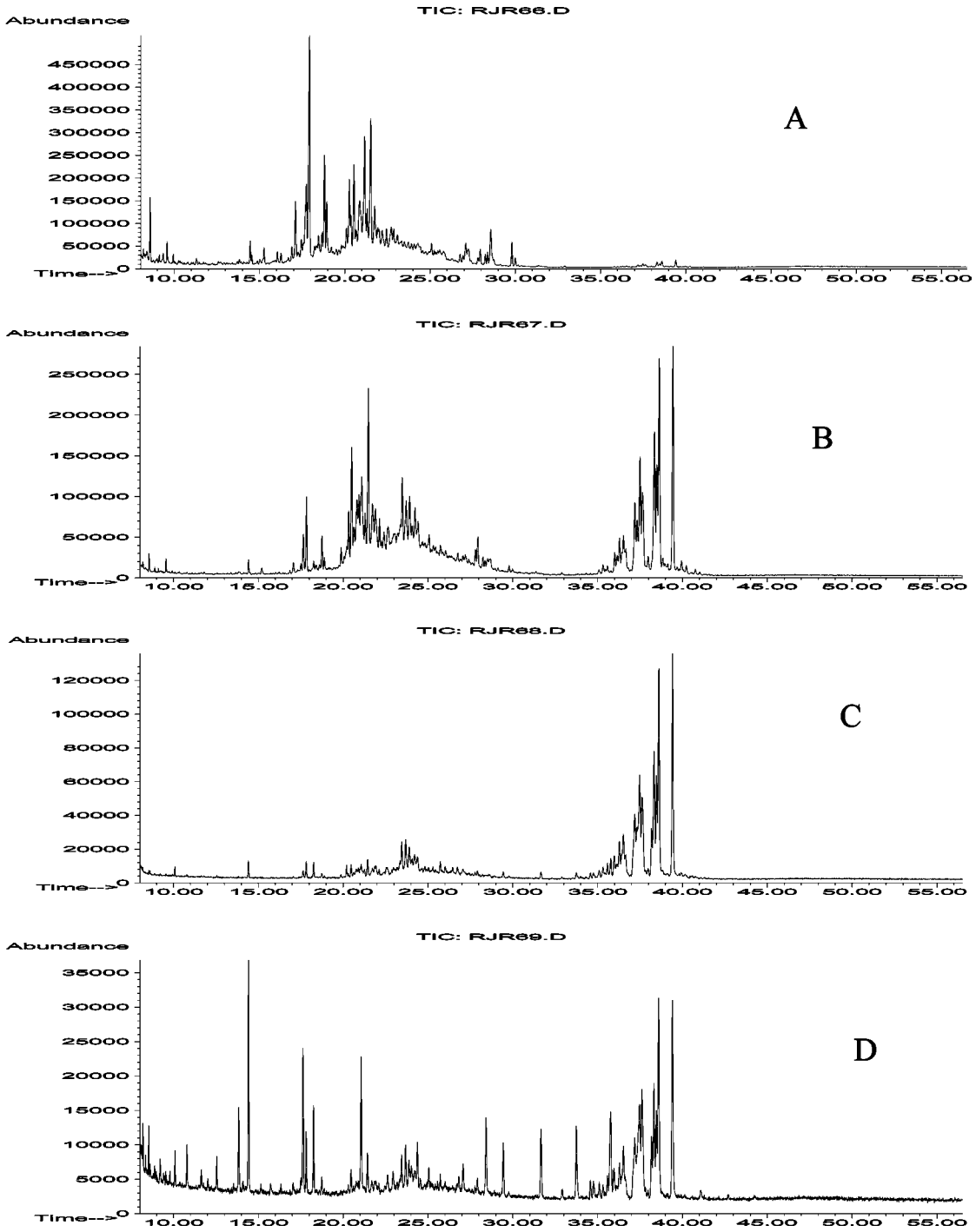

Figure 6. GC/MS profile of isolated SE (e.g., derivatized) originating from fractionation of Turkish tobacco via SFE at different pressures (**A** = 150, **B** = 200, **C** = 350, and **D** = 450 atm) and 60 °C using 2 mL/min of liquid CO_2. Each fraction was extracted for 75 min.

Figure 3 shows the GC/MS of each extract after derivatization of the four hydroxyl groups per SE using 1:1 BSTFA:DMF. As can be observed, all three extraction methods provided similar chromatograms, which suggested that qualitatively the same types of compounds were extracted. However, it is important to note that the SF extract exhibited a much lighter color than the other two extracts. An estimation of the recovery of SE via each technique was undertaken. For this purpose, an internal standard (50 μL of pyrene at a concentration of 100 ng/μL) was added to each extract solution (1 mL) before derivatization. To more accurately compare extraction recovery of SE using each extraction method, five ions from derivatized SE were spectrometrically extracted (443, 457, 471, 485, and 499 amu). These ions were fragments of the various acylated SE tetramethylsilyl ethers. **Table 1** shows the total concentration of each ion from single measurement. All extraction methods provided qualitatively a similar extraction recovery of SE from the tobacco. Despite a qualitatively similar recovery, it is important to note here that Kandra's and Severson's extraction methods apparently removed much more of the polar analytes than supercritical CO_2, which caused the color of these liquid extracts to be much darker after extraction and clean up than supercritical extracts.

A previously developed HPLC method (*6*) was applied to each of the three extracts in order to obtain practically pure fractions of tetra-acyl SE. A total of three fractions of each

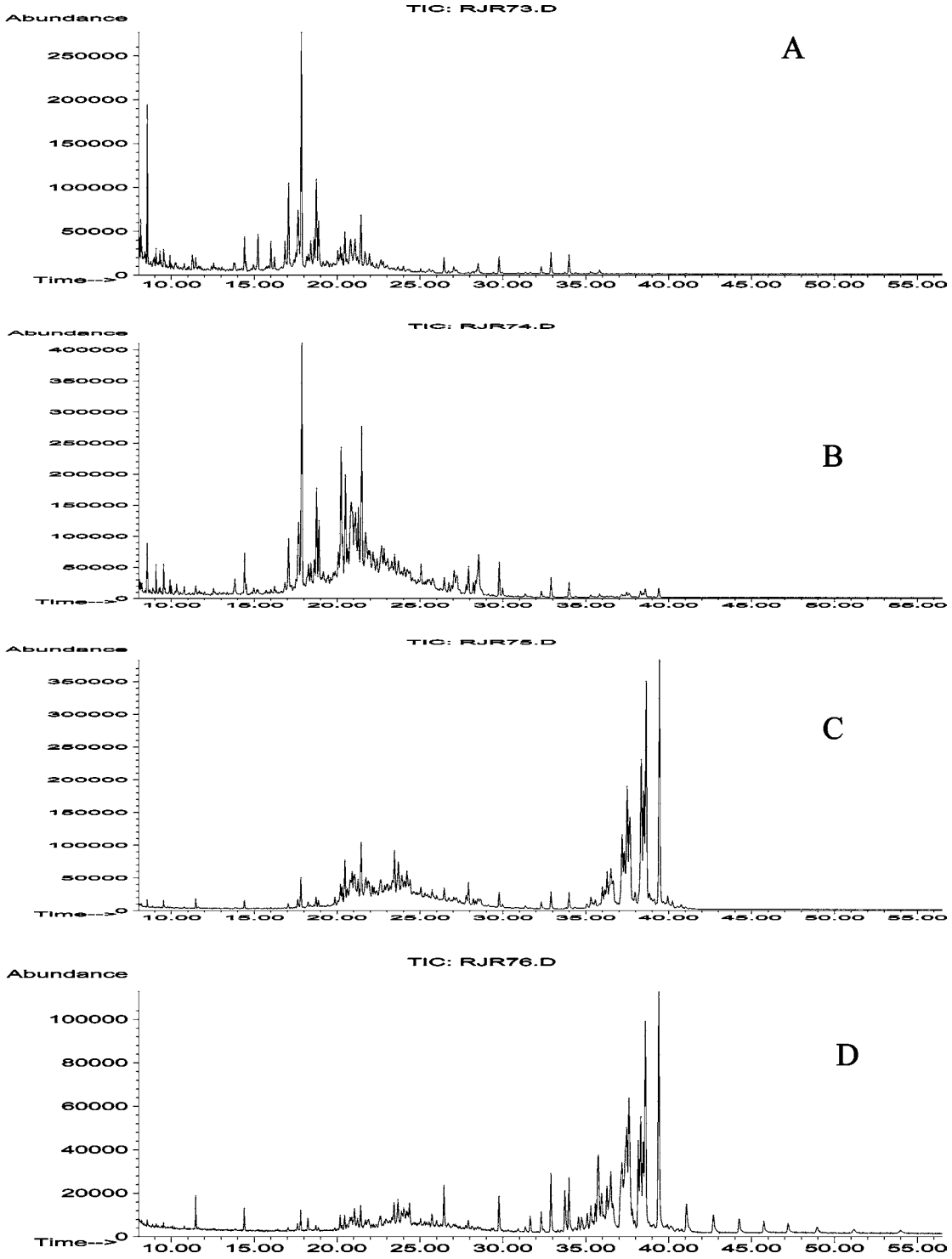

Figure 7. GC/MS profile of isolated SE (e.g., derivatized) originating from fractionation of Turkish tobacco via SFE at different pressures (**A** = 150, **B** = 200, **C** = 350, and **D** = 450 atm) and 100 °C using 2 mL/min of liquid CO_2. Each fraction was extracted for 75 min.

extract were collected in the semipreparative HPLC mode. **Figure 4** shows the HPLC/UV trace of the Severson extract with detection at 214 nm, for example. The time of collection for the first fraction was from 3.5 to 8.1 min, the second fraction was from 8.1 to 17.5 min, and the third fraction (which was believed to contain the SE) was from 17.5 to 30.0 min. Later, GC/MS analysis of each derivatized fraction revealed that (as predicted) only the third fraction contained primarily SE. Unfortunately, GC/MS of the third fraction of each extract showed that some impurities (with retention times from 10 to 30 min) still were observed. Thus, Severson et al. have demonstrated the best isolation of tobacco-derived SE until now, although their procedure is relatively long and tedious and prone to yield impure fractions as we have shown.

Fractionation of SE. Next, we attempted to isolate SE from a Turkish tobacco via selective, optimized supercritical fluid fractionation with no subsequent semipreparative fractionation HPLC step. At the outset, we felt that these tobacco-derived SEs would not be extractable with 100% CO_2 because that are not fully esterified. Octa-acylated SE are known to be CO_2 soluble, but SE from tobacco are known to be only tetra-acylated (*12*). To show the feasibility of this process, 2 g of tobacco

was extracted at different CO_2 pressures and temperatures (**Figure 5**). In this part of the study, the extraction time for each fraction was 75 min using 2 mL/min of liquid CO_2. First, the tobacco sample was extracted at four pressures (150, 200, 350, and 450 atm) and two temperatures. It was decided to not use any modifier since all SE were extractable with pure CO_2. **Figure 6** shows the GC/MS traces of the various derivatized fractions at 60 °C. As can be observed at 150 atm (**Figure 6A**) by the absence of peaks between 35 and 40 min, no SE was extracted. When the CO_2 pressure was systematically increased, however, from 150 to 200 and 350 atm (**Figure 6B**,**C**), a mixture of SE began to appear in the extract, retention time 35−40 min. At 450 atm after continuous extraction of the same sample at the three lower pressures, a relatively small amount of SE was observed in this extract (note the much smaller axis in **Figure 6D** relative to the same axis in **Figure 6A**−**C**) and an even lower amount of coextractives (e.g., retention time 10−35 min) was observed.

Similar extractions were performed on a fresh sample with the extraction temperature increased from 60 to 100 °C. In this series of extractions (as can be observed via GC/MS chromatograms of each derivatized, extracted fraction), no SE was removed at either 150 or 200 atm (**Figure 7A**,**B**). However, at 350 atm and 100 °C, a relatively large amount of SE was extracted (**Figure 7C**) with a minimal yield of coextractives (e.g., retention time 18−28 min). Increasing the pressure from 350 to 450 atm at 100 °C increased the solvating power of the fluid, which caused the extraction of additional SE (**Figure 7D**) with even less interference from coextractives. Integration of the SE peak areas suggested that the majority of the SEs are removed at 350 atm with pure CO_2 if the temperature is 100 °C.

From this study, one can conclude that SE extraction efficiency cannot be affected by temperature since SE are not volatile at these operating temperatures. It has been reported previously (*13*) for environmental samples such as polynuclear aromatic hydrocarbons (PAHs) that an increase in temperature actually enhances extractability even though the increase in temperature is accompanied by a decrease in CO_2 extraction density. This would suggest that the extraction of these PAHs is more kinetically driven than thermodynamically driven. This situation obviously does not prevail with SE in a tobacco matrix because higher solvating power (e.g., greater CO_2 pressure) is required to extract the underivatized SE from tobacco at a higher temperature. For our purposes, this situation worked to our advantage in that the other tobacco coextractives were more extractable at the higher temperature (e.g., greater diffusivity lower pressure). In other words, raising the temperature enhanced the fractionation process because the extraction of SE was driven by CO_2 solvating power, whereas most of the other extractable components were removed by a kinetically driven process. Thus, SE have been isolated in our laboratory for the first time via supercritical CO_2 from other components in the tobacco by simply varying the CO_2 pressure keeping constant temperature. Such great selectivity with a supercritical fluid is not readily achieved with a combination of conventional LSE of dried tobacco followed by semipreparative liquid chromatography.

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