



A comprehensive methodology for the chiral separation of 40 tobacco alkaloids and their carcinogenic E/Z-(R,S)-tobacco-specific nitrosamine metabolites

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ARTICLE INFO

Keywords:

Nicotine
Tobacco
Nitrosamine carcinogens
Isomeric alkaloids
Electrospray ionization liquid chromatography-mass spectrometry
Enantiomer separation

ABSTRACT

The predominant enantiomer of nicotine found in nature is (S)-nicotine and its pharmacology has been widely established. However, pharmacologic information concerning individual enantiomers of nicotine-related compounds is limited. Recently, a modified macrocyclic glycopeptide chiral selector was found to be highly stereoselective for most tobacco alkaloids and metabolites. This study examines the semi-synthetic and native known macrocyclic glycopeptides for chiral recognition, separation, and characterization of the largest group of nicotine-related compounds ever reported (tobacco alkaloids, nicotine metabolites and derivatives, and tobacco-specific nitrosamines). The enantioseparation of nicotine is accomplished in less than 20 s for example. All liquid chromatography separations are mass spectrometry compatible for the tobacco alkaloids, as well as their metabolites. Ring-closed, cyclized structures were identified and separated from their ring-open, straight chain equilibrium structures. Also, E/Z-tobacco-specific nitrosamines and their enantiomers were directly separated. E/Z isomers also are known to have different physical and chemical properties and biological activities. This study provides optimal separation conditions for the analysis of nicotine-related isomers, which in the past have been reported to be ineffectively separated which can result in inaccurate results. The methodology of this study could be applied to cancer studies, and lead to more information about the role of these isomers in other diseases and as treatment for diseases.

1. Introduction

Tobacco smoke has been reported to contain at least 60 carcinogens and several have been directly related to cancer [1]. Tobacco and its derived products constitute a leading preventable cause of death in the United States (US) [2]. The Food and Drug Administration (FDA) regulates all commercial tobacco products via the Family Smoking Prevention and Tobacco Control Act and the extension, the Deeming Rule [3,4]. Recently, the FDA also announced a comprehensive plan for lowering the nicotine (NIC) content in cigarettes to make them less or non-addictive [5]. To facilitate dependence, the reduced amount has been estimated to be 0.05 mg NIC compared to the current range of 0.5–1.5 mg NIC yield in one cigarette [1,6]. One challenge might be that smokers turn to other tobacco products for the higher NIC content compared to reduced NIC content cigarettes, such as smokeless tobacco products, which are connected to oral and esophageal cancers [7]. Smokeless tobacco products, like moist snuff, have been determined to

contain tobacco-specific nitrosamines (TSNAs), which have been shown to be responsible for oral cavity cancer from smokeless tobacco [7]. The most prevalent and toxic TSNAs have been reported as *N*-nitrosonornicotine (NNN) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) [1]. The other main TSNAs, *N*-nitrosoanatabine (NAT) and *N*-nitrosoanabasine (NAB), haven't shown as potent carcinogenicity in laboratory animals [7,8]. In one study, 12 rats were treated with racemic NNN and 96 oral cavity tumors and 153 esophageal tumors were observed [8]. Also, the (S)-NNN enantiomer was determined to be more tumorigenic than (R)-NNN indicating that the stereochemistry of this compound is highly important [8].

In 2017, the FDA proposed, "The mean level of *N*-nitrosonornicotine in any batch of finished smokeless tobacco product not exceed 1 microgram per gram (μg/g) of tobacco (on a dry weight basis) at any time through the product's labelled expiration date as determined by specified product testing." [9]. Current commercial US smokeless tobacco products contain NNN levels ranging from 1 to

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10 µg/g dry weight [10]. NNN is formed by the nitrosation of NIC and nor nicotine (NNIC), which is a tobacco alkaloid native to tobacco, as well as a nicotine metabolite [7]. The level of NNIC is dependent on the leaf senescence and curing process [7,11]. Tobacco strains with less (S)-NNIC have been reported to contain less (S)-NNN [11]. Therefore, genetic engineering efforts have been focused on reducing the inherent amount of NNIC [11]. Also, NNN can be formed endogenously, which was shown when NNN was found in saliva after using NIC replacement therapies [12]. Furthermore, NNN metabolizes to another TSNA, *N*-nitrosonor nicotine-1-*N*-oxide (NNNO), which has been shown to be less carcinogenic than NNN in F344 rats and Syrian golden hamsters [13].

The other major carcinogen found in unburnt tobacco and tobacco smoke is NNK, an achiral TSNA, which is formed from NIC during the curing and processing of tobacco [7]. NNK was found to be the only potent lung carcinogen that formed tumors in rats, mice, and hamsters [14]. Metabolites of NNK and other TSNA are known to bind to DNA once activated, forming adducts that can cause oncogene activation leading to tumor development if they persist [7]. Long-term exposure to these mutation events can lead to cancer and death [7]. NNK is known to metabolize mainly to 4-(methyl nitrosamino)-1-(3-pyridyl)-1-butanol, NNAL, and its glucuronides [15]. Since NNK and NNAL are only found in tobacco and not from any other source, they can be used as highly specific biomarkers of carcinogen exposure, especially second-hand smoke exposure [16]. Also, the ratio of NNAL-glucuronide to NNAL has been used as a biomarker of susceptibility to lung cancer [16].

NNAL has been reported to have similar toxicity as NNK, with a higher tumorigenicity of the (R)-NNAL enantiomer than (S)-NNAL, due to preferential metabolic activation [17]. NNK, NNAL, and NNN were reported to form E/Z isomers [18–20]. The relative level of E isomers was higher than Z isomers [18–20]. Some previous reports have shown the separation of a few TSNA, but most do not report the separation of both their enantiomers and E/Z isomers [20–23]. Thus, some researchers have expressed confusion because the tops of their TSNA chromatographic peaks show splitting [24]. However, TSNA are known to interconvert between E/Z isomers [18–20]. Chiral capillary electrophoresis has been used to separate E/Z-NNK and (R,S)-(E/Z)-NNAL [20]. Also, achiral nitrosamines, other than TSNA, have been separated by LC into their E/Z isomers. For example, fish toxicants like 6',7'-acetylenic nitrosamines were efficiently resolved with an achiral LC method [25]. Using a similar LC method, but with the addition of chiral derivatizing agents, the indirect separation of (R)-(E/Z) and (S)-(E/Z)-TSNA isomers was performed [18,19]. The approach described in this work provides a direct and efficient separation of both E/Z isomers and their enantiomers as well as indicating if isomeric interconversions occur under “ordinary” conditions. In jaundice phototherapy, toxic, unconjugated bilirubin is isomerized to several E/Z configurations [26]. This isomerization makes bilirubin become more soluble in plasma so it can be excreted by the liver [26]. Therefore, E/Z isomers have different physical and biological properties and should be further studied with TSNA.

TSNA are nitrosated metabolites of chiral tobacco alkaloids, which have similar structures as NIC [7]. NIC is predominantly found as the (S)-(-) enantiomer in tobacco plants [27]. The percent (R)-(+)-NIC in tobacco, and medicinal products derived from tobacco was reported to be in the 0.1–1.2% range [27]. The pharmacology of (R)-(+)-NIC has not been an area of great concern, most likely because human exposure and intake of (R)-(+)-NIC is minimal. However, the individual enantiomers have been examined for their use as therapies for neurodegenerative diseases. These studies have reported that NIC enantiomers have different pharmacological effects, such as oxidative stress, weight loss, and binding mechanisms [28–30]. (R)-NIC has been reported as eighty times less cytotoxic than (S)-NIC, when considering their metabolites [30]. A recent study determined new smoking products (e-liquids), which have synthetic NIC (tobacco-free nicotine, TFN), contained 50% of (R)-(+)-NIC [31]. Since new products contain higher

(R)-NIC levels than in tobacco-derived products, it was suggested that the pharmacology of (R)-NIC should be more extensively studied [31]. The binding affinity of (R)-NIC to nicotine acetylcholine receptors was estimated to be 10 times lower than (S)-NIC, which might result with a less stimulating dopaminergic response [30]. New TFN products with higher (R)-NIC might be analogous to commercial products with less addictive NIC levels.

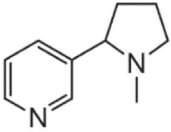
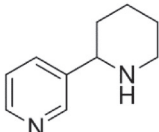
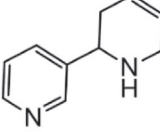
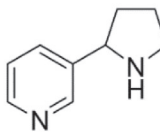
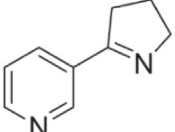
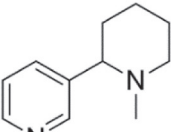
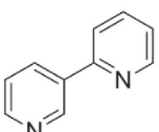
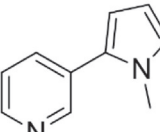
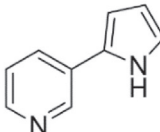
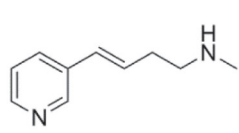
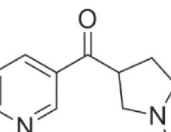
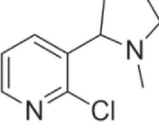
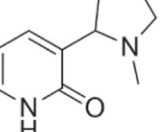
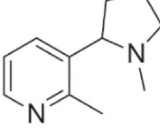
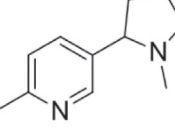
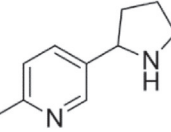
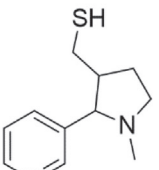
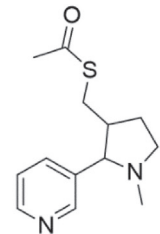
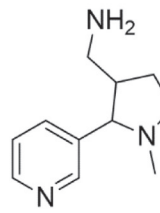
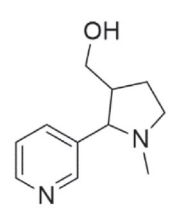
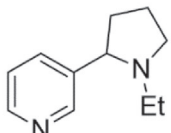
While (S)-NIC is the main alkaloid in tobacco products, minor chiral alkaloids also are present including NNIC, anatabine (AT), and anabasine (AB) [32]. The R-enantiomers of minor tobacco alkaloids have been reported to be present at higher relative levels than (R)-(+)-NIC [32]. Most biomarker strategies utilize tobacco alkaloids or their metabolites, such as the major chiral metabolite, cotinine (COT). COT is used to measure NIC uptake, due to its long half-life, such as in smoking cessation trials and tobacco exposure tests [33]. However, tobacco alkaloids are useful to differentiate the use of tobacco while using NIC replacement therapies [34]. Also, chiral alkaloids have been reported to be useful as therapies for neurodegenerative diseases by mimicking NIC's neuropharmacological and neuroprotective effects [30,35]. Enantiomers are well known to have different pharmacological effects, e.g. (R)-AB was reported to be more toxic and cause more birth defects than (S)-AB [36]. So, if these alkaloids were developed into medicinal products, the FDA would require, in their words, “the pharmacology and toxicology of the enantiomer should be characterized for the principal effects and any other pharmacological effect, with respect to potency, specificity, maximum effect, etc.” [37]. However, most analytical methods do not have the capability to analyze the individual enantiomers of these alkaloids and metabolites, so new more effective methods are needed. To quantitate and perform biological studies, it would be useful if such “chiral methods” were compatible with mass spectrometry (MS).

Some separation approaches for chiral nicotine-related compounds, more importantly the carcinogenic compounds, have been reported, but most have disadvantages that limit the analysis. Most analyses are similar to those of achiral nitrosamine analysis and do not have the capability of separating enantiomers, such as a study which determined the amount of TSNA in replacement liquids for electronic cigarettes [38]. One chiral approach reported the separation of NIC and several alkaloids using a packed liquid chromatography (LC) microcolumn with a β -cyclodextrin mobile phase, but required three hours [39]. Other previous approaches mainly utilized chiral gas chromatography (GC) or chiral derivatization LC [18,23,32]. GC isn't best suited for the biological analysis of these compounds due to the thermal lability of the sample. Chiral derivatization LC methods increase cost and analysis times and rely on the purity of the chiral derivatization agent. The best approach for chiral separations of nicotine-related compounds is using LC chiral stationary phases (CSPs). Enantioseparations of three tobacco alkaloids using LC CSPs have been reported, but they used normal phase solvents, which are not compatible with MS [36,40]. These alkaloids might be possible targets for neurodegenerative therapies, but these methods won't be compatible for biological analysis [30].

Recently, a fast, high efficiency, mass spectrometry compatible, chiral LC approach was developed to analyze NIC in TFN commercial e-liquids [31]. Herein, we examine this approach for applicability for the sensitive identification and enantiomeric quantification of most nicotine-related compounds and metabolites in commercial tobacco products and biological samples. Focus is paid to the LC separation of carcinogenic compounds, like NNN or NNK, and other complex isomeric mixtures that have not been reported to separate previously. This study examines the effectiveness of new and known macrocyclic glycopeptide chiral selectors in resolving the most comprehensive set of chiral nicotine-related compounds yet investigated, including minor tobacco alkaloids, metabolites, synthetic related compounds, and E/Z-TSNA [31,41–44]. Further, only LC-MS compatible formats were considered.

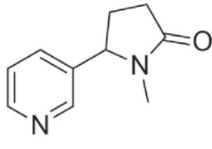
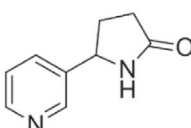
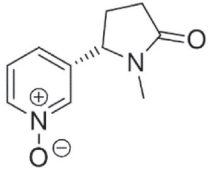
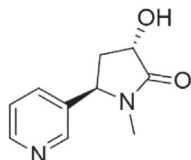
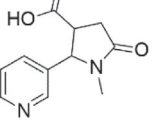
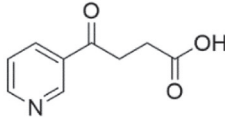
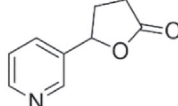
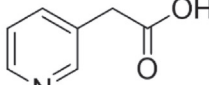
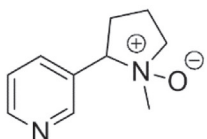
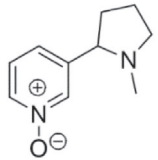
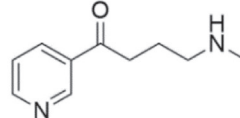
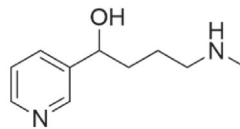
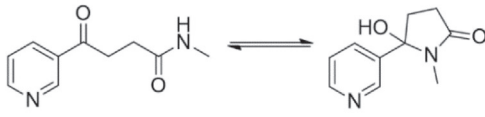
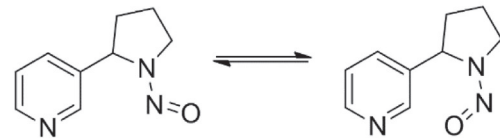
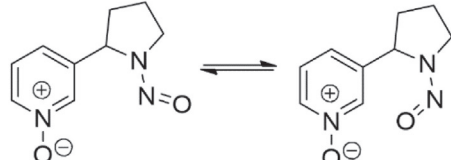
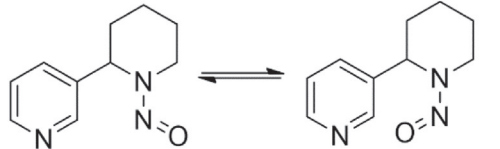
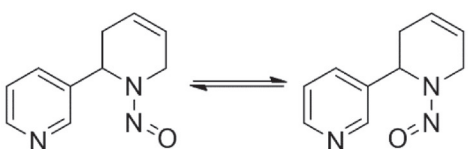
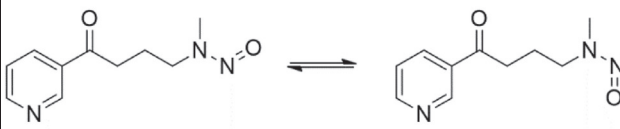
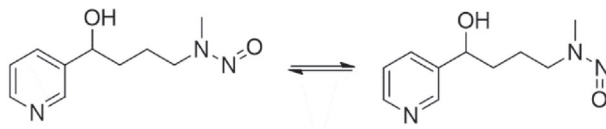
Table 1

Structures of nicotine-related compounds (all chiral compounds denoted by *).

a) Tobacco alkaloids				
Nicotine* (NIC) 	Anabasine* (AB) 	Anatabine* (AT) 	Nornicotine* (NNIC) 	Myosmine (MYS) 
N-methylanabasine* (MAB) 	2,3'-bipyridyl (BPY) 	β-nicotyrine (β -NT) 	β-nornicotyrine (β -NNT) 	Metanicotine (E) (MET) 
b) Synthetic derivatives				
1-methyl-3-nicotinoylpyrrolidine* (1M3NP) 	2-chloronicotine* (2CN) 	2-hydroxynicotine* (2HN) 	2-methylnicotine* (2MN) 	6-methylnicotine* (6MN) 
6-methylnornicotine* (6MNN) 	rac-(2S,3S & 2R,3R)-<i>trans</i>-3'-thiomethyl nicotine* (T3TMN) 	rac-(2S,3S & 2R,3R)-<i>trans</i>-3'-acetylthiomethyl nicotine* (T3ATMN) 	rac-(2S,3R & 2R,3S)-<i>trans</i>-3'-aminomethyl nicotine* (T3AMN) 	rac-(2S,3S & 2R,3R)-<i>trans</i>-3'-hydroxymethyl nicotine* (T3HMN) 
N-ethyl-nornicotine* (NENN) 				

(continued on next page)

Table 1 (continued)

c) Metabolites						
<p>Cotinine* (COT)</p> 	<p>Norcotinine* (NCOT)</p> 	<p>(5S)-Cotinine-N-oxide (CNO)</p> 	<p><i>trans</i>-(3<i>S</i>,5<i>R</i>)-3'-hydroxy-cotinine (T3HC)</p> 	<p><i>rac-trans</i>-(2<i>S</i>,3<i>R</i> & 2<i>R</i>,3<i>S</i>)-cotinine carboxylic acid* (4TCCA)</p> 		
<p>γ-oxo-3-pyridinebutyric acid (OPBA)</p> 	<p>5-(3-pyridyl) tetrahydro-2-furanone* (5THF)</p> 	<p>3-pyridylacetic acid (LAC)</p> 	<p><i>rac</i>-Nicotine-1'-N-oxide* (S,S & R,R) (NNO)</p> 	<p>Nicotine-1-oxide* (NO)</p> 		
<p>4-(methylamino)-1-(3-pyridyl)-1-butanone (NAN)</p> 	<p><i>rac</i>-4-(methylamino)-1-(3-pyridyl)-1-butanol* (NAL)</p> 		<p>N-methyl-γ-oxo-pyridinebutanamine (OPBN) to 5'-hydroxycotinine* (5HCOT)</p> 			
d) (E/Z)-Tobacco-specific nitrosamines						
<p><i>rac</i>-(E/Z)-N'-nitrosanornicotine* (NNN)</p> 		<p><i>rac</i>-(E/Z)-N'-nitrosanornicotine-1-N-oxide* (NNNO)</p> 				
<p><i>rac</i>-(E/Z)-N'-nitrosoanabasine* (NAB)</p> 		<p><i>rac</i>-(E/Z)-N'-nitrosoanatabine* (NAT)</p> 				
<p><i>rac</i>-(E/Z)-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)</p> 		<p><i>rac</i>-(E/Z)-4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol* (NNAL)</p> 				

2. Materials and methods

Native vancomycin (VancoShell, V, 100 × 4.6 mm inner diameter (i.d.)) and teicoplanin (TeicoShell, T, 100 × 4.6 mm i.d. and 150 × 4.6 mm i.d.), hydroxypropyl-β-cyclodextrin (CDShell-RSP, 100 × 4.6 mm i.d.), quinine (QShell, 100 × 4.6 mm i.d. and 150 × 4.6 mm i.d.) and modified macrocyclic glycopeptide (NicoShell, N, 100 × 4.6 mm i.d.) CSPs were bonded to superficially porous particles (SPPs), and obtained from AZYP, LLC. (Arlington, TX, USA). An Eclipse XDB-C18 (C18), 5 μm, 150 × 4.6 mm i.d. column was obtained from Agilent Technologies (Palo Alto, CA, USA). A broad set of nicotine-related compounds were selected (see Table 1 for structures drawn as manufacturer label and acronyms, * denotes chiral compounds) and all chiral compounds were obtained as racemic analytical standards from Toronto Research Chemicals (Toronto, Canada) except cotinine-N-oxide (CNO) and *trans*-3'-hydroxycotinine (T3HC). Also, individual enantiomers of AT, AB, NIC, NNIC, COT, NNN, NAT, and NAB were obtained. An achiral compound, metanictine (MET) was obtained as an E isomer. TSNAs were obtained as racemates, but these compounds are also known to exist as a mix of E/Z isomers and were not labelled accordingly. The standards were diluted with methanol to concentrations of 1 mg/mL and stored 24 h before analysis. High performance LC grade methanol (MeOH) was obtained from Sigma-Aldrich (St. Louis, MO, USA) as well as acetonitrile (ACN), acetic acid (HOAc), ethanol (EtOH), triethylamine (TEA), ammonium hydroxide (NH₄OH), ammonium formate (NH₄Formate), and ammonium trifluoroacetate (NH₄TFA). Water was purified by a Milli-Q water purification system (Millipore, Billerica, MA, USA).

A 1260 high performance LC instrument (Agilent Technologies, Palo Alto, CA, USA) was used in this study. It consisted of a 1200 diode array detector, autosampler, column oven, and quaternary pump. Also, a Shimadzu triple quadrupole LC-MS instrument, LCMS-8040, (Shimadzu, Tokyo, Japan) was used. All MS was operated in positive ion mode with an electron spray ionization source. The parameters were set as follows: nebulizer gas flow, 3 L/min; drying gas flow, 15 L/min; desolvation line temperature, 250 °C; heat block temperature, 400 °C. All separations were carried out at room temperature, unless otherwise noted, using an isocratic method. All analytes were screened, then optimized using a variety of mobile phases in the polar ionic mode (PIM), polar organic mode (POM), and reversed phase (RP) with all stationary phases. The mobile phases were degassed by ultrasonication under vacuum for 5 min. The UV wavelengths 220 and 263 nm were utilized for detection. Chiral separations were optimized (as shown in Table 2) with the following mobile phases: PIM1: 100/0.1 wt%: MeOH/NH₄TFA; PIM2: 100/0.025 wt%: MeOH/NH₄Formate; PIM3: 100/0.5 wt%: MeOH/NH₄Formate; PIM4: 100/0.2 wt%: MeOH/NH₄Formate; PIM5: 100/0.2/0.05: MeOH/HOAc/NH₄OH; POM1: 60/40/0.3/0.2: ACN/MeOH/HOAc/NH₄OH; POM2: 50/50/0.3/0.2: ACN/MeOH/HOAc/NH₄OH; POM3: 100: MeOH; RP1: 90/10: MeOH/16 mM NH₄Formate pH 3.6; RP2: 90/10: EtOH/16 mM NH₄Formate pH 3.6; RP3: 30/70: MeOH/16 mM NH₄Formate pH 3.6; RP4: 30/70: ACN/16 mM NH₄Formate pH 3.6; RP5: 10/90: ACN/16 mM NH₄Formate pH 3.6.

The dead time, t_0 , was determined by the peak of the refractive index change due to the unretained sample solvent. Retention factors (k) were calculated using $k = (t_R - t_0) / (t_0)$, where t_R is the retention time of the first peak. Selectivity (α) was calculated using $\alpha = k_2 / k_1$, where k_1 and k_2 are retention factors of the first and second peaks, respectively. Resolution (R_s) was calculated using the peak width at half peak height, $R_s = 2(t_{R2} - t_{R1}) / (w_{0.5,1} + w_{0.5,2})$. Each sample was analyzed in triplicate. The relative standard deviation (%RSD) was determined to be within ± 1.0% for the resolution of all analytes. Peak area calculations were determined by peak deconvolution according to a previous report [45]. The relative standard deviation (%RSD) was determined to be within ± 3.0% for the area ratios reported.

Table 2

Optimized enantiomeric separations of nicotine-related compounds using macrocyclic glycopeptides.

Class ^{1a}	Name ^{1b}	CSP ²	MP ³	T ⁴	F ⁵	k_1^{6a}	α^{6b}	R_s^{6c}
Tobacco alkaloids	NIC	N	PIM4	45	1.5	0.5	1.60	3.0
	AB	V	POM1	45	1.0	3.2	1.21	2.6
		N	PIM4	45	1.0	3.0	1.16	2.8
	AT	V	RP1	25	1.0	2.0	1.18	2.9
		N	PIM3	45	1.0	1.1	1.45	5.2
	NNIC	V	PIM2	45	0.7	4.1	1.10	1.5
		N	PIM3	45	1.0	2.7	1.14	2.3
	MAB	V	PIM4	25	0.5	1.3	1.38	3.2
		N	PIM5	25	1.0	1.9	1.24	2.5
Synthetic derivatives	1M3NP	N	PIM3	45	1.0	1.7	1.17	2.6
	2CN	V**	RP2	25	0.5	3.4	1.08	1.5
		N	PIM5	45	1.0	0.6	1.55	5.4
	2HN	N	PIM3	30	1.0	2.0	1.30	3.5
	2MN	V	RP1	30	0.7	2.3	1.11	1.7
		N	PIM4	45	1.5	1.1	1.17	2.2
	6MN	V**	PIM5	25	0.3	2.3	1.09	1.5
		N	PIM4	45	1.5	0.6	1.42	3.1
	6MNN	V	PIM1	25	1.0	2.3	1.20	2.7
		N	PIM4	45	2.0	3.7	1.26	3.6
	NENN	V	PIM5	25	1.0	2.3	1.19	2.5
		N	PIM4	45	2.0	0.5	1.76	4.6
	T3ATMN	V**	PIM2	25	0.5	1.3	1.06	1.5
		N	RP1	45	1.0	0.8	1.58	5.7
	T3AMN	V	RP1	45	0.5	5.0	1.15	1.5
		N	PIM4	45	0.5	4	1.21	1.5
	T3HMN	V	RP2	25	0.5	3.9	1.14	1.9
		N	PIM4	25	1.0	0.5	1.42	2.7
	T3TMN	V	RP2	25	0.5	2.7	1.15	1.7
		N	PIM4	25	1.0	0.4	1.72	3.5
Nicotine metabolites	COT	T**	POM3	25	0.5	0.7	1.12	1.5
	NCOT	T	POM3	25	1.0	0.9	2.64	9.3
	4TCCA	T*	RP3	45	0.5	0.9	1.18	2.0
	5THF	T	POM3	25	0.3	0.7	1.15	1.5
	NNO	N	POM2	25	0.7	1.4	1.21	2.2
	NO	V	RP1	45	0.5	1.4	1.12	1.6
		N	PIM3	45	1.0	0.4	2.18	3.0
	NAL	N	PIM4	25	0.5	5.9	1.09	1.8
	5HCOT	V	POM1	25	1.0	0.4	1.58	3.5

^{1a,b} See Table 1 and Section 2.

² Refer to Section 2 for information concerning chiral stationary phases (CSP). * denotes 150 × 4.6 mm (i.d.), ** denotes 2 columns coupled, 200 × 4.6 mm (i.d.).

³ Refer to Section 2 for information concerning mobile phase conditions.

⁴ T: column temperature (°C)

⁵ F: flow rate (mL/min)

^{6a,b,c} Chromatographic parameters calculated according to Section 2.

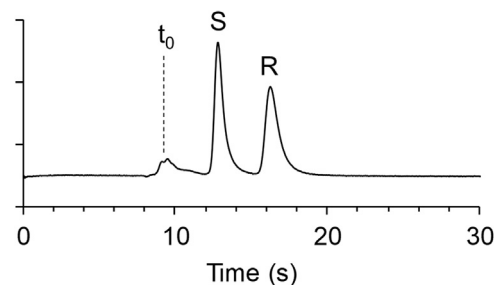


Fig. 1. Ultra-fast LC enantioseparation of nicotine (NIC) using NicoShell, 50 × 4.6 mm (i.d.), PIM4 at 4 mL/min. S: S-NIC; R: R-NIC; t_0 : impurities at dead time. See Section 2 for other acronyms and calculations ($k_1 = 0.7$, $\alpha = 1.64$, $R_s = 2.6$).

3. Results

The enantioseparation of NIC can be obtained in 18 s with a $R_s = 2.6$ (Fig. 1). Table 1 provides the structures and names of 40 nicotine-related compounds analyzed in this study. The optimized, baseline separation conditions for all chiral nicotine-related compounds using

macrocyclic glycopeptides are given in Table 2. NicoShell separated 19 compounds, VancoShell separated 15 compounds, and TeicoShell separated 4 compounds. Racemic AT, AB, and NNIC had higher resolution with NicoShell compared to VancoShell, but were baseline separated with VancoShell. NIC could not be baseline separated with VancoShell and TeicoShell. Addition of methyl groups to either adjacent carbons of the pyridine nitrogen, 2-methylnicotine (2MN) and 6-methylnicotine (6MN), increased resolution with VancoShell in comparison to NicoShell. 2MN and 6MN, in comparison to NIC, decreased selectivity with NicoShell, but increased selectivity with VancoShell. Addition of chlorine groups to NIC, 2-chloronicotine (2CN), increased resolution using NicoShell and VancoShell. Addition of oxygen functionalities to the pyridinium nitrogen, nicotine-1-oxide (NO), compared to NIC, increased selectivity with NicoShell and VancoShell. However, oxygen functionalities added to the pyrrolidinium group, nicotine-1'-oxide (NNO), resulted in less than a baseline separation with VancoShell. Also, NNO had decreased selectivity and longer retention than NIC and NO with NicoShell. Addition of alkyl groups to AB or NNIC, N-methylanabasine (MAB) or N-ethyl-nornicotine (NENN), had different effects depending on the CSP. In comparison to AB, MAB had increased resolution using NicoShell, but similar resolution with VancoShell. When comparing to NNIC, NENN had increased resolution for both NicoShell and VancoShell. Synthetic rac-*trans* nicotine-related compounds were also compared, which differed by peripheral functional groups. All rac-*trans* enantiomers were baseline separated by NicoShell and VancoShell. COT, norcotinine (NCOT), 4-*trans*-cotinine-carboxylic acid (4TCCA), and 5-(3-pyridyl)-tetrahydro-2-furanone (5THF) were only baseline separated by TeicoShell, while 1-methyl-3-nicotinoylpyrrolidine (1M3NP), 2-hydroxynicotine (2HN), 4-(methylamino)-1-(3-pyridyl)-1-butanol (NAL), NIC, and NNO were only baseline separated by NicoShell.

The only macrocyclic glycopeptide that separated 5'-hydroxycotinine, 5HCOT, was VancoShell, but 5HCOT was also separated by CDSHELL-RSP, which is shown in Fig. 2A. Fig. 2A also depicts a third component in the total ion chromatogram and selective ion monitoring chromatogram, which was identified as N-methyl-y-oxo-pyridinebutanamine, OPBN, based on the manufacturer label and mass spectra obtained. The manufacturer label marks 5HCOT and OPBN as equilibrating structures with the same mass, forming ring-open and ring-closed structures. The product ion scans for the two compounds were similar for peaks 2 and 3, but different for peak 1. Peaks 2 and 3 were identified as the chiral compound, 5HCOT, and peak 1 was identified as OPBN. The ratio of racemic HCOT to OPBN was 85:15 at 263 nm. 4-(methylamino)-1-(3-pyridyl)-1-butanone (NAN) was analyzed using CDSHELL-RSP, but wasn't expected to result in multiple peaks because it isn't chiral (Fig. 2B). There were two chromatographic peaks (peaks 4 and 5) at a ratio of 90:10 at 263 nm, and a raised baseline between the two peaks. A raised baseline between two related peaks indicates that there is an interconversion on the chromatographic time scale (Section 4). The additional peak was identified as N'-methylmyosmine (MMYS) based on the mass spectra obtained. The product ion scans had different fragmentation patterns for each peak. The raised baseline was present in the selective ion monitoring of each peak. Fig. 3 shows the separation of 10 tobacco alkaloids and 7 nicotine metabolites. NicoShell enantioseparated 5 chiral tobacco alkaloids in 10 min (Fig. 3A). Also, 3 enantioseparations of chiral nicotine metabolites were obtained with TeicoShell in 14 min (Fig. 3B). For identification, the appropriate *m/z* value was selected, and the ion chromatogram of interest extracted. The components that had the same *m/z* were identified by spiking the sample with a standard.

The separation of NNAL is shown in Fig. 4A, and resulted in two pairs of chromatographic peaks that had similar areas. The ratio of each pair was 75:25 at 263 nm. The separation of NNK is shown in Fig. 4B, with two chromatographic peaks at a ratio of 75:25 at 263 nm. Fig. 4C shows 4 distinct peaks from the separation of racemic NNN with the Q-Shell column. When pure enantiomers were injected, (R)-NNN in

Fig. 4D, each enantiomer showed two peaks at a ratio of 65:35 at 263 nm with a raised baseline between them (Figs. 4C and 4D). Single enantiomers were spiked into the original sample to identify each enantiomeric peak. An enantiomeric impurity was observed in the (R)-NNN sample, and identified as (S)-NNN (Fig. 4D). The elution order of NNN and NNIC enantiomers were different than the other tobacco alkaloids and metabolites (Figs. 3A, 3B, and 4D). Additionally, a reversal of elution for AB was observed when switching between PIM and POM solvents (Figs. S1A and S1B). Racemic NAB was separated into 2 peaks of equal area, while (S)-NAB was separated into 2 peaks at a ratio of 80:20 at 263 nm (Figs. S2A and S2B). NAT was separated into 2 peaks that each had a peak shoulder at a ratio of 80:20 at 263 nm (Fig. S2C). NNNNO was separated into 4 peaks with similar ratios at 263 nm with a raised baseline using NicoShell coupled to TeicoShell (Fig. S2D). The separation of each TSNA resulted with similar MS fragmentation patterns for each respective peak.

4. Discussion

The chiral selectors examined in this study were shown to have both broader and higher selectivity for tobacco alkaloids and their metabolites than other approaches (Table 2) [27,39,40]. Higher selectivities and efficiencies allows the use of shorter columns and higher flow rates, which produces faster analysis times, and often sharper peaks, and better detection (Fig. 1) [32,39,40]. In turn, this can be useful in high throughput screening, and studying biotransformations and the biokinetics and dynamics of low levels of tobacco alkaloid metabolites [46]. For such studies, it is essential for the stereoselective separation methods to be compatible with ESI-MS detection as were all methods herein (Table 2). Separations that didn't work well for one macrocyclic glycopeptide separated with a different related one, which is known as complementary behaviour (Table 2). The "principle of complementary separations" states that a partial separation with one chiral selector can be brought to baseline with one of the other related selectors [47]. Complementary separations were seen with NIC and several derivatives (2HN, 1M3NP, NNO), which had poor resolution using VancoShell, but worked well with NicoShell. Also, TeicoShell baseline separated the metabolites that VancoShell and NicoShell didn't. Usually within a class of structures several functionalities differ, which might enhance the separation using one macrocyclic glycopeptide, but inhibit another. It is unclear why some compounds had poor resolution using NicoShell and VancoShell due to the complex interaction mechanisms of macrocyclic glycopeptides. However, complementary separations offer an effective solution for difficult separations, which has been exploited for high-throughput screening [46]. This significant characteristic provides a high likelihood of baseline separating any structure within a certain class, as in the case of the tobacco alkaloids and their metabolites (Figs. 3A and 3B). Utilizing multiple chiral selectors and chromatographic solvents also gave rise to a reversal of elution order. So, this method could also be applied to situations that require a certain elution profile of enantiomers, such as in determining enantiomeric purity or in preparative separations.

Additional chromatographic peaks were observed in the separation of some tobacco alkaloids (Figs. 2 and 4). Fig. 2 shows the separation of 5HCOT, which has been reported as the chiral cyclized, ring-closed form of the straight-chain structure, OPBN [33,48,49]. 5HCOT was previously reported to be quickly and favourably formed in water, which agrees with our results [49]. Some chromatographic separations have been challenging as indicated in previous reports [50]. The separation approach of this study provides two methods (Table 2 and Fig. 2A). A raised baseline was not observed, but in the separation of NAN a raised baseline was seen between the NAN chromatographic peak and the additional peak, MMYS, indicating interconversion on the chromatographic time scale (Fig. 2B). NAN was previously reported to equilibrate with MMYS by a dehydration/hydration reaction, but little was found in the literature about their roles in metabolism

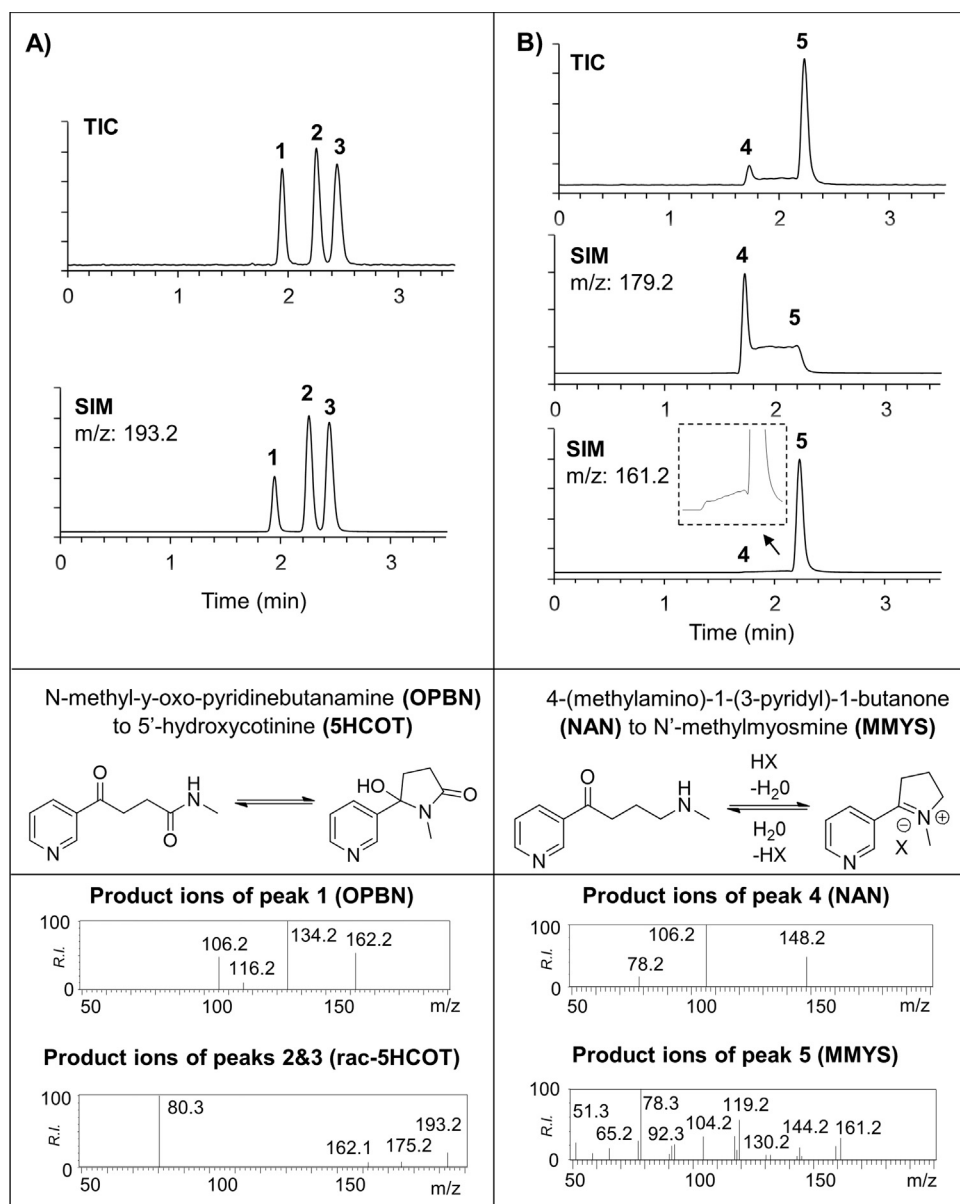


Fig. 2. Separation of ring-closed and ring-open equilibrating tobacco alkaloids. (A) OPBN (peak 1) to 5HCOT (peaks 2,3) (TIC scan from 110 to 220 m/z and product ion scan from 50 to 200 m/z) (B) NAN (peak 4) to MMYS (peak 5) (TIC scan from 150 to 220 m/z and product ion scan from 50 to 200 m/z). Conditions: CDShell-RSP, 100 \times 4.6 mm (i.d.), RP5, 1.0 mL/min, 25 $^{\circ}$ C. R.I.: Relative intensity, TIC: total ion chromatogram, SIM: selective ion monitoring. See Section 2 for other acronyms and information.

[33,48,49,51]. NAN and MMYS might have faster interconversion rates than the other ring-closed and straight-chain equilibrating structures, in Fig. 2A, due to the observed raised baseline at ordinary conditions. The reproducibility of a separation was highly dependent on the time between sample preparation and analysis. It was observed that differences in this time and temperature would change the ratio between the chromatographic peaks. Also, if another solvent was used to dissolve the analyte for analysis, the equilibration time was much different. Furthermore, other equilibrating structures have been reported to exist between these structures [48]. Detection and separation of these components has not been reported in the literature, but with this procedure, it is now possible.

A single enantiomer of NNN was separated into 2 chromatographic peaks at ratio of 70/30, which agrees with a previous report of NNN's E and Z isomeric ratio in tobacco and were labelled accordingly (Fig. 4D) [19]. E/Z isomers in TSNA have not been extensively studied. Reports of some have been performed, such as NNN, NNK, and NNAL, concluding there is generally a higher concentration of the E isomer than the Z isomer [18–20]. Our results indicate that this also is true for NAB and NAT (Figs. 4B and S2). Upon further inspection, a raised baseline was observed between the peaks. This indicates the diastereomeric

interconversion of E and Z isomers on the chromatographic time scale (Fig. 4D). In general, decreasing the temperature of the column lowered the baseline between the converting peaks. On the other hand, higher column temperatures increased the rate of conversion, such that no peak separation was observed. Perhaps, this explains why previous reports that use GC at high temperatures didn't observe E/Z isomers during TSNA analysis [23]. The interconversion rate between E/Z isomers was different because some TSNA had distinct raised baselines, like NNN, while others like NNK didn't (Figs. 4B and 4D). However, a raised baseline was observed at higher temperatures for NNK, so it does interconvert, but slowly at ordinary conditions. Previous reports of some pharmacokinetics of racemic TSNA have been investigated, such as their half-lives. An observed trend was that a short half-life correlated to the carcinogenicity of the TSNA, so more potent TSNA were eliminated faster [52]. However, the half-lives and interconversion rates of single enantiomers have not been reported, which may be different, especially since they contain equilibrating isomers. All equilibrium mixtures in this study had constant ratios under room temperature conditions, but due to the raised chromatographic baseline, there was difficulty in isolating pure E or Z isomers [18,19]. Since E/Z isomers were observed for all TSNA, further investigation of the

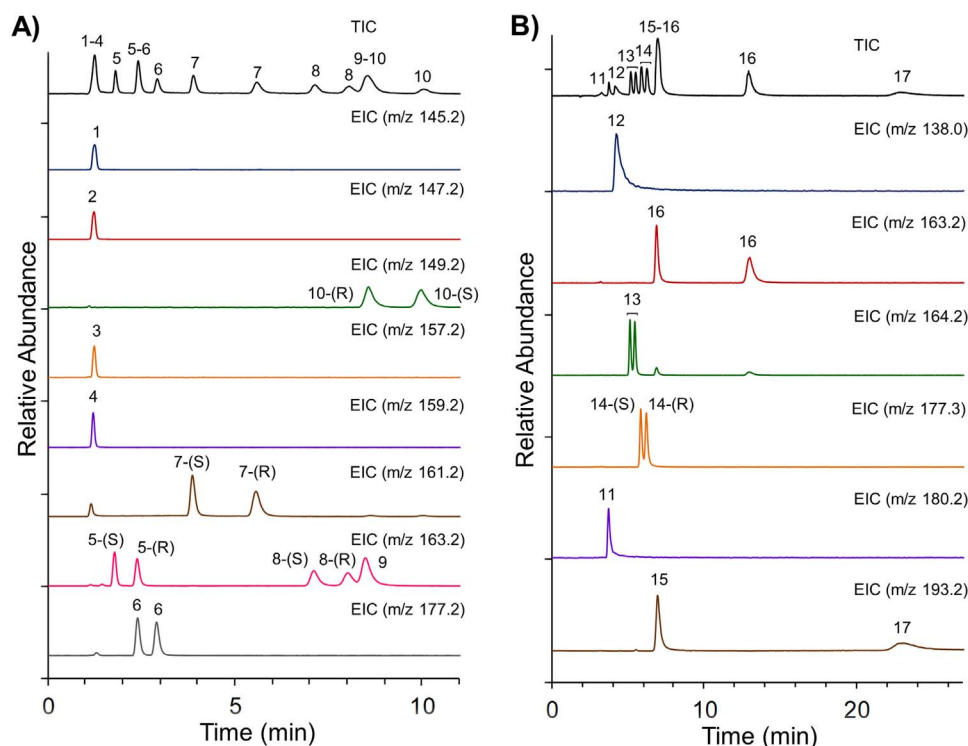


Fig. 3. Chromatographic separation and detection of 10 tobacco alkaloids (A) and 7 nicotine metabolites (B). Total ion chromatograms (TIC) and extracted ion chromatograms (EIC) are shown. (A) Conditions: NicoShell, 100 × 4.6 mm (i.d.), PIM4, 1 mL/min. Tobacco alkaloids: 1. β -NNT, 2. MYS, 3. BPY, 4. β -NT, 5. (S,R)-NIC, 6. MANB, 7. (S,R)-ANT, 8. (S,R)-ANB, 9. MET, 10. (R,S)-NNIC. (B) Conditions: TeicoShell, 150 × 4.6 mm (i.d.), POM3, 0.5 mL/min. Nicotine metabolites: 11. OPBA, 12. LAC, 13. rac-5THF, 14. (S,R)-COT, 15. T3HC, 16. rac-NCOT, 17. (S)-CNO. See Section 2 and Table 1 for all acronyms, structures, and other information.

enantiomers and their respective isomers as well as other TSNA metabolites, such as their half-lives, might be useful to determine their stereoselective roles and routes in metabolism.

Since the FDA has issued the mandatory regulation of NNN levels in smokeless tobacco products, manufacturers will have to quantify the amount of NNN in their finished products [9]. This may lead to

confusion, as some manufacturers might use achiral LC methods, which might broaden or split their chromatographic peaks [24]. Since these splits are most likely E/Z isomers of the TSNA, it is important that they be included in the quantification required by the FDA. However, E/Z isomers are known to have different physical and biological properties, so studies might be needed to evaluate whether these E/Z isomers

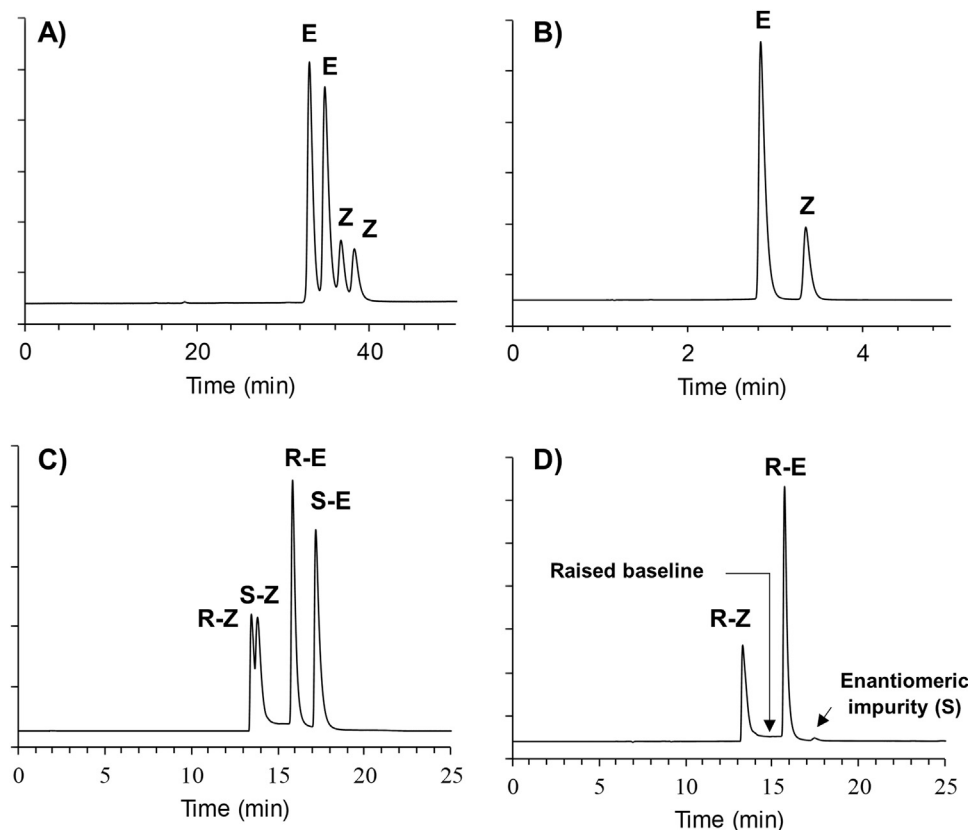


Fig. 4. The direct separation of tobacco-specific nitrosamines. R = R-NNN, S = S-NNN, E = E isomer, Z = Z isomer based on previous reports (Section 4), see Section 2 and Table 1 for other acronyms used. (A) rac-NNAL, TeicoShell, 100 × 4.6 mm (i.d.), RP3, 0.3 mL/min, 25 °C. (B) NNK, CDShell-RSP, 100 × 4.6 mm (i.d.), RP4, 1.0 mL/min, 25 °C. (C) rac-NNN, QShell, 250 × 4.6 mm (i.d.) 150 × 4.6 mm (i.d.) coupled to 100 × 4.6 mm (i.d.), RP3, 0.3 mL/min, 25 °C. (D) R-NNN, QShell, 250 × 4.6 mm (i.d.) 150 × 4.6 mm (i.d.) coupled to 100 × 4.6 mm (i.d.), RP3, 0.3 mL/min, 25 °C.

contribute differently to cancer and other diseases [26]. The methodology of this study can be applied, as the results of this study clearly demonstrate a comprehensive approach for the analysis and enantioseparation of these nicotine-related compounds. Further investigation is ongoing, but with the methods presented in this study, more pharmacological information concerning individual enantiomers and other isomers of nicotine-related compounds can effectively and quickly be obtained. These studies would lead to a more complete knowledge about tobacco alkaloids and their metabolites and their roles or therapeutic use for cancer and other diseases.

Acknowledgements

We thank AZYP, LLC, for their technical support and HPLC chiral column technology.

Funding

This work was supported by the Robert A. Welch Foundation (Y0026).

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.talanta.2017.12.060>.

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