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Assessment of toxicological validity using tobacco emission condensates: A comparative analysis of emissions and condensates from 3R4F reference cigarettes and heated tobacco products

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Abstract

The tobacco emission condensate, hereafter "tobacco condensate," is crucial for evaluating tobacco product toxicity. This condensate, derived from tobacco emissions, provides an optimized liquid concentrate for storage and concentration control. Thus, the validation of its constituents is vital for toxicity assessments. This study used tobacco condensates from 3R4F cigarettes and three heated tobacco product (HTP) variants to quantify and contrast organic compounds (OCs) therein. The hazard index (HI) for tobacco emissions and condensates was determined to ascertain the assessment validity. The total particulate matter (TPM) for 3R4F registered at 17,667 μg cig^{-1}, with its total OC (TOC) at 3777 μg cig^{-1}. HTPs' TPM and TOC were 9342±1918 μg cig^{-1} and 5258±593 μg $stick^{-1}$, respectively. 3R4F's heightened TPM likely arises from tar, while HTPs' OC concentrations are influenced by vegetable glycerin (2236–2688 μg $stick^{-1}$) and propylene glycol (589–610 μg $stick^{-1}$). During condensation, a 71.5% reduction in OC was observed for 3R4F versus

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HTPs' near-zero loss of –1.6%, albeit with OC-type variations. The HI was marginally higher for emissions, ranging from 7.92% (HTPs) to 18.6% (3R4F). Meticulous OC recovery from smoke condensates enables tobacco condensates to provide reliable toxicological data on tobacco products.

Keywords: tobacco emission condensate; hazard index; whole cigarette smoke condensate (WCSC); heated tobacco product aerosol condensate (HTPAC); organic compound (OC)

1. Introduction

Cigarette smoke comprises over 7000 organic compounds, of which more than 250, including benzene, are recognized as harmful (Chai et al., 2020; Dusautoir et al., 2021; Lim et al., 2023). This complex composition has been linked to the onset of multiple diseases, thereby adversely affecting human health (Jia et al., 2021; Lugg et al., 2022; Tatsuta et al., 2019). To counteract these negative implications, the advent and dissemination of safer tobacco alternatives, such as various electronic nicotine delivery systems (ENDS) encompassing electrically heated tobacco products, e-vapor devices, and hybrid variants, have been propelled (Benowitz et al., 2021; Caruso et al., 2021; Herbst et al., 2022; O'Connor et al., 2022). This burgeoning sector, valued at $15 billion in 2020, has witnessed a proliferation in product diversity, with the global tally of brand models surging from 184 in 2020 to 269 in 2022–a growth of over 45% over two years (Ali et al., 2023; Berg et al., 2022; Bigwanto et al., 2022; Xiao et al., 2022). Given this relentless expansion and innovation, it is imperative to develop prompt and precise assessment methodologies to assess the potential harm caused by these emerging products.

A thorough hazard evaluation is mandated before the commercial introduction of a new tobacco product (Cao et al., 2021; Lempert and Glantz, 2021). This often entails exposing animals or cell

cultures to cigarette smoke and subsequently observing the toxicological responses (Adesanya et al., 2020; da Silva Araújo et al., 2020; Jaccard et al., 2019). This direct exposure methodology not only incurs significant costs because of the requisite specialized apparatus and expertise but also because it poses constraints on the handling and storing of smoke samples (Go et al., 2022; Kogel et al., 2021; Park et al., 2020). The essential apparatus for these procedures included smoke generators, concentration regulators, and experimental chambers (Chang et al., 2020; Lee et al., 2021; Lim et al., 2021). Another limitation is the concurrent necessity for smoke production and inhalation assessments, which complicates the preservation of smoke samples for future analyses.

Cigarette smoke condensate (CSC) is a liquid matrix derived from tobacco smoke captured via filters or solvents and subsequently subjected to preprocessing techniques, notably solvent extraction and concentration (Ellis et al., 2014; Haghighi et al., 2022; Kim et al., 2018; Kim and Kim, 2023; Steele et al., 1995). First introduced in the 1950s, CSCs have been used to ascertain the carcinogenicity of tobacco (Rubin, 2001; Thun and Burns, 2001; Vineis et al., 2004). Since then, its application has expanded to gauge the impact of tobacco smoke on the onset of diverse ailments, encompassing chronic obstructive pulmonary disease, atherosclerosis, renal afflictions, and oral conditions, among others (Kim et al., 2023; Messner et al., 2012; Odoni et al., 2002; Semlali et al., 2014). Because of its liquid form, CSC is a preferable alternative to direct tobacco smoke exposure, facilitating efficient sample procurement for toxicological evaluation, precise concentration modulation, and convenient storage and conservation (Lin et al., 2021; Rehder Silinski et al., 2020; Wang et al., 2021). Consequently, CSC enables a streamlined assessment of potential hazards

associated with diverse tobacco products. CSC is utilized in the evaluation of tobacco toxicity through both in vitro and in vivo experiments (Haghighi et al., 2022). In vitro assessments expose specific cells to CSC to observe various toxic impacts. In vivo experiments involve injecting CSC into animals' lungs to gauge inhalation toxicity. The dosage of CSC, usually under 1 mL, is tailored to the species and weight of the animals. The exposure's frequency and duration are adjusted based on the specific toxic effects being studied, as documented in various research papers including those by Kim et al. (2022) and Driscoll et al. (2000).

The use of CSC has provided significant insight into the deleterious effects of tobacco products. However, concerns have persistently arisen regarding the reliability of toxicity data owing to a lack of standardized production methodologies for CSC. This challenge has been substantially addressed by developing a standardized condensation protocol for tobacco constituents introduced by our research team in 2023 (Kim and Kim, 2023a). Kim and Kim (2023a) utilized a Cambridge filter pad combined with Dulbecco's phosphate-buffered saline (DPBS) to concurrently capture both the particulate (total particulate matter, TPM) and gaseous phases of smoke from a standard 3R4F reference cigarette. Once trapped by the Cambridge filter pad, TPM from smoke underwent solvent extraction with subsequent nitrogen purging to eliminate the solvent, resulting in an isolated TPM residue. When amalgamated with DPBS containing the gaseous phase of cigarette smoke, this TPM residue yielded whole cigarette smoke condensate (WCSC), encompassing both particulate and gaseous components. The triplication of this procedure guaranteed that the concentration profiles of the primary organic compounds (OCs: nicotine, glycerin, acetaldehyde, and acetone) within the

generated WCSC maintained outstanding consistency, as evidenced by the relative standard deviations (RSDs) being consistently within a 5% range. Notably, the comparative mass concentration of these focal OCs in TPM closely mirrored that observed in cigarette smoke, registering at 90.4±29.4%. In a subsequent study (Kim and Kim, 2023b), a novel methodology was formulated to produce aerosol condensate of heated tobacco products (HTPs), referred to as heated tobacco product aerosol condensate (HTPAC), and its effectiveness was robustly validated. Through these methodological advancements in both conventional cigarettes and HTPs, our team has significantly enhanced the credibility of toxicity evaluations anchored on tobacco emission condensates. However, it is imperative to note that our preliminary investigations were confined to examining primary OCs within tobacco emission condensates. For a comprehensive and precise toxicity evaluation of a broader spectrum of tobacco products that use tobacco emission condensates, future studies will require qualitative and quantitative assessments of the encompassed OCs.

Through gas chromatography (GC), it is possible to detect more than 100 distinct OCs in cigarette smoke using singular analysis (Amaral et al., 2019; Dewulf et al., 2002; Reese et al., 2019). Employing mass spectrometry (MS) as the detection mechanism and leveraging library searching in the electron ionization mode enables comprehensive qualitative elucidation of these compounds (Overdahl et al., 2021; Perez-Riverol et al., 2022; Tiwary et al., 2019). Nevertheless, quantitative assessment requires acquiring standard samples of the identified OCs to formulate calibration curves (el Manouni el Hassani et al., 2020; Güzel and Canli, 2020; Mustafa and Milina, 2019; Nerdy et al., 2023). Considering the extensive array of OCs, securing standard samples for each compound poses

significant logistical challenges (Kim et al., 2014). Consequently, quantifying all OCs identified in cigarette smoke via GC-MS using an external calibration method with standard samples remains complex (Kim et al., 2014). To address this impediment, our team introduced an adequate carbon number (ECN) method that facilitates quantitative determination without standard samples (Kim et al., 2014). The ECN method paves the way for qualitative and quantitative assessments of an expansive array of OCs in tobacco smoke condensates.

In this research, we utilized GC-MS and the ECN method for qualitative and quantitative analysis of OCs in tobacco emission condensate (WCSC and HTPAC) (hereafter referred to as tobacco condensate). We compared quantitative data of OCs in tobacco condensate with those from tobacco emissions (derived from previous studies on cigarette smoke or HTP aerosols) to assess the relative concentrations of OCs during their transition from emissions to condensates. Additionally, OC quantification, combined with available toxicity data, was used to calculate and compare the hazard indices (HI) of tobacco emissions and condensates. The primary aim was to assess tobacco product toxicity, hence minimal toxicity indicators (hazard quotient (HQ) and HI) were determined and evaluated across various tobacco samples. This approach enabled a thorough assessment of the validity of hazard evaluations based on tobacco condensates for tobacco-related products.

2. Materials and methods

2.1. Preparation and analysis of tobacco condensates (WCSC and HTPAC)

In this study, we used 3R4F cigarettes (University of Kentucky, Lexington, KY, USA) and three

distinct HTPs to prepare the WCSC (sample code:3R4F) and HTPACs (sample codes: HTP-A, HTP-B, and HTP-C). The HTPs for this study was strategically based on their dominant market presence and rising sales (Hwang, 2022). These HTPs, chosen for analysis, were identified as the top three in market share within the South Korean market in 2017, as detailed by Hwang et al. (2021). This methodology ensures that the study examines products that are not only widely used by consumers but also hold considerable relevance in today's market, providing a more accurate reflection of current trends and usage patterns in the field.

For tobacco condensate generation, 3R4F cigarettes and HTPs emissions were initiated using an SG-300 smoke-generation device (Sibata, Japan). The emissions were collected through a Cambridge filter pad (44 mm, GE Healthcare, Buckinghamshire, UK) targeting the particulate phase. After the Cambridge filter process, the gaseous phase was absorbed into DPBS (1X, Gibco™, Life Technologies, USA). The particulate matter retained on the Cambridge filter pad was subjected to methanol-based solvent extraction and N_2 evaporation, resulting in a TPM residue. Combining this residue with the gaseous phase in DPBS, we obtained tobacco condensate WCSC for 3R4F and HTPACs for HTPs. As detailed in previous publications, our team developed and standardized this tobacco condensate preparation method (Kim and Kim, 2023a; Kim and Kim, 2023b). Tobacco condensates were evaluated both qualitatively and quantitatively using instrumental analysis. Further details of this method are provided in the supplementary material. The essential parameters for the preparation and analysis of the tobacco condensates are listed in Table 1.

Table 1
Preparation and analysis of tobacco condensates (WCSC and HTPAC).

Tobacco condensate type:	**WCSC**	**HTPAC**		
Sample code:	3R4F	HTP-A	HTP-B	HTP-C
A. Generation of cigarette smoke and HTP aerosol (modified HCI regimen (HCI, 1999))				
Puff number:	8	12	9	8
Puff volume (mL):	55	55	55	55
Puff duration (sec):	2	2	2	2
Puff interval (sec):	30	30	30	30
Filter vent blocking (%):	100	100	100	100
Cigarette (or stick) number:	30	80	80	80
B. Collection of TPM in smoke and aerosol samples (modified ISO regimen (ISO, 1999))				
Sampler for particulate phase:	44 mm Cambridge filter			
Sampler for gaseous phase:	49.5 mL DPBS			
C. Pretreatment of TPM collected on Cambridge filter				
Solvent for extraction:	5 mL methanol			
N_2 evaporation:	2 L min^{-1} for 3 h			
Surfactant[a]:	0.5 mL DMSO			
D. Preparation of WCSC and HTPACs				
Mixing samples:	Pretreated TPM (methanol-removed TPM residue + 0.5 mL DMSO) + 49.5 mL DPBS absorbing gaseous phase of cigarette smoke or HTP aerosol			
E. Analysis of WCSC and HTPACs				
Instrument:	GC-MS system (refer to Table S2)			
F. Quantification of OCs in the tobacco condensate samples				
Calibration method:	Analysis of liquid working standards containing 29 OCs (refer to Table S1)			
Quantification of CLASS:	ECN method[b] (Kim et al., 2014)			

*Abbreviations: compounds lacking authentic standards or surrogates (CLASS), dimethyl sulfoxide (DMSO), Dulbecco's phosphate buffered saline (DPBS), effective carbon number (ECN), gas chromatography (GC), heated tobacco product (HTP), heated tobacco product aerosol condensate (HTPAC), mass spectrometry (MS), organic compound (OC), total particulate matter (TPM) and whole cigarette smoke condensate (WCSC).
[a]0.5 mL DMSO was added to the methanol-removed TPM residue for mixing with DPBS to absorb the gaseous phase of smoke or aerosol. The mixture was stirred for 1 min at 500 rpm.
[b]The ECN method was used to calculate the concentration of OCs due to the absence of standard material (i.e., authentic compounds) or the complexity involved in standard preparation.

2.2. Preparation of working standards for calibration curve acquisition

In this study, aiming to establish calibration data for the quantification of OCs in tobacco condensates, we initially formulated working standards comprising 29 OCs, each characterized by diverse functional groups: (1) two alkanes: pentane and hexane, (2) four aldehydes: acetaldehyde,

propionaldehyde, butyraldehyde, and valeraldehyde, (3) two ketones: acetone and methyl isobutyl ketone, (4) three acetates: methyl acetate, butyl acetate, and ethyl hexanoate, (5) three alcohols: ethyl alcohol, isobutyl alcohol, and 1-hexanol, (6) four aromatics: toluene, p-xylene, m-xylene, and o-xylene, (7) six carboxylic acids: acetic acid, propionic acid, butyric acid, valeric acid, hexanoic acid, and heptanoic acid, (8) two phenols: o-cresol and m-cresol, (9) one amine: trimethyl amine, (10) two sulfides: dimethyl sulfide and dimethyl disulfide. To improve the precision of the ECN-based quantitative inference methodology, we selected the 29 OCs with a range of functional groups as the target analytes. This approach leverages a correlation analysis between the physicochemical characteristics of the OCs and their experimental response factors (RFs). The variety in functional groups significantly enhances the refinement and robustness of the quantitative inference formula (Kim et al., 2014).

Except for trimethylamine (purity = 25.0%), all 29 OCs were procured as reagent-grade chemicals (RGCs) with purity exceeding 97% (Sigma-Aldrich, USA). The RGCs were systematically diluted in methanol (purity >99.95%; JT Baker, USA) using gravimetric techniques to derive the working standards. A primary standard (PS) was assembled from the 29 RGCs by dilution in methanol and subsequently stratified into three categories based on the OCs' functional groups (PS-1: alkene, aldehyde, ketone, acetate, alcohol, and aromatic; PS-2: carboxylic acid; and PS-3: phenol, amine, and sulfide). The concentration of OCs in the formulated PSs was documented as follows: mean concentration = 10,113±935 ng μL^{-1} (PS-1), 10,611±576 ng μL^{-1} (PS-2), and 9548±1096 ng μL^{-1} (PS-3). A first working standard was derived by amalgamating PS-1, -2, and -

3 in methanol, achieving a 510±44.7 ng μL^{-1} concentration. The culminating working standard intended for calibration was formulated by diluting the first working standard in methanol, establishing a 5-point concentration gradient (mean concentrations = (1) 5.10±0.45 ng μL^{-1}, (2) 10.2±0.89 ng μL^{-1}, (3) 25.5±2.23 ng μL^{-1}, (4) 51.0±4.47 ng μL^{-1}, and (5) 102±8.93 ng μL^{-1}). An in-depth methodology for preparing the working standards encapsulating 29 OCs is presented in Table S1.

2.3. Instrumental analysis

A GC-MS system with a liquid autosampler was used to analyze OCs in the tobacco condensates. A volume of 1 μL of the tobacco condensate was introduced into the GC injector, heated to 230°C, via a 10 μL syringe (Shimadzu, Japan) from the auto-sampler. Following injection, the sample was carried through a DB-heavy wax column (length:60 m, diameter:0.25 mm, film thickness:0.25 μm; Agilent, USA) using helium (purity > 99.999%) as the carrier gas at a flow rate of 2 mL min^{-1}. The GC oven temperature program began at 40°C, held for 5 min, then ramped up at 5°C min^{-1} to 230°C and sustained for 10 min, resulting in a total run time of 53 min. Once separated by GC, the OCs were channeled to an MS for further qualitative and quantitative analyses. Both the interface and the ion source temperatures in the MS were maintained at 230°C. Quantitative measurements were performed using the total ion chromatogram mode, spanning a mass-to-charge ratio (m/z) range of 35–600. The specific instrumental settings and conditions are listed in Table S2.

2.4. Quantitative determination of OC concentrations in tobacco condensates (WCSC and HTPAC)

For this study, we selected 29 previously prepared OCs as final working standards for external calibration (refer to Table S1). As described in previous studies, calibration data from these standards facilitated the derivation of predictive equations using the ECN method (Kim and Kim, 2013; Kim et al., 2014; Szulejko et al., 2013). Given the lack of standard materials (such as authentic compounds) and the challenges associated with standard preparation, the ECN method proved crucial for determining the concentration of each OC. The OCs identified in the WCSC and HTPAC samples that could not be quantified via external calibration were determined using the ECN method. The OC concentrations were obtained from predictive equations built upon a linear regression relationship between the RF values of the 29 target standards and their respective ECNs (Fig. S1). Each ECN was ascertained by tallying the number of atoms (C, H, O, N, and S) and functional group components (e.g., aldehyde, ester, ketone, carboxyl, alcohol, cyclic, and acyclic groups) in terms of carbon number equivalents. This approach considers the approximate relative contribution of each element or group to the sensitivity of (RF) in the MS system. A comprehensive breakdown of the calculation process is presented in Table S3.

2.5. Preliminary tobacco emission data for comparative analysis with tobacco condensate

To evaluate the relative concentration (also referred to as recovery) of OCs in tobacco condensates versus tobacco emissions, we collated the OC concentration data from tobacco

emissions, focusing on 3R4F cigarette smoke and HTP aerosols, as outlined in several studies (Bekki et al., 2021; Cancelada et al., 2019; Forster et al., 2018; Jaccard et al., 2019; Li et al., 2019; Savareear et al., 2018; Savareear et al., 2019; Uchiyama et al., 2018). Tobacco emissions data were derived from preliminary research utilizing products identical to those used to produce tobacco condensate. Emission data were procured for the 3R4F, HTP-A, and HTP-C samples, excluding the HTP-B sample (Table S4). The cited studies measured tobacco emissions using both the HCI and ISO methodologies. For a coherent comparison between the data from these prior studies and our present investigation on tobacco condensate, we standardized the emission data units to showcase the mass of OCs emitted per cigarette or stick, denoted as μg cig^{-1} or μg $stick^{-1}$. The numbers of data points for OC concentrations from the studies above were as follows: 3R4F = 99 (HCI) and 83 (ISO); HTP-A = 58 (HCI) and 31 (ISO); and HTP-C = 91 (HCI) and 15 (ISO).

2.6. Gathering toxicity data for OCs

To compare the toxicity between tobacco emissions and condensates, we collected toxicity data related to OC content in tobacco samples through a literature review of 143 references. These references include international academic journals and government agency reports (Table S5). The toxicity data obtained included the following metrics: no observed adverse effect concentration (NOAEC, n=96), lowest observed adverse effect concentration (LOAEC, n=41), lethal concentration for 50% killing (LC_{50}, n=57), lethal concentration at 0% (LC_0, n=7), no observed effect level (NOEL, n=2), lowest published toxic dose (TD_{Lo}, n=4), and lowest lethal concentration

(LC_{Lo}, n=9). Among the NOAEC data, the values for eight OCs (2-phenylethanol, benzyl alcohol, dimethoxydimethylsilane, heptanoic acid, nicotine, propionaldehyde, propylene glycol, and pyridine) were converted from their oral toxicity values (no observed adverse effect level, NOAEL), as described by Choi et al. (2024). The recorded toxicity effects were primarily general symptoms in the experimental animals. The specific criteria for toxicity effects (systemic, local, neurotoxicity, and reproductive/developmental effects) are listed in Table S5.

3. Results and Discussion

This study's pivotal discoveries, including the newly identified concentration profiles of OCs in 3R4F and HTP emissions and their differential toxicity, are concisely summarized in Table 2, organized by theme. Detailed elaborations of the comprehensive tobacco research undertaken are provided in the subsequent subsections.

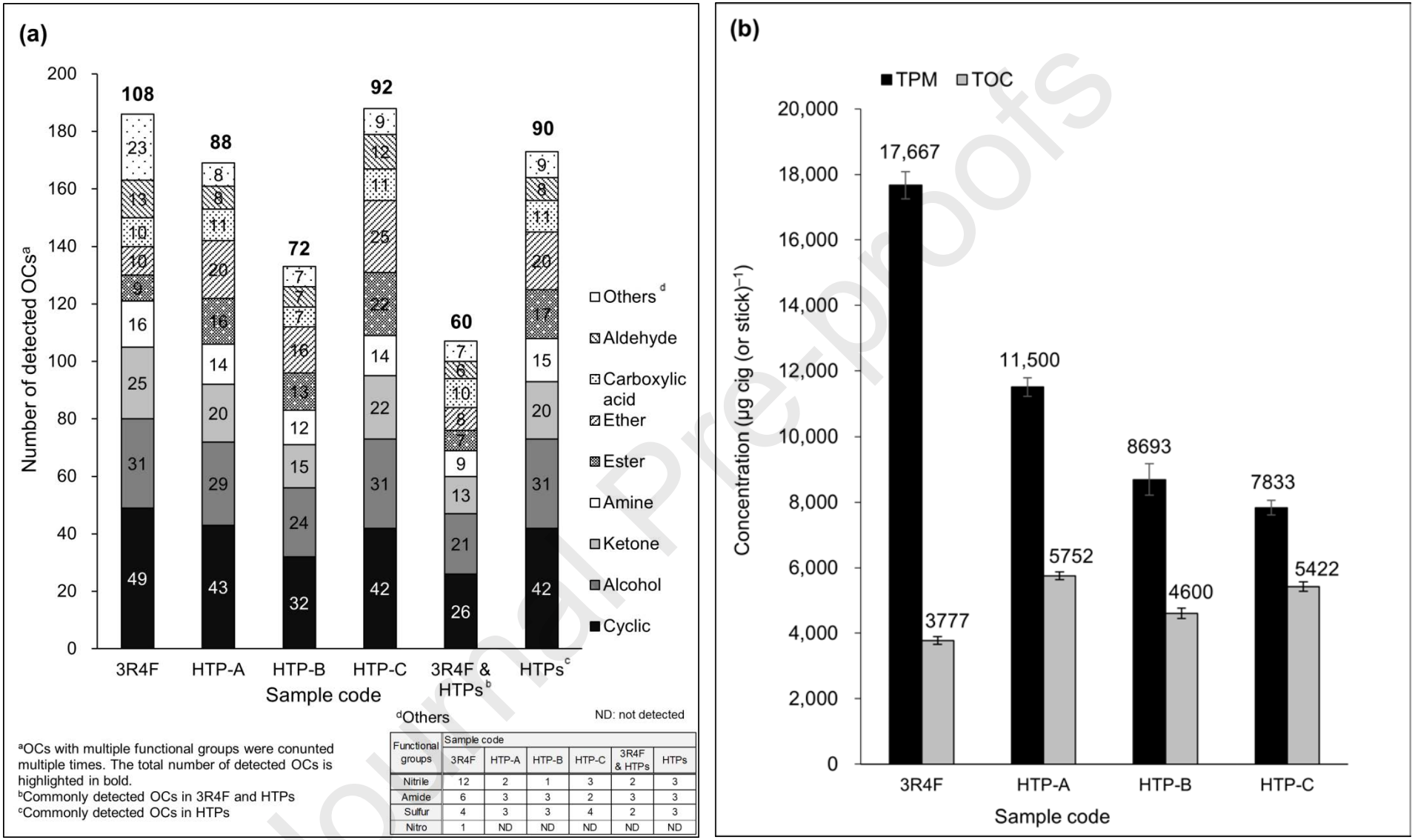
3.1. Qualitative and quantitative assessment of OCs in tobacco condensates

This study evaluated OC types and concentrations in tobacco condensates from 3R4F, HTP-A, HTP-B, and HTP-C. Chromatographic overlays of these condensates are depicted in Fig. S2. We calculated the number of OCs, their cumulative concentration (total OC, TOC), and their relative concentrations by functional group, as shown in Fig. 1. Relative concentrations (F/T, %) were computed as: Sum of OC concentrations by functional groups (μg cig (or stick)$^{-1}$) / Sum of TOC concentration (μg cig (or stick)$^{-1}$) × 100%.

Table 2. Comprehensive overview of principal discoveries from this research.

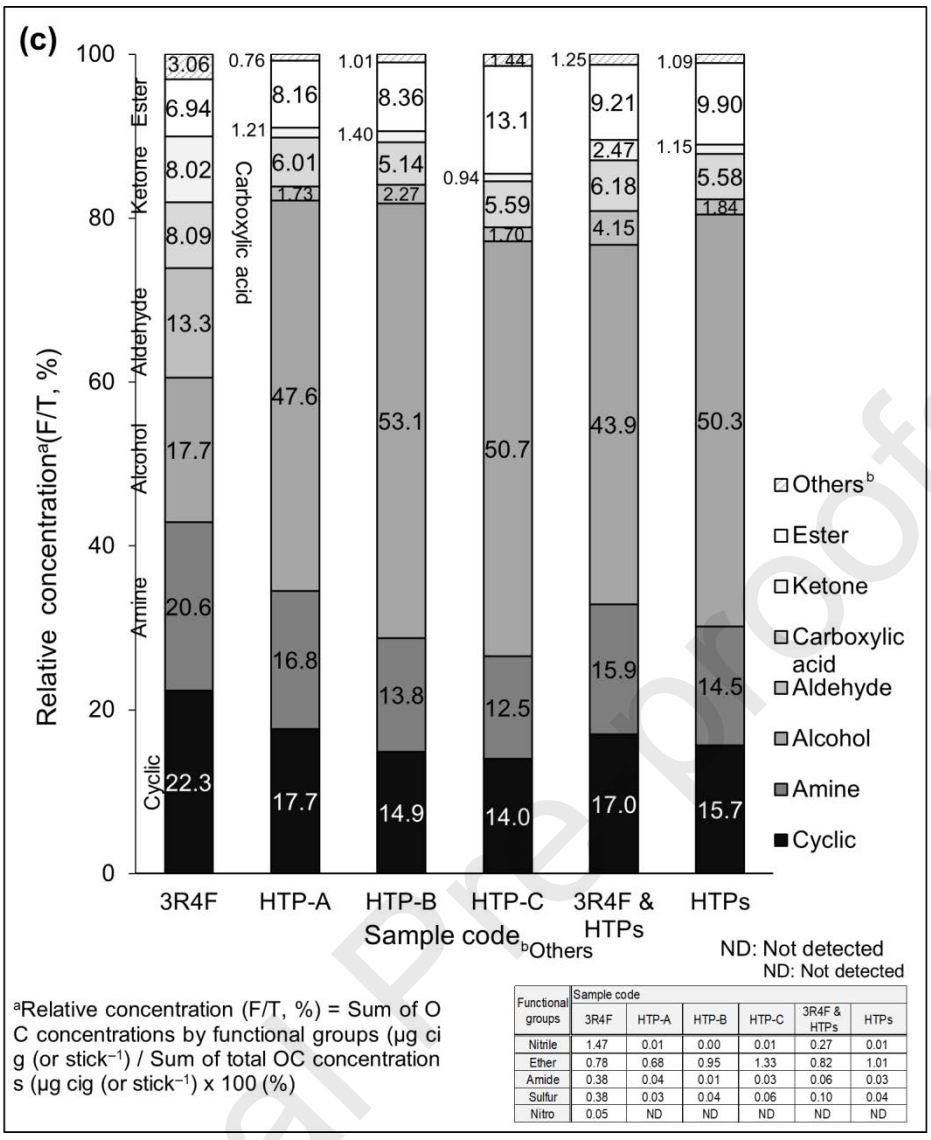
Order	Key findings	Content
3.1. Qualitative and quantitative assessment of OCs in tobacco condensates		

1		OCs), but over half of the OCs were common to both.
2	Functional group	Cyclic, alcohol, and ketone compounds were prevalent across all samples. Notably, nitrogen-bearing OCs were found in all samples, indicating their stability at HTPs' lower temperatures.
3	TOC levels	HTP-A recorded the highest TOC (5752 μg stick^{-1}), surpassing HTP-C, HTP-B, and the lower TOC in 3R4F. However, 3R4F showed a higher TPM concentration than HTPs.
4	Relative concentrations (F/T)	In 3R4F, cyclic and amine compounds were predominant, whereas HTPs had higher alcohol and lower aldehyde and ketone levels.
5	Absolute concentrations	Nicotine represented over 90% of cyclic and amine concentrations in all samples. HTPs had increased alcohol concentrations due to vegetable glycerin and propylene glycol, while 3R4F had higher aldehyde and ketone levels, primarily from acetaldehyde and acetone.
6	Nicotine and aldehyde comparisons	Nicotine concentrations were similar in all samples, but aldehyde levels were notably higher in 3R4F compared to HTPs.
3.2. Comparative analysis of OC concentrations in tobacco emissions and condensates		
1	Comparative study	This research compared 29 OCs in tobacco emissions and condensates using HCI and ISO protocols.
2	OC variations	Higher concentrations of certain OCs, like vegetable glycerin, were observed in emissions compared to condensates, with a notable 2.7-fold increase in the 3R4F sample's emissions.
3	Differences between emissions and condensates	Fifteen OCs registered higher concentrations in emissions, with some showing a significant decrease (C/E < 20%) during condensation.
4	Relative concentrations (C/E)	In the 3R4F sample, substances such as nicotine, propylene glycol, and benzyl alcohol had higher concentrations in smoke, whereas HTP samples showed higher concentrations in condensates. Notably, compounds like triacetin and acetic acid were much more concentrated in HTP-C condensates.
5	Pattern variability	Compounds like acetone, 2-furan-methanol, and phenol exhibited inconsistent concentration patterns across different sample types.
6	OC concentration factors	The study identified no direct link between OC concentration changes and intrinsic characteristics like functional groups or molecular weights. However, the tobacco sample type significantly influenced the relative concentration differences between emissions and condensates.
7	Statistical findings	A paired t-test revealed no marked differences in OC concentrations between emissions and condensates, except in the 3R4F sample, where significantly lower concentrations were noted in condensates.
8	Regimen Impact	Contrary to previous findings suggesting higher OC concentrations in HCI regimen emissions than ISO regimen emissions, this study found inconsistencies, with some OCs in HTP-C samples being higher under ISO conditions.
3.3. Hazard assessment of tobacco products using tobacco emission and condensate data		
1	HI values	The HI was highest in the 3R4F tobacco condensate sample at 45.2, with HTPs ranging from 30.3 (HTP-A) to 40.0 (HTP-C). Tobacco emissions generally had higher HI values, with 3R4F and HTP-A exhibiting greater HIs in emissions compared to condensates.
2	HQ values	Nicotine had the highest HQ in the 3R4F condensate (17.5), followed by methyl vinyl ketone and acetaldehyde. HTP variants also showed high HQs for nicotine, vegetable glycerin, propylene glycol, formic acid, and triacetin.
3	Dominant Hazard of Nicotine	Across all tobacco condensate samples, nicotine consistently displayed the highest HQ, significantly impacting the HI.
4	HQ variability	There was significant variability in the HQs of vegetable glycerin and propylene glycol, particularly higher in HTPs than in 3R4F. This variability complicates definitive conclusions on their increased toxicity in HTPs.
5	Tobacco emission hazard analysis	Nicotine and vegetable glycerin maintained high HQs in tobacco emissions, aligning with condensate data. Acetaldehyde consistently showed higher HQs in emissions than condensates.
6	Toxicity Analysis	A paired t-test revealed no significant differences in overall toxicity between emissions and condensates in most samples. However, for HTP-C, a one-tailed p-value suggested a notable difference in toxicity levels.



Functional groups	Sample code					
	3R4F	HTP-A	HTP-B	HTP-C	3R4F & HTPs	HTPs
Nitrile	12	2	1	3	2	3
Amide	6	3	3	2	3	3
Sulfur	4	3	3	4	2	3
Nitro	1	ND	ND	ND	ND	ND

Fig. 1. (a) Classification of detected OCs by function, (b) concentrations of TPM and TOC (μg cig (or stick)$^{-1}$), and (C) relative OC concentrations (F/T, %) stratified by function and according to tobacco sample type.



Functional groups	Sample code					
	3R4F	HTP-A	HTP-B	HTP-C	3R4F & HTPs	HTPs
Nitrile	1.47	0.01	0.00	0.01	0.27	0.01
Ether	0.78	0.68	0.95	1.33	0.82	1.01
Amide	0.38	0.04	0.01	0.03	0.06	0.03
Sulfur	0.38	0.03	0.04	0.06	0.10	0.04
Nitro	0.05	ND	ND	ND	ND	ND

Fig. 1. (Continued) (a) Classification of detected OCs by function, (b) concentrations of TPM and TOC (μg cig (or stick)$^{-1}$), and (C) relative OC concentrations (F/T, %) stratified by function and according to tobacco sample type.

The 3R4F sample contained the highest diversity of OCs with 108 distinct types, while the HTP samples ranged from 72 to 92 OCs, averaging a 22.2±9.80% decrease in OCs compared to 3R4F (Fig. 1). Sixty OCs were common to both 3R4F and HTPs, constituting over half of the total OCs in each sample. Notably, 90 OCs were consistently present in all HTP samples, suggesting similar OC profiles across HTP variants. The most prevalent OC functional groups were cyclic, alcohols, and ketones. Nitrogen-bearing OCs were consistently found in all samples, indicating that

significant nitrogen compounds can form in HTPs, despite the lower temperatures compared to traditional cigarette combustion, as observed in the 35 nitrogen compounds in 3R4F versus an average of 18.0±1.73 in HTPs (Ishizaki and Kataoka, 2019; Meikopoulos et al., 2022; Xia et al., 2021).

The TOC concentration observed in each tobacco condensate sample was highest in HTP-A, registering at 5752 μg stick^{-1}, which was followed by HTP-C and HTP-B, with 5422 μg stick^{-1} and 4600 μg stick^{-1}, respectively. In contrast, the 3R4F sample exhibited the lowest TOC concentration, measuring 3777 μg stick^{-1}. Notably, although 3R4F contained the greatest diversity of detected OCs, it also had the lowest TOC concentration. However, when captured on a Cambridge filter pad and subsequently weighed, the total particulate matter (TPM) concentration from the 3R4F conventional cigarette was 17,667 μg cig^{-1}. This value is roughly 1.89 times greater than the average TPM concentration in the HTPs, which stood at 9342±1918 μg stick^{-1}. These findings imply that constituents such as tar, not identified during the GC-MS analysis, are more abundant in 3R4F cigarette smoke (Lu et al., 2021; Pennings et al., 2020; Uchiyama et al., 2015). In contrast to 3R4F, which is produced via combustion, HTP aerosols are generated by a heating process, indicating that the observed differences in TPM and TOC concentrations cannot be ascribed solely to tar levels. We postulate that the variance in TPM and TOC concentrations in HTP aerosols is largely due to water content. As reported by Hidayat and Alayyannur (2021), water accounts for 46.4% of TPM in HTP aerosols. In our investigation, the variation between TPM and TOC concentrations in HTP aerosols was noted to be between 30.8% (HTP-C) and 50.0% (HTP-A), which is consistent with the

proportion of water to TPM in HTP aerosols as delineated by Hidayat and Alayyannur (2021).

In the 3R4F sample, the relative concentrations (F/T) of OCs categorized by functional group were as follows: cyclic compounds at 22.3%, amines at 20.6%, alcohols at 17.7%, aldehydes at 13.3%, carboxylic acids at 8.09%, ketones at 8.02%, and esters at 6.94%, while groups like nitriles and ethers were below 3%. The HTP samples displayed comparable levels for cyclic (15.5±1.92% F/T) and amine (14.4±2.21% F/T) compounds, but a notably higher alcohol concentration (50.5±2.75% F/T). In contrast, aldehyde and ketone concentrations in HTPs were significantly lower (1.90±0.32% and 1.18±0.23% F/T, respectively) than in 3R4F. This pattern suggests higher alcohol concentrations in HTP samples, while 3R4F is richer in aldehydes and ketones. Absolute OC concentrations in tobacco condensates, broken down by functional group and principal components, are presented in Fig. 2.

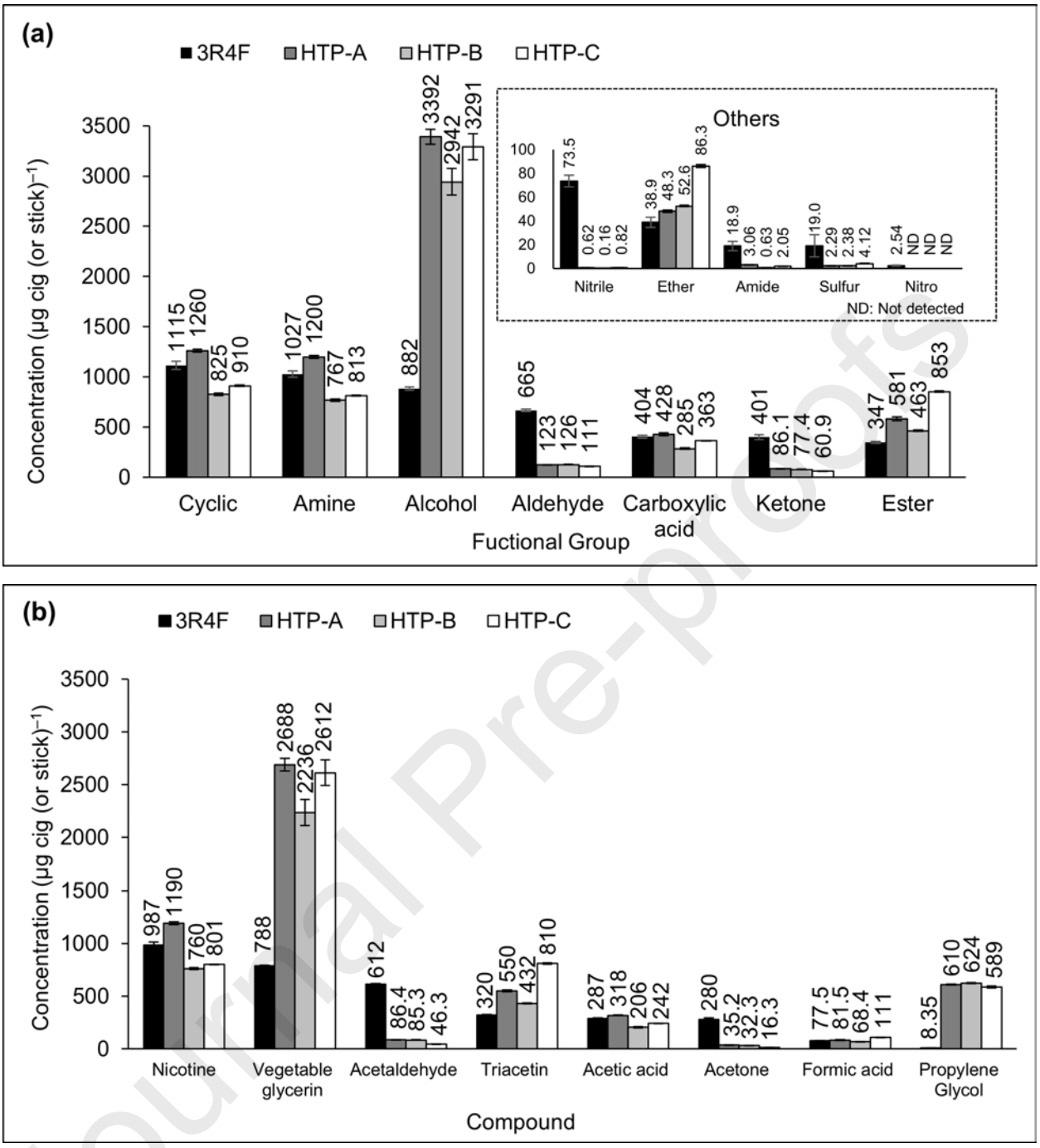


Fig. 2. Concentrations of detected OCs in tobacco samples: (a) categorized by functional group and (b) for predominant compounds (n=8).

Analysis of Fig. 2(a) reveals that the 3R4F sample had cyclic and amine compound concentrations of 1115 μg cig^{-1} and 1027 μg cig^{-1}, respectively, with nicotine (987 μg cig^{-1}) (Fig. 2(b)) comprising over 90% of these. In HTP samples, nicotine (917±237 μg stick^{-1}) also accounted for over 90% of these compounds. Alcohol concentrations, primarily from vegetable glycerin and propylene glycol,

were notably higher in HTP samples (2512±242 μg stick^{-1} and 608±17.6 μg stick^{-1}) compared to 3R4F (788 μg cig^{-1} and 8.35 μg cig^{-1}). The 3R4F sample had a higher aldehyde concentration (665±20.0 μg cig^{-1}) than HTPs (120±8.09 μg stick^{-1}), with acetaldehyde being a major contributor (92.3% in 3R4F, 62.5% in HTPs). Furfural was more concentrated in HTPs (25.9±5.01 μg stick^{-1}) than in 3R4F (9.07±0.92 μg cig^{-1}). Carboxylic acid levels were similar in both, predominantly comprising acetic acid (over 70% of the total concentration). Formic acid levels were 77.5±4.78 μg cig^{-1} in 3R4F and 87.0±21.8 μg stick^{-1} in HTPs. Ketone concentrations were about five times higher in 3R4F (401±41.2 μg cig^{-1}) than in HTPs (74.8±12.8 μg stick^{-1}), with acetone being the primary ketone in 3R4F and nearly equal concentrations of acetone and hydroxyacetone in HTPs. Ester concentrations were roughly double in HTPs (633±200 μg stick^{-1}) compared to 3R4F (347±17.3 μg cig^{-1}), mainly composed of triacetin (over 90% of esters).

In the present study, nicotine concentrations consistently ranged between 760 and 1190 μg cig^{-1} (or stick^{-1}) for 3R4F and HTP samples. In contrast, the aldehyde concentrations in the 3R4F samples were markedly elevated, reaching up to ten times that of the HTP, with a peak value of 612 μg cig^{-1}. For alcohols, namely vegetable glycerin and propylene glycol, the HTP samples demonstrated concentrations of 2512±242 μg stick^{-1} and 608±17.6 μg stick^{-1}, respectively, which are approximately 3 to 80 times greater than their counterparts in the 3R4F samples. A comprehensive list of all the OCs identified in the tobacco condensate samples and their corresponding concentrations is presented in Table S6.

3.2. Comparative analysis of OC concentrations in tobacco emissions and condensates

Tobacco condensate is a liquefied form of tobacco smoke. Some OCs from the tobacco emissions may have been lost during this liquefaction process. Hence, assessing the OC concentrations within tobacco condensates, in comparison to the original tobacco emissions, is imperative. For this purpose, the concentrations of 29 OCs, which were ubiquitously detected in both the condensate and emissions (e.g., vegetable glycerin, acetaldehyde, nicotine, triacetin, and others), are delineated in Fig. 3. The relative concentrations (C/E, %) of the tobacco condensate and emissions for individual tobacco samples are shown in Fig. 4. This relative concentration (C/E) was computed using the formula: Relative concentration (C/E, %) = OC concentration in tobacco condensates (μg cig (or stick)$^{-1}$) / OC concentration in tobacco emissions (μg cig (or stick)$^{-1}$) × 100%.

Tobacco emission data were obtained from the HCI and ISO regimens. Given that the condensate samples were derived using the HCI regimen, this section's comparative analysis between emissions and condensates primarily utilizes the OC concentration data from the HCI regimen. The OC concentration data obtained under the ISO regimen served as auxiliary information for comparison. The common OCs identified in both the emissions and condensates amounted to 27 for the HCI and 24 for the ISO regimens.

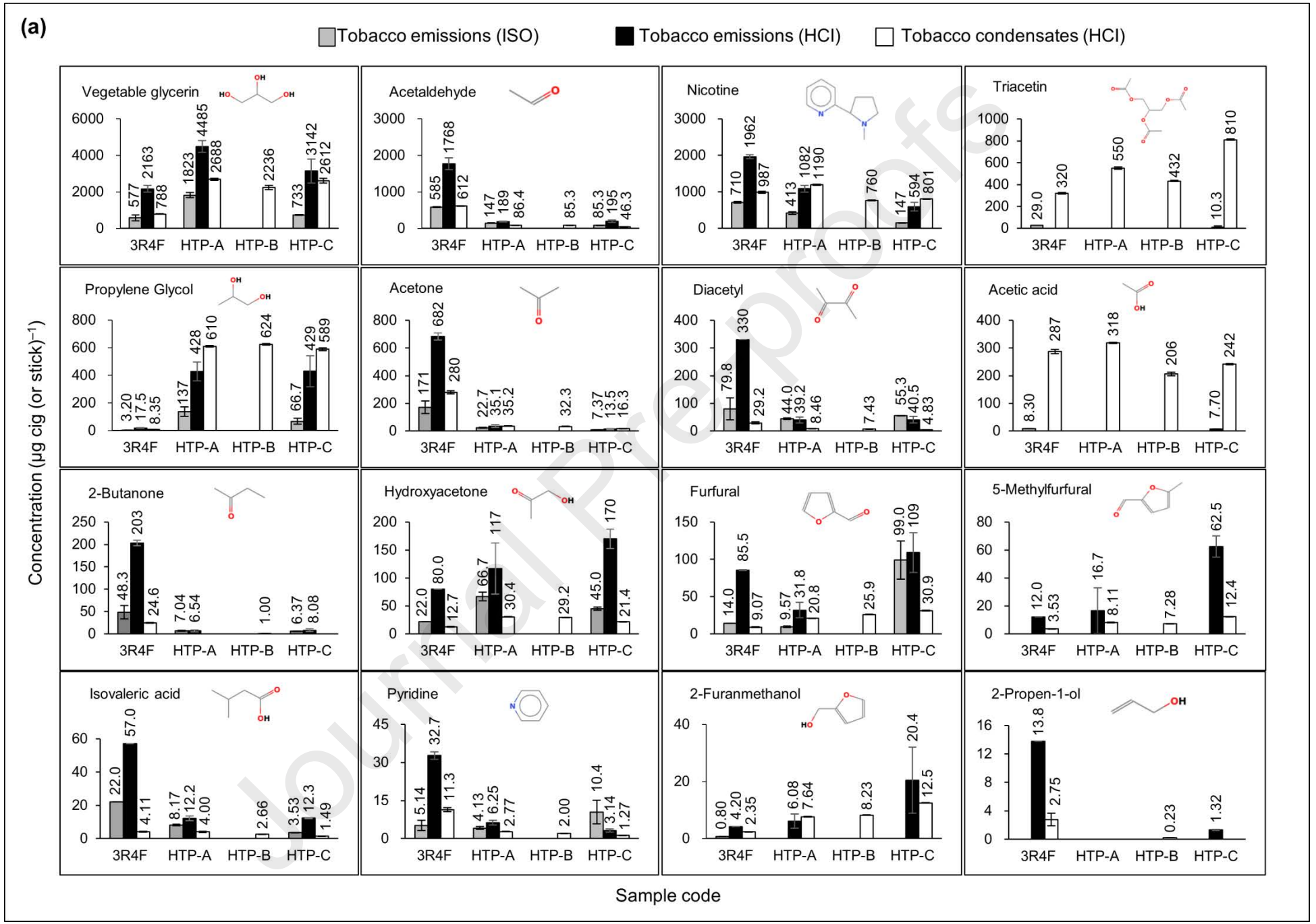


Fig. 3. Comparative analysis of OC concentrations between tobacco emissions and condensates.

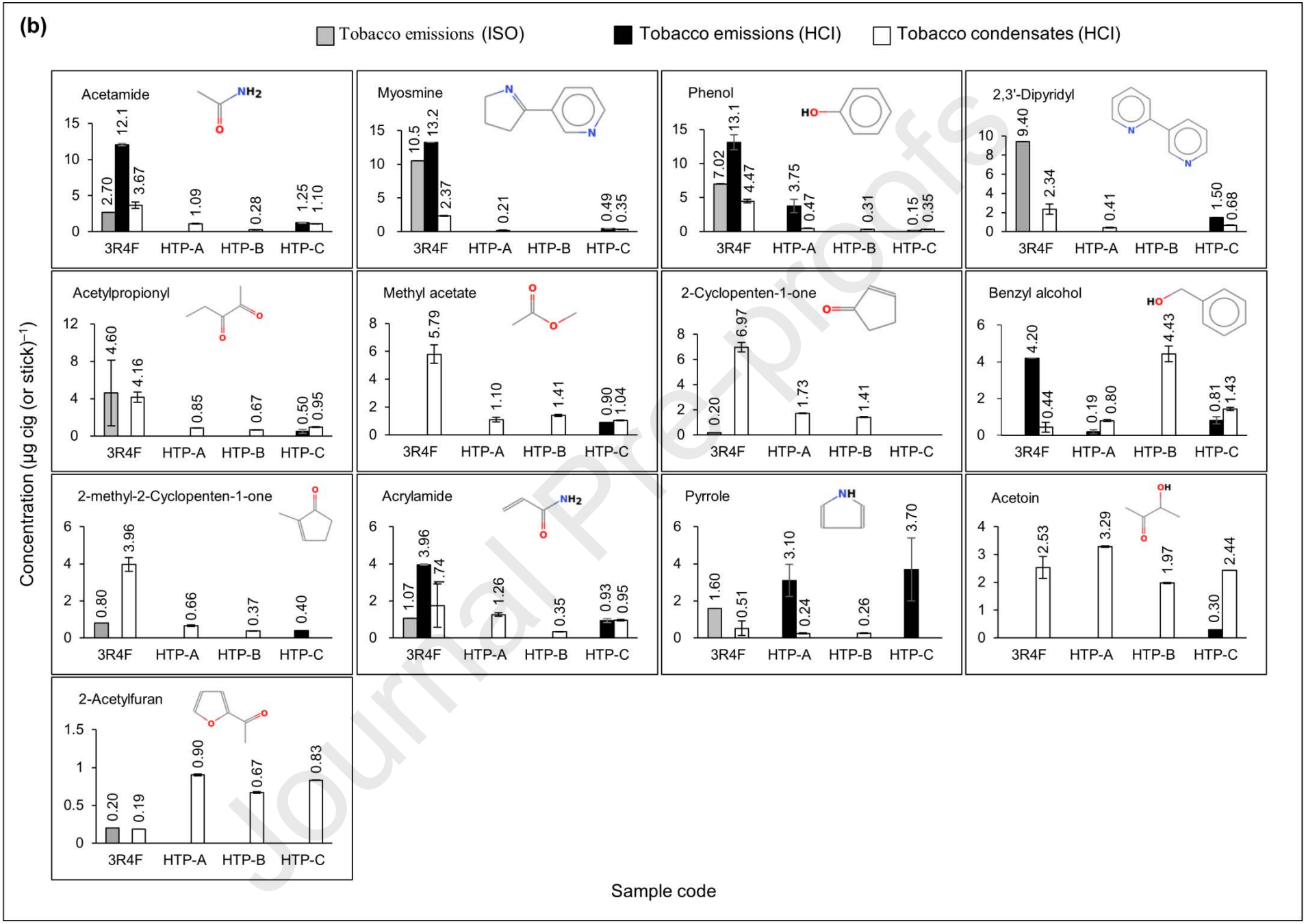


Fig. 3. (Continued) Comparative analysis of OC concentrations between tobacco emissions and condensates.

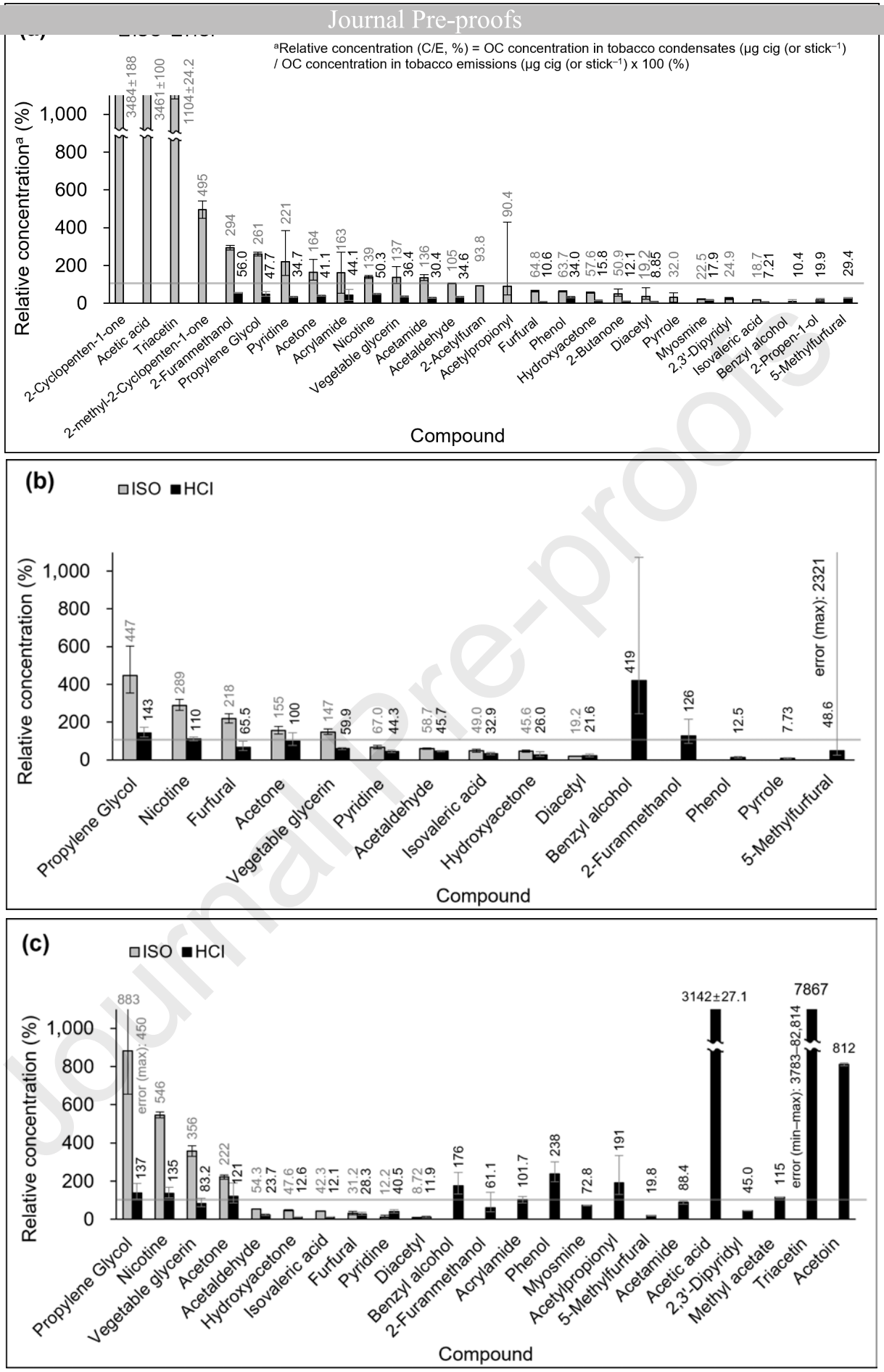


Fig. 4. Comparative relative OC concentrations between tobacco condensates and emissions for (a) 3R4F, (b) HTP-A, and (c) HTP-C samples.

Fig. 3 demonstrates that vegetable glycerin concentrations were consistently higher in tobacco emissions than in condensates. For the 3R4F sample, the emission concentration (HCI) was 2163±315 μg cig^{-1} compared to 788±12.2 μg $stick^{-1}$ in the condensate, a roughly 2.7-fold difference. The smallest discrepancy was observed in the HTP-C sample, with emissions at 3142±1598 μg $stick^{-1}$ and condensates at 2612±210 μg $stick^{-1}$.

Fifteen other OCs, including acetaldehyde, diacetyl, and pyridine, also showed higher concentrations in emissions than condensates. Notably, certain compounds like 2-propen-1-ol and pyrrole were found only in specific samples. Relative concentrations (C/E) of several OCs were under 20%, indicating significant reduction from emission to condensation.

Nicotine, propylene glycol, and benzyl alcohol in the 3R4F sample had higher smoke concentrations, while HTP samples showed the opposite. For instance, nicotine's C/E in 3R4F was 50.3±39.5%, but it was over 100% in HTP-A and -C. Some compounds, such as triacetin and acetic acid, had notably higher concentrations in HTP-C condensates than emissions, with C/E ratios reaching 7864% and 3143%, respectively. However, acetone, 2-furan-methanol, and phenol didn't show a consistent pattern across samples, with a mean C/E of about 79.7±20.2%, indicating a slight concentration variation between emissions and condensates. While substances like nicotine and propylene glycol exhibited slightly elevated concentrations in HTPAC relative to HTP aerosol, these differences are within the margin of error, complicating definitive conclusions about higher concentrations in HTPAC. Notably, triacetin and acetic acid, identified in markedly higher concentrations in tobacco condensates compared to emissions, necessitate detailed comparative

analysis in subsequent research.

In the studied tobacco samples, OC concentrations were generally higher in emissions, with 15 occurrences noted. However, eight OCs in tobacco condensates showed concentrations exceeding those in emissions, with relative concentrations (C/E) over 100%, particularly in HTP-C samples. The C/E ratios varied widely, from 7.73% for pyrrole to 7867% for triacetin. Compounds like vegetable glycerin, nicotine, and propylene glycol showed minimal concentration variation between emissions and condensates, with a C/E of around 75.0±27.4%. The variation in OC concentrations between emissions and condensates did not correlate significantly with functional groups or molecular weights. For instance, the 15 OCs with C/E below 100% had an average molecular weight of 87.5±31.3 g/mol, compared to 116±55.6 g/mol for those with C/E above 100%. Under the HCI regimen, the 3R4F sample showed the lowest average relative concentration (28.5±15.4%), whereas HTP-A and HTP-C were higher, at 84.2±102% and 588±1715%, respectively (see Fig. 4). The large deviation in HTP-C's C/E, particularly due to high values for triacetin and acetic acid, reduced to 120%–171% when these compounds were excluded.

As previously mentioned, the variation in OC concentrations between the emissions and condensates did not predominantly correlate with the inherent properties of OC. However, the type of tobacco sample played a pivotal role in demarcating the difference in the relative concentrations (C/E) of OCs between the emissions and condensates. The HTP samples registered relative concentrations (C/E) close to 100%, underscoring the negligible concentration discrepancies between the HTP aerosols and the condensate concerning the evaluated OCs. Contrarily, 3R4F, with

its relatively diminished relative concentration (C/E) of 28.5±15.4%, is posited to display lowered OC relative concentrations, potentially due to the substantive adsorption (or retention) of OCs by tar during the cigarette smoke condensation process (Smart et al., 2022; Stephens et al., 2019).

A paired t-test was employed to assess the statistical significance of the concentration differences in OCs between tobacco emissions and condensates, contingent on the tobacco sample type. The p-value (from a two-tailed test) was more significant than 0.05 for all examined tobacco samples, indicating no significant disparity in OC concentrations between emissions and condensates at a significance threshold 0.05. Notably, the 3R4F sample yielded a p-value of 0.03 in the one-tailed test, implying that OC concentrations in its condensate were substantially reduced compared to its smoke. In contrast, the HTP samples revealed a one-tailed test p-value exceeding 0.3, suggesting the absence of a marked concentration divergence between the HTP aerosol and its condensate.

Previous studies have posited that OC concentrations stemming from the HCI regimen in tobacco emissions may be elevated relative to those originating from the ISO regimen (Li et al., 2019; Salman et al., 2019; Uchiyama et al., 2015). In the case of the 3R4F samples, the OC levels under HCI conditions were invariably higher than their ISO counterparts. Nevertheless, in the context of HTP-C, the diacetyl and pyridine concentrations under ISO, 55.3±1.53 μg stick^{-1} and 10.4±8.03 μg stick$^{-1,}$ respectively, surpassed those observed under HCI, 40.5±23.9 μg stick^{-1} and 3.14±1.85 μg stick^{-1}. However, given the consistency in the concentration variability of diacetyl and pyridine in tobacco emissions with differences between HCI and ISO concentrations, it remains inconclusive to decisively claim a pronounced elevation in their concentrations under the ISO regimen relative

to HCI.

3.3. Hazard assessment of tobacco products using tobacco emission and condensate data

This section describes assessing tobacco sample hazards based on OC concentrations and pertinent toxicity data. The hazard, quantified as the HQ, was derived from OC concentrations with the aggregate measure referred to as the HI, as illustrated in Fig. 5. HQ quantifies the ratio between the potential exposure to a substance and the threshold at which no ae effects are expected. An OC with an HQ greater than or equal to 1 is considered potentially harmful to humans (Elfikrie et al., 2020; Luo et al., 2021; Wei et al., 2022). The primary assessment in this section focuses on OCs that register an HQ of 1 or higher. The computation methodologies for HQ and HI are outlined as Hazard Quotient (HQ) = Exposure concentration (μg mL^{-1}) / Reference concentration (μg mL^{-1}); Reference concentration (μg mL^{-1}) = No Observed Adverse Effect Concentration (NOAEC, μg mL^{-1}) / Uncertainty Factor (UF); Hazard Index (HI) = Summation of HQ values. For the NOAECs, the UF remained constant at 1, barring values resulting from interspecies extrapolation. This section also presents a comparative analysis of tobacco emissions and condensates hazards, utilizing samples procured under HCI regimen conditions. Additional data on tobacco emissions ascertained under the ISO regimen served as an ancillary reference.

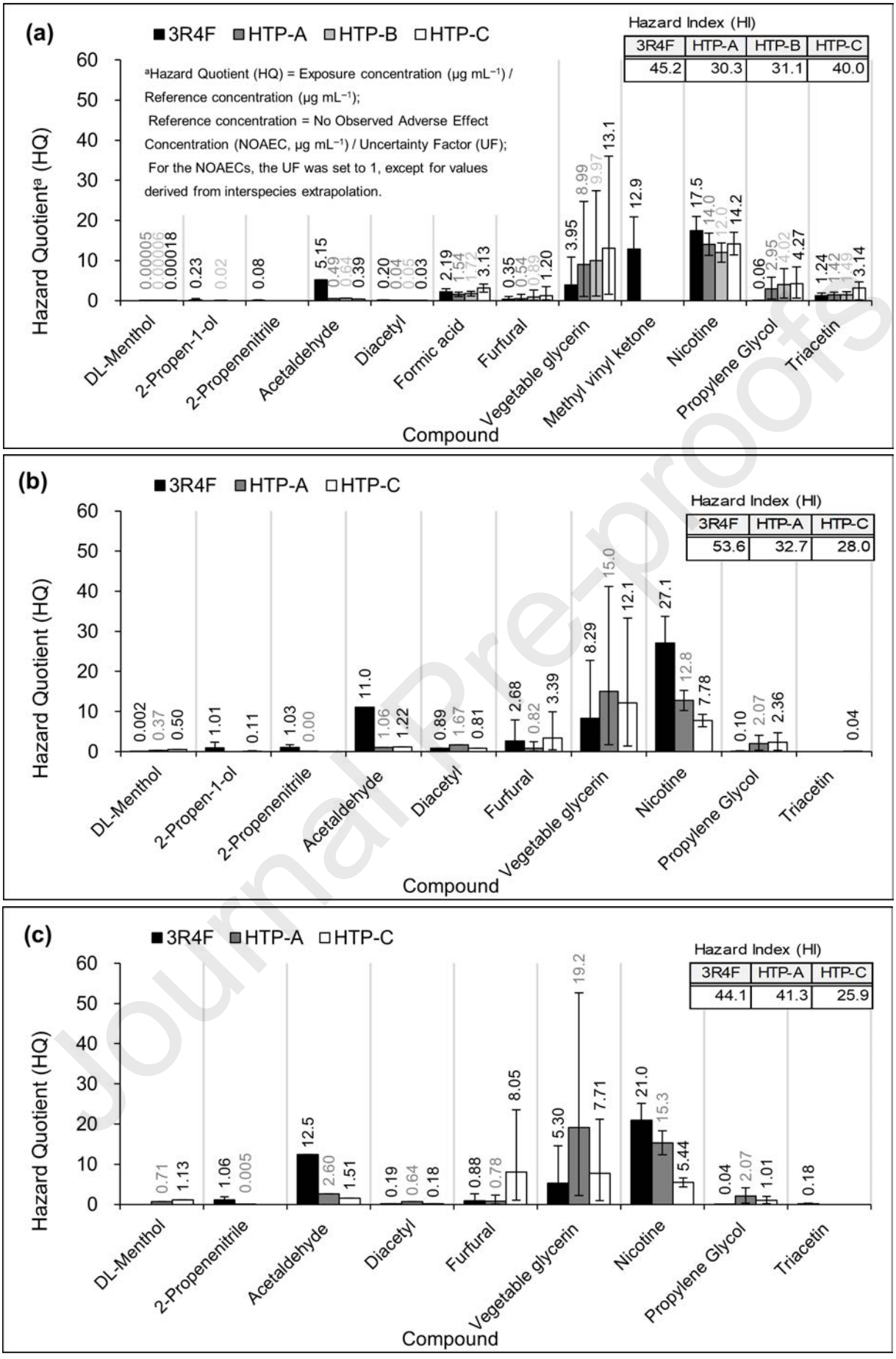


Fig. 5. Tobacco samples' Hazard quotient (HQ) and index (HI). (a) tobacco condensate (HCI), (b) tobacco emission (HCI), and (c) tobacco emission (ISO).

In this study, the HI of tobacco condensate was highest in the 3R4F sample (45.2), with HTPs ranging from 30.3 (HTP-A) to 40.0 (HTP-C). For tobacco emissions (HCI), HI values were 53.6 for HTP-A and 28.0 for 3R4F under HCI conditions, indicating higher HIs in emissions than condensates for 3R4F and HTP-A. The average HIs for emissions under ISO conditions were slightly lower than under HCI. In the 3R4F condensate, six OCs had a HQ exceeding 1, with nicotine recording the highest (17.5). HTP variants showed five OCs with HQs over 1, including nicotine (12.0–14.2) and vegetable glycerin (8.99–13.1). Nicotine consistently had the highest HQ in all condensate samples, significantly influencing the HI. The 3R4F sample had a notable HQ for vegetable glycerin (3.95), compared to an average HQ of 10.7±2.15 in HTPs. However, due to high variability, definitive conclusions about its increased toxicity in HTPs remain uncertain. Methyl vinyl ketone, exclusive to 3R4F, showed a high HQ (12.9), surpassed only by nicotine. In contrast, acetaldehyde had a lower average HQ in HTPs (0.50±0.13) compared to 5.15 in 3R4F. Propylene glycol's HQ was minimal in 3R4F (0.06) but higher in HTPs (3.74±0.70). In tobacco emissions, nicotine and vegetable glycerin had elevated HQs, consistent with condensate findings. The HQ for acetaldehyde was consistently higher in emissions than in condensates across all samples. Notably, triacetin, found only in HTP-C emissions, had a much lower HQ (0.04) compared to its condensate value (3.14).

A paired t-test was performed to determine whether the hazard disparities based on HQ values between tobacco emissions and tobacco condensate exhibited statistical significance among the various tobacco sample categories. The mean p-value across the tobacco samples was 0.49,

suggesting the absence of statistically significant toxicity differences between tobacco emissions and condensate at a threshold of 0.05. Nevertheless, the one-tailed p-value for HTP-C registered at 0.1, rendering it relatively significant when juxtaposed with 3R4F and HTP-A, which documented p-values of 0.33 and 0.30, respectively.

3.4. Challenges and considerations in the quantitative hazard assessment of tobacco emission and condensate

This study focused on the quantitative assessment of the concentration and hazards of tobacco condensate, specifically targeting OCs amenable to detection using the GC-MS analytical framework. Given this focus, several OCs that were elusive using the selected GC-MS methodology were excluded from the hazard appraisal. Formaldehyde is a salient example of such a compound. Drawing from the tobacco emission (HCI) dataset, the HQ for formaldehyde across tobacco specimens spanned from 9.07±12.5 (in HTP-C) to 99.4±137 (in 3R4F). The observed broad variability in the toxicity metrics for formaldehyde translates to an HQ range for tobacco examples, from marginally below 1 to as high as 300. Although formaldehyde has substantial relevance in the qualitative hazard profiling of tobacco, issues surrounding its reproducibility and reliability merit attention.

In addition to formaldehyde, this study did not quantify pivotal noxious OCs, such as polycyclic aromatic hydrocarbons (PAHs) and tobacco-specific nitrosamines (TSNAs). These entities are implicated in triggering malignancies and other pathologies, such as chronic obstructive

pulmonary disease (COPD), lung carcinoma, esophageal neoplasms, and oral malignancies (Rahman et al., 2022; Scherer et al., 2022; Zhang et al., 2022). The paucity of accessible NOAEC metrics for most PAHs and TSNAs complicates HQ computation. For the discernible data, the HQ of benzo[a]pyrene, a representative PAH, within tobacco emissions was ascertained to be below unity:0.54 for 3R4F and 0.02 for HTP-C. The diminished concentration thresholds of PAHs in tobacco emissions, manifesting at sub μg cig (or stick)$^{-1}$, rationalize these subdued HQ figures. In addition, the toxicological implications of tar, which is predominantly present in conventional cigarettes, warrant mention. Tar is intrinsically multifaceted and exhibits variability in its toxicological attributes, contingent on its genesis. The consequent physical detriment of human inhalation further convolutes its quantitative representation, akin to that of HQ.

In the face of these inherent challenges in achieving an exhaustive hazard assessment of tobacco specimens, the present study sought to judiciously extrapolate hazards confined to the ambit of deleterious constituents identifiable by the GC-MS modus operandi.

4. Conclusions

In this study, a quantitative analysis of the OCs present in tobacco condensates was performed. Tobacco condensate, which serves as an essential medium for swift toxicity assessments of tobacco derivatives, was sourced from s3R4F cigarettes and three distinct HTP variants: HTP-A, HTP-B, and HTP-C. The determined OC concentrations in the tobacco condensate were juxtaposed with those observed in the tobacco emissions. By leveraging the associated toxicity

datasets, we discerned the differential hazard profiles of the two entities—this comparative analysis aimed to validate the suitability of tobacco condensates for comprehensive tobacco toxicity evaluation.

In the scrutinized tobacco condensate samples, OC varieties ranged from 72 (HTP-C) to 108 (3R4F). Notably, the 3R4F showcased the broadest OC spectrum yet exhibited the lowest TOC concentration of 3777 μg cig^{-1}. This starkly contrasted with the HTP samples, averaging 5258±593 μg $stick^{-1}$. The noted disparity can be traced to elevated concentrations of propylene glycol (608±17.6 μg $stick^{-1}$) and vegetable glycerin (2512±242 μg $stick^{-1}$) in the HTP aerosols. In a contrary observation, the 3R4F presented a TPM concentration of 17,667 μg cig^{-1}, nearly double that of the HTP variants. This increase is likely attributed to the tar content of 3R4F cigarette emissions. The presence of tar was postulated to dampen the efficiency of OC concentration during the condensation process. When evaluating the OC levels in emissions versus condensates, 3R4F showed a mere 30% recovery in its condensed form, with HTP recoveries ranging from 80% to 120%. This results in considerable losses during the 3R4F condensation phase, which is likely steered by tar-mediated OC adsorption. However, the HI for both the 3R4F smoke and condensate deviated by less than 20% (53.6 and 45.2, respectively). Concurrently, HTP HI metrics fluctuated between 30.3–40.0 (condensate) and 28.0–32.7 (aerosol).

Our findings underscore the marked congruence in OC concentrations and HI between HTP aerosols and their corresponding tobacco condensate (HTPAC), thus emphasizing the robustness of toxicity assessments based on HTPAC. While the 3R4F condensate's OC composition

constituted approximately 30% of its emitted counterpart, the ensuing HQ disparity remained within 20%, indicating the potential utility of such extracts for toxicity assessments. This study illustrates that rigorous OC quantification is pivotal for effectively harnessing conventional tobacco condensate (WCSC) in toxicity evaluations. Expanding the quantitative and hazard analyses to encompass other deleterious compounds not addressed in this study would augment the fidelity of toxicity datasets rooted in tobacco condensates. In conclusion, the results of this investigation demonstrate the swift and precise execution of toxicity assessments across an array of tobacco products via their condensate proxies.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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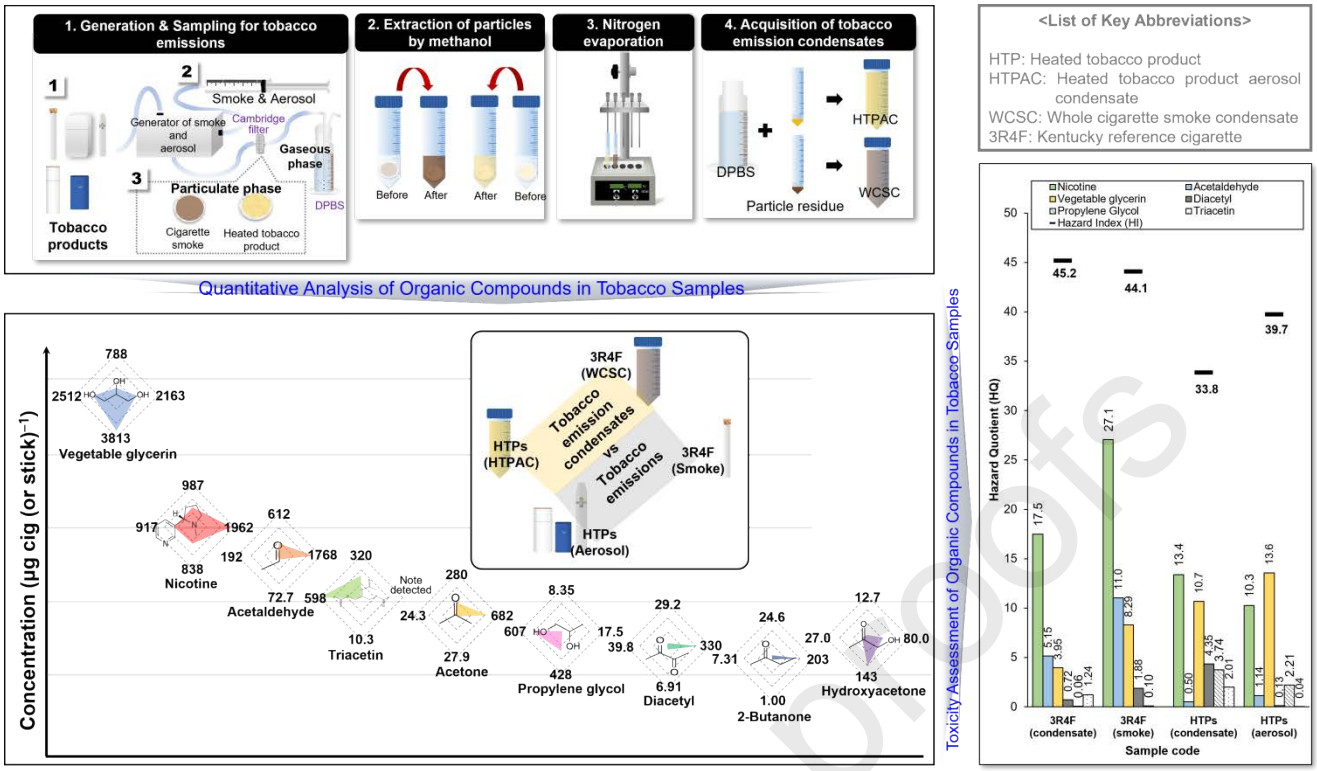
Graphical Abstract



Highlights (maximum 85 characters, including spaces per bullet point)

- Tobacco emission condensates enable rapid toxicity assessment of tobacco products
- OC concentration varied by an average of 5% between HTP aerosol and condensate
- The hazard index (HI) for tobacco emission was 53.6 (3R4F) and 30.4 (HTP)
- The HI values of the tobacco emission condensates were 45.2 (3R4F) and 33.8 (HTP)
- Using tobacco condensate ensures a reliable evaluation of tobacco product toxicity

Author Statement

- Sung-Hwan Kim: Formal analysis, Investigation, Data curation, Writing – Original draft preparation.
- Yong-Hyun Kim: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original draft preparation, Writing – Review & Editing, Supervision.

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: