DETERMINATION OF PRIMARY AROMATIC AMINES IN SMOKELESS TOBACCO PRODUCTS

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Introduction

The list of harmful and potentially harmful constituents (HPHCs) published by the FDA in 2012 features six primary aromatic amine (PAA) compounds — *o*-toluidine, *o*-anisidine, 2,6-dimethylaniline, 1-aminonaphthalene, 2-aminonaphthalene, and 4-aminobiphenyl. Of these six PAAs, five have been deemed to be carcinogenic or potentially carcinogenic in humans by the International Association for Research on Cancer (IARC)[1] (Table 1). PAAs in mainstream smoke are thought to be combustion products formed from the tobacco's nitrogen-containing constituents. Smokeless tobacco products (STPs), therefore, might be expected to be relatively free from PAAs, but some tobacco curing processes may introduce PAA contaminants through exposure to smoke and heat.

In this study, we sought to determine if PAAs are present in a range of STPs and if there is any correlation to the tobacco types used in the product. Using a procedure that incorporated elements from our own in-house methods and a draft CORESTA method for the analysis of PAAs in mainstream cigarette smoke by GC-MS[2], we screened a variety of reference and market products for their PAA content.

Table 1. PAA compounds and their IARC classifications.

PAA	Abbreviation	IARC Classification
ortho-Toluidine	o-TOL	1
ortho-Anisidine	o-AND	2B
2,6-Dimethylaniline (2,6-xylidine)	DMA	2B
1-Aminonaphthalene	1-ANP	3
2-Aminonaphthalene	2-ANP	1
4-Aminobiphenyl	4-AMB	1

Methodology

Primary aromatic amines (PAAs) are extracted from a 0.5 g sample of tobacco using a 1.6 N hydrochloric acid solution. After shaking for 1 hour, samples are spiked with internal standards, filtered, basified by the addition of 5.5 N sodium hydroxide, and then extracted into hexanes. The hexanes layer is taken and dried over Na_2SO_4, and subsequently derivatized with pentafluoropropionic acid (PFPA). Derivatized extracts are then cleaned up by Florisil SPE and analyzed by GC-MS/CI in negative mode. The concentration of each PAA in the sample extract is quantified using the internal standard method and reported on a ng/g basis.

Validation Summary

The method was validated using guidelines issued by the International Conference on Harmonization (ICH).[3] Each PAA analyte was quantitated against a calibration curve generated using non-labelled Guide 34 standards, with the respective deuterated analogue used as the internal standard. All calibration curves were linear with 1/x weighting and R^2 values >0.995. Extraction efficiency was optimized using a sample of ground dark fire-cured (DFC) tobacco that was found to contain relatively high levels of each PAA analyte. Accuracy was determined through low, mid, and high-level recovery spikes in 3R4F tobacco filler, which contains low levels of the analytes. Repeatability and intermediate precision were determined by three different analysts over three days, each performing a set of mid-level spike recoveries in 3R4F tobacco filler and a set of DFC tobacco extractions (five replicates of each set).

Table 2. Summarized validation data.

	o-TOL	o-AND	DMA	1-ANP	2-ANP	4-AMB
Internal Standard	o-Tol-d_7	o-AND-d_3	DMA-d_{11}	1-ANP-d_7	2-ANP-d_7	4-AMB-d_9
Instrument LOQ (ng/mL)	0.012	0.012	0.024	0.008	0.004	0.004
Method LOQ (ng/g)	0.12	0.12	0.24	0.08	0.04	0.04
Spiked 3R4F						
Native levels (ng/g)	0.9	2.0	0.3	<0.03	<0.01	<0.01
Av. Accuracy ± StDev (%)	100 ± 5	102 ± 4	102 ± 5	96 ± 3	97 ± 2	93 ± 3
Av. Repeatability ± StDev (%)	1.5 ± 0.5	2.7 ± 0.6	2.2 ± 0.7	4.4 ± 4.1	1.6 ± 0.4	1.8 ± 0.5
Intermediate Precision (%)	1.9	2.6	2.2	6.0	1.8	2.1
Dark fire-cured tobacco						
Native levels (ng/g)	30.1	6.3	1.5	1.9	2.4	0.3
Av. Repeatability ± StDev (%)	2.3 ± 0.4	2.3 ± 0.7	7.3 ± 2.1	3.7 ± 1.3	3.5 ± 2.2	6.1 ± 1.5
Intermediate Precision (%)	5.1	3.9	8.4	9.1	7.3	7.4

Chromatography

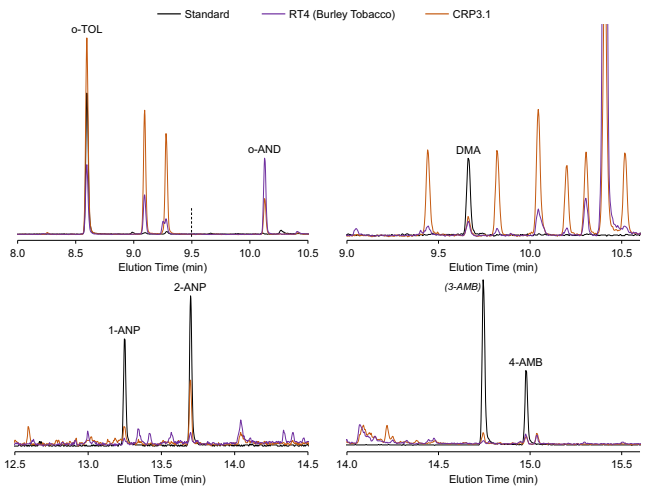

Figure 1. SIM chromatograms for each PAA showing a mid-level standard, a reference Burley tobacco (RT4), and a reference smokeless tobacco product (CRP3.1). Note the SIM chromatograms for o-TOL and o-AND are shown combined despite their different monitoring ions (*m/z* 233 and 249, respectively), the dotted line indicates the change in monitored ion. [Note: 3-aminobiphenyl (3-AMB) was validated for this method but is not discussed here since it is not part of the FDA's HPHC list]

Screening Results

To establish the range of PAA levels present in tobacco products, we screened a number of reference and market STPs. Five ground reference tobaccos (RT) were also obtained from the Center for Tobacco Reference Products (CTRP, University of Kentucky)[4] to explore how the tobacco curing process may affect the resulting PAA content. Three replicates of each product were analyzed and the results are summarized in Table 3.

Table 3. Measured PAA levels in various reference tobacco types and reference and marketed smokeless tobacco products. Values are the average of three (3) replicates. Market products 1 and 2 are dry snuffs; market products 3–8 are moist snuffs. (* indicates values above the method limit of detection, but below the method LOQ)

	o-TOL (ng/g)	o-AND (ng/g)	DMA (ng/g)	1-ANP (ng/g)	2-ANP (ng/g)	4-AMB (ng/g)
CTRP Reference Tobaccos						
Flue-cured (RT2)	0.98 ± 0.168	2.19 ± 0.139	0.37 ± 0.060	0.18 ± 0.047	0.06 ± 0.011	<0.01
Sun-cured (RT3)	0.80 ± 0.115	1.78 ± 0.046	0.15 ± 0.025	0.17 ± 0.006	0.07 ± 0.003	<0.01
Burley (RT4)	3.25 ± 0.107	5.19 ± 0.201	0.59 ± 0.057	0.04 ± 0.014*	0.07 ± 0.013	0.13 ± 0.017
Dark air-cured (RT9)	4.43 ± 0.019	4.68 ± 0.039	0.38 ± 0.032	0.17 ± 0.007	0.06 ± 0.002	0.10 ± 0.008
Dark fire-cured (RT10)	4.99 ± 0.253	3.06 ± 0.158	0.60 ± 0.057	0.24 ± 0.019	0.62 ± 0.141	0.11 ± 0.013
Reference Smokeless Tobacco Products						
CRP1.1	0.58 ± 0.258	0.57 ± 0.034	0.14 ± 0.042	0.05 ± 0.022*	0.06 ± 0.036	0.04 ± 0.005
CRP2.1	2.90 ± 0.017	0.96 ± 0.059	0.24 ± 0.044	0.13 ± 0.005	0.36 ± 0.093	0.09 ± 0.012
CRP3.1	9.15 ± 0.150	2.48 ± 0.017	0.84 ± 0.108	0.28 ± 0.027	0.45 ± 0.029	0.11 ± 0.006
CRP4.1	1.34 ± 0.338	0.65 ± 0.040	0.42 ± 0.118	0.12 ± 0.094	0.11 ± 0.093	0.07 ± 0.020
Market Smokeless Tobacco Products						
Market Product 1	8.55 ± 0.034	1.49 ± 0.020	0.36 ± 0.021	0.05 ± 0.015*	0.09 ± 0.008	0.03 ± 0.003*
Market Product 2	7.86 ± 0.061	1.51 ± 0.075	0.20 ± 0.060	0.03 ± 0.008*	0.11 ± 0.011	0.04 ± 0.004
Market Product 3	2.67 ± 0.211	0.80 ± 0.009	0.38 ± 0.012	0.15 ± 0.007	0.58 ± 0.039	0.06 ± 0.005
Market Product 4	3.52 ± 0.130	1.03 ± 0.017	0.43 ± 0.019	0.18 ± 0.008	0.60 ± 0.022	0.08 ± 0.003
Market Product 5	2.61 ± 0.152	1.46 ± 0.092	0.27 ± 0.033	0.17 ± 0.013	0.31 ± 0.015	0.08 ± 0.006
Market Product 6	4.05 ± 0.069	1.51 ± 0.112	0.37 ± 0.024	0.25 ± 0.010	0.50 ± 0.022	0.08 ± 0.004
Market Product 7	3.29 ± 0.070	0.93 ± 0.031	0.20 ± 0.050	0.20 ± 0.025	0.33 ± 0.027	0.09 ± 0.016
Market Product 8	3.77 ± 0.042	1.09 ± 0.168	0.30 ± 0.019	0.23 ± 0.014	0.39 ± 0.002	0.09 ± 0.010

Discussion

The analysis of the various tobaccos and smokeless tobacco products reveal that the six HPHC-listed PAAs are present to varying degrees, with o-TOL and o-AND the most abundant. As expected, the levels are significantly lower than those generated during smoking, with the exception of o-AND. For example, a 1.5 g "pinch" of market product 5 (M5) contains a similar amount of o-AND to that delivered by a single 3R4F cigarette smoked under ISO 3308 conditions (2.2 ng vs 2.3 ng, respectively), whereas all other PAAs would be less than one-fifth the level.

Among the reference tobaccos screened, the highest PAA levels were found in the air- and fire-cured tobaccos (RT4, RT9, and RT10). While higher levels might be expected in the DFC tobacco due to the use of smoke during curing, this would not account for the levels in air-cured tobaccos. One possibility is that the curing duration may play a part since these three tobacco types are typically cured for multiple weeks and have a longer exposure to elevated temperatures. Of note is that 2-ANP appears to be a characteristic feature of the DFC tobacco PAA profile, whereas it is barely quantifiable in the other tobacco types.

For the CORESTA reference products, the PAA levels appear to follow the trends seen for their constituent tobacco types, especially when corrected for tobacco content (Figure 2). The highest levels are seen for CRPs 2.1 and 3.1, whose blends are composed entirely of air- and fire-cured tobaccos, with DFC tobacco being the major component for both (>60%). CRP4.1, exclusively air-cured tobacco, is intermediate between these and CRP1.1 (23% air-cured tobacco, with the remainder flue- and sun-cured tobaccos).

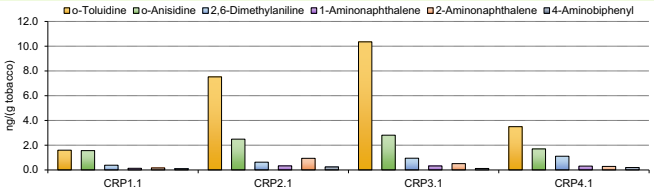

Figure 2. PAA levels in CORESTA reference products adjusted for tobacco content. Total tobacco content was determined using information on each product made available by CORESTA.[5]

For the marketed STPs, information on the tobacco blends used is not available, however, both air- and fire-cured tobaccos are commonly used in moist and dry snuff products and the PAA levels found are consistent with this observation. Furthermore, the presence of 2-ANP suggests the tobacco blends contain a high proportion of DFC tobacco.

Lastly, it can be seen that some of the STPs have higher PAA levels than the reference tobaccos screened, even before correction for actual tobacco content. This is likely due to variations within the tobacco types and can be clearly seen in the two DFC tobaccos that were analyzed. The DFC tobacco used for validation presented significantly higher PAA levels (Table 2) than the RT10 reference tobacco (Table 3) and may be due to differences in the curing procedure. Virginia and Tennessee/Kentucky DFC tobaccos, for example, have different smoke exposure levels during curing.

Conclusions

We have developed and validated a robust method for the determination of primary aromatic amines in smokeless tobacco products. Screening of reference tobacco types showed that the curing process appears to affect the PAA content and may even lead to a characteristic profile for some tobacco types. PAA levels in STPs were found to generally reflect those of the constituent tobaccos within the blend, suggesting that the curing process is the major source of PAAs rather than any post-curing treatments. Despite the relatively low amounts of PAAs in STPs compared to mainstream smoke, the varied usage and consumption patterns within these products warrants further investigation into assessing their PAA levels and the associated risks. Further exploration into the origin of PAAs in cured tobaccos would also be of interest.

References

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