Determination of the levels of NNAL, iso-NNAL and NNA in contemporary US and Swedish smokeless tobacco products

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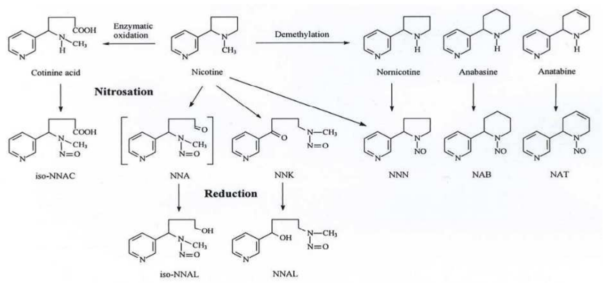
1. Introduction:

There has been considerable interest in recent years in the chemical composition of smokeless tobacco products (STPs), primarily based around health concerns associated with their use. IARC Monograph 89 (1) summarised the presence of 28 chemical agents in STPs including Benzo(a)pyrene (B(a)P), trace metals, aflatoxins, and a number of nitrosated species such as N-nitrosonornicotine (NNN), 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) and 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL). IARC has classified STPs as carcinogenic to humans (Group 1).

Over the last 20-30 years numerous studies have characterised the contents of NNN and NNK in STPs, and research has shown changes in levels of these compounds over this time period. However, other nitrosamines, including NNAL, have received significantly less attention.

The mechanistic formation of NNAL, and other nitrosation reactions occurring in tobacco, have been described by Hoffmann et al (1995) (2)



In addition to NNAL a number of other nitrosamine species are involved in the nitrosation reactions occurring in tobacco, including 4-(N-methyl-N-nitrosamino)-4-(3-pyridyl)-1-butanol (iso-NNAL) and 4-(N-methyl-N-nitrosamino)-4-(3-pyridyl)-butanal (NNA). There have been few studies of the levels of NNAL and iso-NNAL in smokeless tobaccos, and NNA has been described as a very reactive aldehyde which has never been quantified in tobacco or tobacco smoke (1).

We have recently reported levels of NNN and NNK in contemporary smokeless tobacco products (3). However, given the lack of pertinent information in this area, a survey of the levels of NNAL, iso-NNAL and NNA was considered necessary to more fully characterise the chemistry of currently available STPs. The current study focused on the TSNA profiles of contemporary STPs from the USA and Sweden.

2. Smokeless Tobacco Products (4,5):

The STPs analysed in the current work were Swedish pouched and loose snus, US snus, US chewing tobacco, pellet (hard and moist), dry snuff, moist snuff and plug. The differences between these product styles are described below:

Dry Snuff: Powdered tobacco, with a significant proportion of fire cured styles and around 10% moisture content, consumed by placing a pinch of powder between gum and cheek.

Moist snuff: ("Dipping tobacco") cut air-cured and fire-cured tobaccos, blended and fermented, processed into fine particles (fine-cut) or strips (long cut), with a high moisture content of 50-60%. They can be used as small portions or pinches of loose material or in small sachets. They are consumed by positioning in the lower part of the mouth, sucking, and with occasional expectoration.

Plug: The moist plug tobacco examined in this study has a moisture content around 20%, is composed of mild Burley, Virginia and Philippines tobaccos, is finely ground, soaked in honey and pressed into blocks or "plugs". This product style is consumed by chewing.

Chewing tobacco: Loose leaf chewing tobacco is manufactured from air cured tobaccos which are shredded and cased with sugars and flavours; they have a moisture content around 20-30%.

Tobacco Pellets: Two forms of tobacco pellets were examined, a hard pellet form containing fine ground tobacco and inorganic materials, with a moisture content of around 5-10%, which is consumed by allowing it to dissolve in the mouth (hard pellet); and a moister (around 20%) small cylindrical pellet made from single leaf flavoured tobacco (moist pellet) which is kept between cheek and gum until the taste has gone.

Snus: Air-cured and sun-cured tobaccos blended and heat treated, presented as either a loose form (loose snus) or in small sachets (portion snus), with a moisture content normally in the range of 45-60%, although some lower moisture content products have been observed. They are consumed by positioning in the upper part of the mouth, without expectoration.

3. Public health concerns over NNAL, iso-NNAL and NNA:

IARC Monograph 89 did not consider the carcinogenicity of NNAL, iso-NNAL or NNA due to the limited data available on these compounds. However, NNAL is a potent pulmonary carcinogen in rats and mice (6), and Sleiman et al (7) recently called for research to assess human intake and health implications of NNA exposure; iso-NNAL has been reported to be inactive in lung tumorigenesis assays in female *N* mice (8).

4. Methods:

74 STPs available on the Swedish and US markets were sampled in August 2010, consisting of 30 Swedish loose and pouched snus products and 44 US products including chewing tobacco, dry snuff, pellets, moist snuff, US snus, and plug. STPs were sampled to include products from all major manufacturers and represented significant market share brands for all categories.

Method: NNAL, iso-NNAL and NNA were determined at BAT's analytical laboratory using methods developed and validated for this study. Analyses were conducted in triplicate. STP samples were fortified with deuterated internal standards of NNAL, iso-NNAL and NNA, hydrated, allowed to equilibrate and extracted with methanol.

NNA was analysed without further clean-up due to reactivity issues encountered with SPE columns. NNA levels were quantified by LC-MS/MS using an Agilent 1200 series liquid chromatograph, degasser, binary pump, column compartment and autosampler connected to an AB Sciex API 5000 mass spectrometer. Two different MS/MS transitions were examined (MRMs; each for 200 ms): m/z 208.0 → 121.1 for NNA and 211.0 → 124.0 for NNA-d3.

For NNAL and iso-NNAL, the methanolic extracts were acidified and cleaned-up using ion exchange SPE (Oasis MXB-500). NNAL and iso-NNAL levels were quantified using the instrumentation described above but with a different LC method. Four different MS/MS transitions were examined (MRMs; each for 100 ms): m/z 210.1 → 180.1 for NNAL; 210.1 → 121.5 for iso-NNAL; 213.1 → 183.1 for NNAL-d3 and 213.1 → 124.5 for iso-NNAL-d3

The lower limits of quantification (LLOQ) were determined by the analysis of fortified matrix samples with a performance standard of 100±20% recovery. Effective limits of detection (eLOD) were calculated as 30% of the LLOQ combined with visual inspection of the chromatogram to support peak assignment. The following LLOQ and eLOD values were determined:

Nitrosamine	Units	LLOQ	eLOD
NNAL	ng/g (wet weight basis)	28	8.4
iso-NNAL	ng/g (wet weight basis)	29	8.7
NNA	ng/g (wet weight basis)	1151	345

5. Disclosure: The study was funded by British American Tobacco.

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6. Results:

The analysis exercise showed very low levels of the three nitrosamines in most samples, with levels of NNAL up to 2400 ng/g, iso-NNAL up to 540 ng/g, and NNA up to 4300 ng/g on a wet weight basis.

The majority of STPs did not contain detectable levels of iso-NNAL and NNA , but NNAL was detected in all but two samples.

No clear correlations were observed between the three nitrosamines, nor between the nitrosamines and moisture content.

For presentation purposes, in the graphs below, values below LLOQ are represented as 50% of LLOQ, and values below eLOD are represented as 50% of eLOD.

Nitrosamine	Samples with Quantifiable Nitrosamine Values	Samples with Nitrosamine contents below the Quantification limit (Below LLOQ)	Samples where the Nitrosamine was Not Detected (Below eLOD)
NNAL	42	30	2
iso-NNAL	11	8	55
NNA	2	13	59

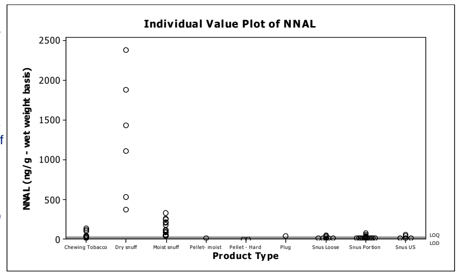
6.1 NNAL Contents of US and Swedish STPs:

The highest levels of NNAL were observed in the dry snuff samples; these levels were higher than for any of the other product categories examined.

The pellet, plug and snus products examined had similar and low NNAL contents; there were few observable differences in NNAL contents of Swedish loose and portion snus and US snus.

NNAL contents of moist snuff and chewing tobaccos were intermediate between these two extremes.

The two products with no detectable NNAL were hard pellet products.



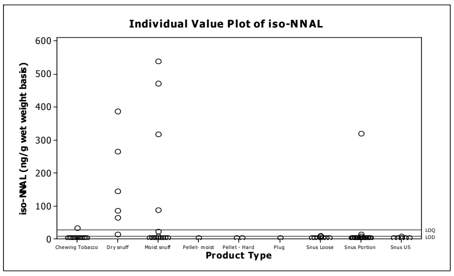
6.2 iso-NNAL Contents US and Swedish STPs:

Although contents up to 540ng/g were recorded in this survey, some samples used for analytical validation had contents up to 900 ng/g of iso-NNAL

The highest levels of iso-NNAL in the survey samples were observed in the moist and dry snuff products; one Swedish snus product was also found to have a relatively high iso-NNAL level.

Plug, pellet, US snus, Swedish loose snus and all but one pouched snus product had similar and relatively low levels of iso-NNAL.

The widest range of measured values was found in moist snuff samples; with both the highest measured values and a significant number of samples with levels below the eLOD for the method.

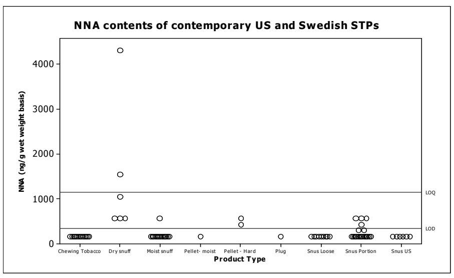


6.3 NNA Contents US and Swedish STPs:

During method development NNA was found to be reactive, with degradation of added ('spiked') NNA occurring within STPs, and NNA extracted from STPs degrading in the SPE column. An appropriate method was developed without SPE clean up, however, interference from other peaks in the samples meant that relatively high LLOQ values were obtained.

As far as we are aware, this is the first reported observation or quantification of NNA in tobacco.

NNA was detected in 15 samples and quantified in 2; both of these samples were dry snuff products. NNA was also observed in some moist snuff, hard pellet and portion snus products.



A method which offers lower LLOQ's would possibly enable the detection or measurement of NNA in a greater number of STP samples.

7. Conclusions:

This study is the first reported instance of NNA being detected in tobacco samples; it was observed in almost a quarter of the samples analysed.

Clear differences were observed in the NNAL, iso-NNAL and NNA contents of different product styles. Dry snuff samples were found to contain the highest levels of NNAL and NNA. Highest levels of iso-NNAL were found with dry and moist snuff samples. On the whole Swedish snus, US snus, plug, pellet and chewing tobacco samples contained lower levels of these nitrosamines.

Overall, similar levels of these nitrosamines were found with Swedish loose snus and US snus samples; some pouched snus levels were higher than observed with the other snus products.

No clear correlations were observed between the three nitrosamines, nor between the nitrosamines and moisture content.

The analytical methods reported here add to the available techniques for determination of nitrosamines in tobacco.

8. References:

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