A Rigorous Extraction Methodology for Snus: Application of ISO 10993-12 Guideline to Permit Thorough *In Vitro* Toxicology Testing

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Introduction

Smokeless tobacco products, and in particular Snus, are being increasingly considered as Potential Reduced Exposure Products (PREPs) and in some quarters (notably Sweden) as cessation products. Although epidemiological evidence from Sweden suggests that Snus use is substantially less hazardous than cigarette smoking, Snus is not considered as harmless and, with the exception of Sweden, is banned in the European Union.

The challenges for *in vitro* toxicology testing of Snus include the fact that the material to be assessed is insoluble (but absorbent) plant matter, and that there is no standard method for applying Snus, or extracts of Snus, to *in vitro* test systems. The objective of the work described was to establish an appropriate extraction methodology for Snus such that robust *in vitro* safety assessment of Snus products could be conducted in the Ames, Mouse Lymphoma and *in vitro* Micronucleus assays for genotoxicity and the Neutral Red Uptake assay for cytotoxicity.

ISO guideline 10993 already exists for the biological evaluation of insoluble medical devices. This has been used as a starting point for an extraction methodology, but certain modifications to the sample preparation recommendations made in ISO 10993-12 were considered warranted to provide a sufficiently rigorous extraction methodology to afford a thorough hazard assessment of Snus in the various *in vitro* toxicity assays.

The ISO 10993-12 guideline specifies a number of variables for sample preparations, many of which are related to the size and nature of the material to be extracted. A standard extraction temperature and time of 37°C for 24 hours was selected as the most appropriate for Snus, and extraction concentration was the major variable assessed. Parameters such as recoverable extraction volume and extraction efficiency were measured for a range of concentrations, and the resulting extracts were assessed in the various *in vitro* toxicity assay systems at the maximum volume additions that each will tolerate, to determine whether dose-limiting cytotoxicity levels could be achieved.

Materials and Methods

Snus samples were extracted for 24 hours at 37°C in both water and dimethyl sulphoxide (DMSO), at varying concentrations up to 500 mg/mL (equivalent) (Figure 1). All Snus samples were provided by Swedish Match.

Extracts of moist snuff/Snus were prepared using the following procedure at concentrations of 200, 300, 400 or 500 mg of tobacco product per mL, and conducted using sterile containers and solutions, in order to minimise any contamination from external sources.

- Moist snuff/Snus was weighed and mixed with appropriate volumes of sterile purified water or DMSO to produce the required w/v concentration. If the tobacco was not finely divided then brief homogenisation was performed.
- Extractions were performed for 24 hours at 37°C, with shaking.
- At the end of the extraction period, extracts were centrifuged at approximately 1800g for 30 minutes, and heavy particulates removed by decanting off the supernatant.
- The supernatant was further centrifuged at 25,000g for 30 minutes, and fine particulates removed by decanting off the supernatant.
- The final supernatant was adjusted to pH 7.4±0.2 with Hydrochloric acid or Sodium hydroxide (water extracts only).
- The resulting extracts were filter sterilised using a 0.2 µm pore size filter (pre-filtering using a larger pore size was performed where required).
- Aliquots of extracts were stored at approximately -80°C, and used within 3 months of extraction.

Figure 1. Extraction conditions.

The extraction efficiency (percentage nicotine recovery) of each extract was assessed by comparing the achieved nicotine concentration with the known nicotine content of the Snus product being extracted. Nicotine concentrations were determined using high-performance liquid chromatography (HPLC) and UV detection methodology, adapted from that described in USP 27, 2004.

The ISO 10993-12 guideline indicates that there are no standardised methods for testing absorbents, but suggests that the volume of extraction vehicle that 0.1 g of material absorbs should be determined, and extractions performed at 0.1 or 0.2 g of material per mL of extract vehicle but with the appropriate additional volume of extract vehicle that the material will absorb also added. The volume of extract vehicle absorbed by each Snus product used was not assessed in advance, but assessment was made by measuring the volume of extract that could be recovered from each extract concentration. As all extractions were performed at 200 mg/mL (equivalent) or above, without further 'dilution' of the extraction with additional absorbance volume, all extractions were performed at higher concentrations than those specified in the ISO 10993-12 guideline.

Assessments of the cytotoxicity of the Snus extracts were performed in Ames, Mouse Lymphoma, *in vitro* Micronucleus and Neutral Red Uptake assay systems, using methodologies consistent with standard regulatory guidelines for these assays (OECD and ICH). In each case, extracts were tested at the highest volume additions that can be employed with each vehicle within each assay system, namely 0.5 mL/plate (Ames) or 10% v/v (IVM, MLA, NRU) for Snus extractions performed with water, and 0.1 mL/plate (Ames) or 1% v/v (IVM, MLA, NRU) for Snus extractions performed with DMSO.

Results and Discussion

A broadly linear increase in nicotine content compared to extract concentration occurred for the Snus extracts over the assessed extraction concentration range of 200 to 500 mg/mL (equivalent) (Figure 2). The nicotine concentration of the extracts rose from 1.26 to 2.77 mg/mL over this extraction concentration range, and the extraction efficiency was only slightly reduced at the higher concentrations. 500 mg/mL (equivalent) was therefore considered to be an acceptable and appropriate concentration to provide extracts for rigorous assessment of *in vitro* toxicity of the Snus samples.

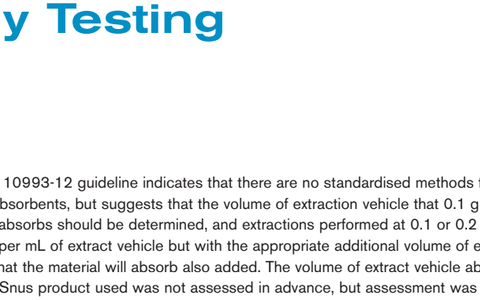

Figure 2. Effect of extract concentration on nicotine content/yield for aqueous Snus extracts.

As expected, increasing the Snus concentration in the aqueous extracts increased the volume of extraction vehicle absorbed, resulting in reduced recoverable volumes of extract (Figure 3). At 500 mg/mL (equivalent), just 21-25% of the extraction vehicle was recovered as final extract, which is equivalent to ≤1 mL of final extract per 2g of sample material. The ISO 10993-12 guideline suggests an extraction concentration of 100 or 200 mg/mL (equivalent) for absorbent materials, but then further diluted to allow for the volume of extract vehicle that this amount of sample material will absorb, giving a recovered extract volume of 10-20 mL per 2g of sample material. Extracting at a concentration of 500 mg/mL (equivalent) therefore provides a considerably more concentrated extract than required by the ISO 10993-12 guideline. Furthermore, the recovered volume of extract is so low that this extraction concentration is considered to be the highest that can practicably be employed with Snus, and therefore permits the most robust *in vitro* toxicity assessment of the Snus samples.

Extract Concentration (mg/mL equivalent)	Extraction Vehicle Recovery (%)
200	45-68
300	25-48
400	21-39
500	21-25

Figure 3. Aqueous extract vehicle recovery.

The following cytotoxicity and genotoxicity results were obtained with 500 mg/mL (equivalent) Snus extracts in each of the assay systems employed:

Bacterial Mutation (Ames) Assay

- No clear evidence of toxicity with either water or DMSO extracts.
- Some small increases in revertant numbers seen: only occurred with water extract treatments in strain TA100 under the extreme assay conditions of concentrations up to 250,000 µg/plate (equivalent) (Figure 4).

Mouse Lymphoma Assay (MLA)

- Cytotoxic effects approaching or within 10-20% Relative Total Growth (RTG) occurred with some maximum achievable treatment concentration treatments: only occurred following 24-hour treatments with water extract in the absence of a metabolic activation system (S9) and 3-hour treatments with DMSO extract in the absence and presence of S9 (Figure 5). Subsequent work (not reported) showed less toxicity following DMSO extract treatments.

In Vitro Micronucleus Assay

- Extreme cytotoxicity (≥60% reduction in Replication Index [RI]) was observed with both water and DMSO extracts at varying concentrations, but only following 20-hour treatments (Figure 6).
- Cytotoxicity approaching the limit level (60% RI) occurred following 3-hour treatments with DMSO extract at the highest treatment concentration (10,000 µg/mL (equivalent); achieved using 2% v/v additions) (Figure 6).

Neutral Red Uptake Assay

- No cytotoxic effects approaching the limit level of 50% survival were observed with any water or DMSO extract treatments (Figure 7).

Pappas *et al* (2008)[1] demonstrated that extractable levels of certain toxic/genotoxic metals such as cadmium, cobalt and nickel can be more efficiently extracted by using artificial saliva. However, Covance has recently performed Snus extractions in artificial saliva[2], and these provided comparable results (not reported) in the various *in vitro* toxicology assays to those achieved using water extracts.

Other workers[3] have performed genotoxicity testing of Snus using lower extract concentrations, but subsequently concentrating the extracts by evaporation or distillation and resuspension. We have performed no comparisons between our extraction process and these concentration methodologies, but we do have concerns that employing such concentration techniques may alter in some way the complex mixture of chemicals in the initial extracts, and therefore not permit a truly representative *in vitro* toxicology assessment.

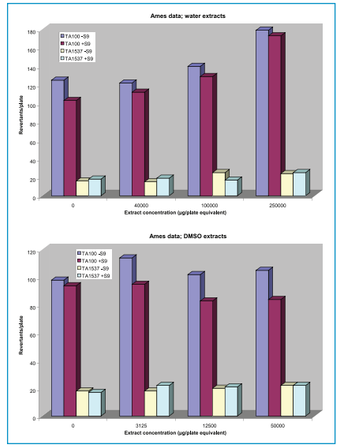

Figure 4. Ames data.

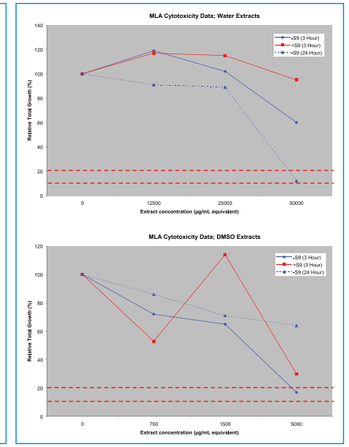

Figure 5. MLA cytotoxicity data.

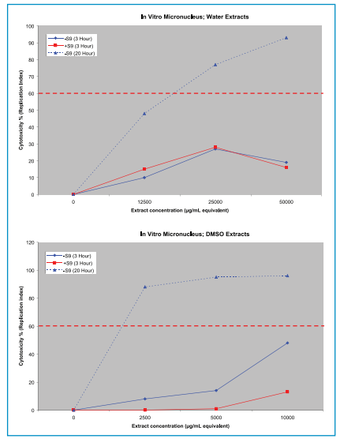

Figure 6. *In vitro* micronucleus data.

Extract Vehicle	Treatments Concentration (µg/mL equivalent)	Percent Survival	
Water	50,000	86%	83%
DMSO	5000	89%	78%

Figure 7. Neutral Red assay; cytotoxicity data.

Conclusions

Due to recovery levels of extraction vehicle (as final extract), 500 mg/mL (equivalent) is considered to be the highest extraction concentration that can practicably be employed for Snus. This is at least 2.5 times more concentrated and least 10 times more rigorous (based on the recovered extraction volume) than ISO 10993-12 specifies for absorbent materials.

Extraction efficiency (based on nicotine concentrations) is not notably affected for Snus extractions performed up to 500 mg/mL (equivalent).

500 mg/mL (equivalent) extracts are considered capable of providing a thorough and rigorous safety evaluation testing of Snus in Ames, Mouse Lymphoma, *in vitro* Micronucleus and Neutral Red assays for the following reasons:

- The extraction concentration far exceeds that required by ISO 10993-12.
- Exposure concentrations required by standard regulatory guidelines applicable for these assays are met or exceeded.
- Treatments up to dose-limiting cytotoxicity levels are achievable with at least some treatment conditions in the *in vitro* Micronucleus and Mouse Lymphoma assays.

Acknowledgements

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