

Final Summary Report

Study Title	Determination of appropriate extraction methodology for Swedish moist snuff (Snus) and toxicity evaluations of resulting extracts in various assay systems
Test Articles	Kentucky Reference Moist Snuff, Batch 2S3 Swedish Moist Snuff (Snus) CPS Swedish Moist Snuff (Snus) CDM
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Covance Study Number	1138/16
Report Issued	October 2010
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Study Director Signature



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25 October 2010
Date

STUDY RESULTS SUMMARY

<u>CLE STUDY NUMBER</u>	1138/16.
<u>TEST ARTICLE DETAILS</u>	The test articles were extracts from the following moist snuff samples:
Name / Batch	Kentucky Reference Moist Snuff, Batch 2S3 Swedish Moist Snuff (Snus) CPS Swedish Moist Snuff (Snus) CDM.
Appearance:	Brown fibres (for the Snus samples, provided in damp sachets).
Storage conditions:	1-10°C in the dark.
<u>STUDY TYPE</u>	Determination of appropriate extraction methodology to permit thorough and robust testing of test articles in various assay systems.
<u>EXTRACTION VEHICLES</u>	Water or Dimethyl sulphoxide (DMSO).
<u>EXTRACTION CONDITIONS</u>	Extractions were performed under the following conditions, nominally split into experimental (extraction) phases 1, 2 and 3, which comprised the assessment and validation process.

Phase	Batch(es) extracted	Extraction solvent	Extraction concentration. (mg/mL equivalent)	Extraction duration (hours)	Extraction temperature
1	CPS, CDM	Water	200	2	Room Temperature
2	CPS	Water	200	24	37°C
	CDM	Water	200	24	37°C
	Kentucky	Water	200	24	37°C
3.1	CPS	Water	200 (x 3)	24	37°C
3.2	CPS	Water	300, 400, 500	24	37°C
3.3	CPS	DMSO	200, 300, 400, 500	24	37°C

Further details of the extraction conditions employed are presented in Appendix 1.

Representative (rather than all) Snus batches were selected for extraction and assessment in some phases, so not all batches were assessed in each phase.

Due to absorbency of the Snus, for all extractions the amount of extract recovered was less than the amount of extraction vehicle recovered, and the recovery rate of the extraction vehicle (volume recovered/volume added) reduced with increasing extract concentration as follows:

200 mg/mL equivalent aqueous extract:	45-68% recovery
300 mg/mL equivalent aqueous extract:	25-48% recovery
400 mg/mL equivalent aqueous extract:	21-39% recovery
500 mg/mL equivalent aqueous extract:	21-25% recovery.

ASSAY SYSTEMS

Bacterial Mutation Assay:

Extracts of moist snuff/Snus were assessed in a bacterial mutation assay system, using *Salmonella typhimurium* strains TA100 and TA1537 using a plate incorporation methodology in the absence and presence of S-9, as described in OECD guideline 471 (adopted 1997), UKEMS Guidelines (1990) and ICH Harmonised Tripartite Guideline (1997). Treatments were assessed for both mutation induction (increases in revertant numbers) and toxicity (background bacterial lawn effects and/or reductions in revertant numbers).

Mouse Lymphoma Assay:

Extracts of moist snuff/Snus were assessed in L5178Y cells in a mouse lymphoma assay (MLA), as described in OECD Guideline 476 (adopted 21 July 1997), UKEMS Guidelines (1990) and ICH Harmonised Tripartite Guideline (1997). Treatments were conducted using 3 hour and 24 hour treatment in the absence of S-9 and 3 hour treatment in the presence of S-9. Treatments were assessed for cytotoxicity only (no assessment for mutation induction was performed) using relative total growth (RTG) evaluations.

***In Vitro* Micronucleus Assay:**

Extracts of moist snuff/Snus were assessed in Chinese hamster V79 cells in an *in vitro* micronucleus assay, in line with IWGT recommendations and draft OECD guideline 487 (2004). Treatments were conducted using 3 hour (pulse) treatments in the absence and presence of S-9, followed by a 17 hour recovery period (3+17) and 20 hour (continuous) treatment in the absence of S-9 and 3 hour treatment in the presence of S-9. Treatments were assessed for cytotoxicity only using Replication Index (RI) evaluations.

Neutral Red Cytotoxicity Assay:

Extracts of moist snuff/Snus were assessed in Balb/c 3T3 cells in the neutral red

cytotoxicity assay. Cytotoxicity was measured by assessment of neutral red uptake after 24 hours of chemical exposure.

Nicotine Determination:

Samples of extracts of moist snuff/Snus were analysed for nicotine content by HPLC using the written analytical procedure Covance CLE 1138/016A, which is presented in Appendix 2 of this report.

CONTROLS

Extraction blanks were used as negative controls in each assay system.

**TREATMENT
CONCENTRATIONS**

As extracts of the test articles were used in this validation study, all treatment concentrations are equivalent concentrations, based on the concentration of test article in the extraction mix.

For the Ames assay, treatments were performed using additions of 0.5 mL water extracts per plate or 0.1 mL of DMSO extracts per plate.

For the mouse lymphoma, *in vitro* micronucleus and neutral red cytotoxicity assays, treatments were performed using 10% v/v additions of water extracts and 1% or 2% v/v additions of the DMSO extracts.

**METABOLIC
ACTIVATION
SYSTEM**

Aroclor induced mammalian liver post-mitochondrial fraction (S-9) employed (as required) for each assay.

RESULTS

Detailed results presented in specific assay sections. Summarised results are as follows:

Bacterial Mutation (Ames) Assay:

No evidence of toxicity in the assay system when treated at 0.5 mL per plate (aqueous extracts) or 0.1 mL per plate (DMSO extracts), with extracts at concentrations up to 500 mg/mL equivalent. Therefore treatments could be performed up to these maximum Snus extract treatment concentrations in this assay system without exceeding toxicity limit levels.

Mouse Lymphoma Assay (MLA):

Following 3 hour treatments using 10% v/v additions with aqueous Snus extracts at concentrations up to 500 mg/mL equivalent, no toxic effects approaching the toxicity limiting level of 10-20% RTG were evident, either in the absence or presence of S-9.

3 hour treatments using 1% v/v additions with DMSO Snus extracts at concentrations of 500 mg/mL equivalent resulted in RTG values approaching or within the toxicity limiting level of 10-20% RTG both in the absence and presence of S-9, but no such toxicity was observed following treatments using a lower DMSO Snus extract concentration (200 mg/mL equivalent).

24 hour treatments in the absence of S-9 using water and DMSO Snus extracts (10% v/v and 1% v/v additions respectively) at concentrations of 500 mg/mL equivalent resulted in RTG values approaching the toxicity limiting level of 10-20% RTG for the water extract, but not for the DMSO extract. No toxicity approaching the limit level was observed following treatments using a lower aqueous Snus extract concentration (200 mg/mL equivalent).

It was therefore considered that treatments in this assay system with aqueous and DMSO Snus extracts at a concentration of 500 mg/mL equivalent could be performed without exceeding toxicity limit levels, but toxicity may approach these levels when employing 24 hour treatments.

***In Vitro* Micronucleus Assay:**

Following 3 hour treatments using 10% v/v additions with aqueous Snus extracts at concentrations up to 500 mg/mL equivalent, toxic effects did not approach the limiting level of 50% cytotoxicity (as assessed by RI values), either in the absence or presence of S-9.

3 hour treatments using 2% v/v additions of DMSO Snus extract at 500 mg/mL resulted in cytotoxicity at approximately the limit level of 50%. Similar treatments using 200 mg/mL equivalent DMSO extracts did not result in any toxicity approaching the limit level in this assay system.

20 hour treatments in the absence of S-9 using 10% v/v additions of aqueous Snus extracts at 200, 400 and 500 mg/mL equivalent or 2% v/v additions of DMSO Snus extracts at 200 and 500 mg/mL equivalent all demonstrated extreme toxicity (>90% cytotoxicity) that exceeded the toxicity limit level for this assay (~50% cytotoxicity).

It was therefore considered that no dose limiting toxic effects were likely to occur when using 3 hour treatments in the absence and presence of S-9 in this assay system with aqueous Snus extracts at concentrations up to 500 mg/mL equivalent (as little toxicity occurred when tested up to 400 mg/mL equivalent). 20 hour treatments resulted in extreme cytotoxicity exceeding the dose limiting level with both aqueous and DMSO Snus extracts at 200 mg/mL equivalent and above, and therefore dilution of these extracts to lower concentrations is likely to be required in order to achieve

acceptable toxicity limit levels in this assay system when using this treatment period.

Neutral Red Uptake (NRU) Assay:

Following treatments using 10% v/v additions with aqueous Snus extracts and 1% v/v additions with DMSO Snus extracts at concentrations up to 500 mg/mL equivalent, no reproducible toxicity was observed that approached the 50% cytotoxicity level. It was therefore considered that treatments using aqueous or DMSO Snus extracts at 500 mg/mL equivalent would not provide notable cytotoxicity in this assay system.

Nicotine Determination:

Good consistency in nicotine levels was obtained between separate 200 mg/mL aqueous Snus extracts, indicating that the extraction methodology provided consistent levels of extracted material. Estimated nicotine recovery levels (based on information provided on nicotine levels of the Snus batch used) from 200, 300, 400 and 500 mg/mL equivalent aqueous extracts were 84%, 78%, 76% and 73%, indicating that nicotine content does continue to increase proportionately over these increasing extraction concentrations, with only a relatively small reduction in recovery efficiency.

CONCLUSIONS

Based on nicotine determination data, proportionately increasing amounts of nicotine occur in aqueous Snus extracts prepared at concentrations of 200 to 500 mg/mL equivalent using the methodology employed for this study. Over this extract concentration range, the nicotine recovery level reduces only slightly (from 84% at 200 mg/mL equivalent to 73% at 500 mg/mL equivalent). However, the extract vehicle volume recovery decreases significantly over these extract concentrations, such that at 500 mg/mL equivalent as little as 21% of the initial extract volume may be recovered as final extract, making extractions at higher concentrations prohibitive.

When 500 mg/mL equivalent Snus extracts were tested in the assay systems assessed in this study at the maximum achievable treatment volume (10% v/v for aqueous extracts and 1% v/v for DMSO extracts in the MLA, NRU and *in vitro* micronucleus assays, or 0.5 mL per plate and 0.1 mL per plate respectively in the Ames assay), toxicity approaching or exceeding limit levels only occurred in the MLA and *in vitro* micronucleus assays. In both these assay systems, cytotoxicity approaching or exceeding dose limiting levels only occurred following short (3 or 4 hour) treatments with DMSO extracts at 500 mg/mL equivalent, or with continuous (20 or 24 hour) treatments using either aqueous or DMSO extracts.

It is considered that 500 mg/mL equivalent is a maximum practicable (and therefore appropriate) Snus extraction concentration to generate extracts for testing in the assay systems assessed in this study. It is further considered that by using the extraction

methodology employed in this study, extracts at these concentrations contain proportionate amounts of extractable material from the Snus to those extracted at lower concentrations. Consequently, use of these Snus extracts to perform treatments in the assay systems assessed in this study, at the maximum tolerated volumes for each assay system, permit testing at the highest practicable and achievable concentrations, and therefore provide a thorough and robust assessment in each assay system. For some treatment conditions in some assays, this maximum achievable concentration approached or exceeded a cytotoxicity limiting level.

BACTERIAL MUTATION ASSAY

TOXICITY RESULTS

- Phase 1: Strain TA100 and TA1537 assayed in the absence and presence of S-9 using 200 mg/mL equivalent water extracts of Snus batches CPS and CDM, allowing treatments up to final concentrations of 100,000 µg/plate equivalent. No clear evidence of toxicity was observed.
- Phase 2: Strain TA100 and TA1537 assayed in the absence and presence of S-9 using 200 mg/mL equivalent water extracts of Snus batches CPS, CDM and Kentucky 2S3, allowing treatments up to final concentrations of 100,000 µg/plate equivalent. No clear evidence of toxicity was observed.
- Phase 3: Only phases 3.2 and 3.3 were run in this assay system. Strains TA100 and TA1537 were assayed in the absence and presence of S-9 using water and DMSO extracts of Snus batch CPS at 200 to 500 mg/mL equivalent, allowing treatments up to final concentrations of 250,000 µg/plate equivalent (water extracts) or 50,000 µg/plate equivalent (DMSO extracts). No clear evidence of toxicity was observed.

MUTAGENICITY RESULTS

- Phase 1: Small increases in revertant numbers occurred following CDM extract treatments at 100,000 µg/plate equivalent in strain TA100 in the absence and presence of S-9, which may have been indicative of some mutagenic activity.
- Phase 2: Small increases in revertant numbers occurred following treatments with all 3 Snus batches at 100,000 µg/plate equivalent in strain TA100 in the absence and presence of S-9, which may have been indicative of some mutagenic activity.
- Phase 3: Small increases in revertant numbers occurred following some CPS water extract treatments at the higher concentrations tested in strain TA100 in the absence and presence of S-9, which may have been indicative of some mutagenic activity. No notable increases were observed following any DMSO extract treatments.

Phase 1 results

**Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H₂O): summary of mean revertant colonies (-S-9)
 Phase 1**

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	114 ± 15	6 ± 2
SNUS-CPS	5000	112 ± 8	10 ± 3
	10000	93 ± 4	8 ± 2
	20000	112 ± 17	8 ± 4
	100000	128 ± 7	8 ± 4
Positive controls	Compound	NaN ₃	AAC
	Dose Level	2 µg	50 µg
	Mean ± SD	525 ± 60	217 ± 76

SD Standard deviation

NaN₃ Sodium azide

**Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H₂O): summary of mean revertant colonies (+S-9)
 Phase 1**

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	104 ± 16	13 ± 5
SNUS-CPS	5000	89 ± 10	11 ± 4
	10000	98 ± 5	10 ± 2
	20000	102 ± 8	11 ± 3
	100000	128 ± 6	12 ± 2
Positive controls	Compound	AAN	AAN
	Dose Level	5 µg	10 µg
	Mean ± SD	1208 ± 199	195 ± 17

SD Standard deviation

AAN 2-Aminoanthracene

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H2O) Phase 1

Table 1

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	136	120	95	111	106
5000	121	109	107		
10000	93	97	90		
20000	97	130	108		
100000	136	125	124		
Positive	470	515	589		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	113.60	5		15.44			
5000	112.33	3	0.99	7.57	0.05 NS	-0.00	-0.11 NS
10000	93.33	3	0.82	3.51	0.59 NS	-0.00	-2.38 NS
20000	111.67	3	0.98	16.80	0.15 NS	-0.00	-0.22 NS
100000	128.33	3	1.13 [^]	6.66	0.48 *	0.00	1.67 NS
Positive	524.67	3	4.62	60.09			
M Statistic = 1.285							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H2O) Phase 1

Table 2

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	97	132	93	106	93
5000	99	80	89		
10000	94	98	103		
20000	103	109	93		
100000	123	126	135		
Positive	1349	1294	980		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	104.20	5		16.42			
5000	89.33	3	0.86	9.50	0.50 NS	-0.00	-1.88 NS
10000	98.33	3	0.94	4.51	0.25 NS	-0.00	-0.68 NS
20000	101.67	3	0.98	8.08	0.02 NS	-0.00	-0.27 NS
100000	128.00	3	1.23 [^]	6.24	0.70 ***	0.00	2.86 *
Positive	1207.67	3	11.59	199.07			
M Statistic = 1.224							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H₂O) Phase 1

Table 3

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	13	9	10	10	6
5000	7	5	7		
10000	16	7	6		
20000	7	16	8		
100000	7	12	14		
Positive	110	312	190		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	9.60	5		2.51			
5000	6.33	3	0.66	1.15	0.65 NS	-0.00	-1.35 NS
10000	9.67	3	1.01	5.51	0.05 NS	-0.00	-0.11 NS
20000	10.33	3	1.08	4.93	0.14 NS	0.00	0.20 NS
100000	11.00	3	1.15 [^]	3.61	0.23 NS	0.00	0.50 NS
Positive	204.00	3	21.25	101.73			
M Statistic = 1.366							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H₂O) Phase 1

Table 4

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	17	11	12	14	12
5000	6	15	22		
10000	20	16	8		
20000	6	6	9		
100000	15	19	13	M	
Positive	109	147	169		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	13.20	5		2.39			
5000	14.33	3	1.09	8.02	0.13 NS	0.00	0.11 NS
10000	14.67	3	1.11	6.11	0.14 NS	0.00	0.31 NS
20000	7.00	3	0.53	1.73	0.44 NS	-0.00	-2.12 NS
100000	15.67	3	1.19 [^]	3.06	0.17 NS	0.00	0.69 NS
Positive	141.67	3	10.73	30.35			
				M Statistic = 1.487			

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

M : Plate counted manually
^ : Maximum increase over control

**Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H₂O): summary of mean revertant colonies (-S-9)
Phase 1**

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	121 ± 9	10 ± 3
SNUS-CDM	5000	107 ± 14	6 ± 1
	10000	100 ± 2	10 ± 6
	20000	95 ± 8	10 ± 5
	100000	169 ± 2 (M)	11 ± 4
Positive controls	Compound	NaN ₃	AAC
	Dose Level	2 µg	50 µg
	Mean ± SD	540 ± 12	204 ± 102

SD Standard deviation

NaN₃ Sodium azide

M : Plate counted manually

Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H₂O): Summary of mean revertant colonies (+S-9) Phase 1

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	92 ± 8	13 ± 2
SNUS-CDM	5000	89 ± 2	14 ± 8
	10000	98 ± 15	15 ± 6
	20000	109 ± 16	7 ± 2
	100000	195 ± 23 (M)	16 ± 3
Positive controls	Compound	AAN	AAN
	Dose Level	5 µg	10 µg
	Mean ± SD	1312 ± 35	142 ± 30

SD Standard deviation

AAN 2-Aminoanthracene

M : Plate counted manually

Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H2O) Phase 1

Table 5

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	122	114	112	124	135
5000	91	112	118		
10000	98	99	102		
20000	86	99	100		
100000	168 M	168 M	171 M		
Positive	528	551	540		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	121.40	5		9.15			
5000	107.00	3	0.88	14.18	0.59 NS	-0.00	-2.29 NS
10000	99.67	3	0.82	2.08	0.74 NS	-0.00	-3.44 NS
20000	95.00	3	0.78	7.81	0.75 NS	-0.00	-4.25 NS
100000	169.00	3	1.39^	1.73	0.81 ***	0.00	6.65 ***
Positive	539.67	3	4.45	11.50			
M Statistic = 0.660							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not Significant

Key to postfixes:

M : Plate counted manually

^ : Maximum increase over control

Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H2O) Phase 1

Table 6

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	81	90	92	94	104
5000	90	90	87		
10000	98	113	84		
20000	127	103	98		
100000	169 M	205 M	211 M		
Positive	1322	1273	1341		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	92.20	5		8.26			
5000	89.00	3	0.97	1.73	0.25 NS	-0.00	-0.37 NS
10000	98.33	3	1.07	14.50	0.25 NS	0.00	0.70 NS
20000	109.33	3	1.19	15.50	0.58 *	0.00	1.94 NS
100000	195.00	3	2.11^	22.72	0.96 ***	0.00	10.00 ***
Positive	1312.00	3	14.23	35.09			
M Statistic = 1.416							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

M : Plate counted manually
^ : Maximum increase over control

Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H₂O) Phase 1

Table 7

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	8	6	4	4	8
5000	7	11	12		
10000	8	6	9		
20000	8	12	5		
100000	11	9	4		
Positive	188	160	303		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	6.00	5		2.00			
5000	10.00	3	1.67 [^]	2.65	0.71 *	0.00	2.00 NS
10000	7.67	3	1.28	1.53	0.36 NS	0.00	0.94 NS
20000	8.33	3	1.39	3.51	0.26 NS	0.00	1.17 NS
100000	8.00	3	1.33	3.61	0.08 NS	0.00	0.97 NS
Positive	217.00	3	36.17	75.78			
M Statistic = 0.907							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H₂O) Phase 1

Table 8

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate					
Solvent	19	12	M	16	12	5
5000	14	7		12		
10000	8	11		10		
20000	8	12		14		
100000	14	10		13		
Positive	201	176		209		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	12.80	5		5.26			
5000	11.00	3	0.86	3.61	0.21 NS	-0.00	-0.52 NS
10000	9.67	3	0.76	1.53	0.35 NS	-0.00	-0.96 NS
20000	11.33	3	0.89	3.06	0.17 NS	-0.00	-0.38 NS
100000	12.33	3	0.96	2.08	0.07 NS	0.00	-0.00 NS
Positive	195.33	3	15.26	17.21			
M Statistic = 1.154							

Key to significance:

* p ≤ 0.05 ** p ≤ 0.01 *** p ≤ 0.005 NS Not significant

Key to postfixes:

M : Plate counted manually

Phase 2 results

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H₂O): summary of mean revertant colonies (-S-9) Phase 2

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	95 ± 8	8 ± 2
SNUS-CPS	5000	94 ± 7	10 ± 1
	10000	93 ± 9	9 ± 7
	20000	91 ± 13	9 ± 3
	100000	127 ± 14	9 ± 3
Positive controls	Compound	NaN ₃	AAC
	Dose Level	2 µg	50 µg
	Mean ± SD	594 ± 93	153 ± 8

SD Standard deviation

NaN₃ Sodium azide

AAC 9-Aminoacridine

**Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H₂O): summary of mean revertant colonies (+S-9)
 Phase 2**

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	73 ± 7	14 ± 2
SNUS-CPS	5000	66 ± 3	16 ± 2
	10000	70 ± 6	17 ± 4
	20000	70 ± 6	14 ± 8
	100000	120 ± 8	9 ± 1
Positive controls	Compound	AAN	AAN
	Dose Level	5 µg	5 µg
	Mean ± SD	880 ± 15	43 ± 5

SD Standard deviation

AAN 2-Aminoanthracene

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H₂O) Phase 2

Table 9

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	104	87	91	90	103
5000	89	91	102		
10000	97	99	82		
20000	91	103	78		
100000	130	139	112		
Positive	487	659	636		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	95.00	5		7.91			
5000	94.00	3	0.99	7.00	0.07 NS	-0.00	-0.13 NS
10000	92.67	3	0.98	9.29	0.14 NS	-0.00	-0.33 NS
20000	90.67	3	0.95	12.50	0.21 NS	-0.00	-0.64 NS
100000	127.00	3	1.34 [^]	13.75	0.80 ***	0.00	4.15 ***
Positive	594.00	3	6.25	93.38			
M Statistic = 0.997							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H2O) Phase 2

Table 10

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	70	67	83	68	78
5000	67	63	68		
10000	73	63	73		
20000	75	72	63		
100000	129	115	116		
Positive	871	871	897		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	73.20	5		6.98			
5000	66.00	3	0.90	2.65	0.56 NS	-0.00	-1.65 NS
10000	69.67	3	0.95	5.77	0.31 NS	-0.00	-0.80 NS
20000	70.00	3	0.96	6.24	0.15 NS	-0.00	-0.73 NS
100000	120.00	3	1.64 [^]	7.81	0.93 ***	0.00	9.35 ***
Positive	879.67	3	12.02	15.01			
M Statistic = 0.497							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H2O) Phase 2

Table 11

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
	7	8	12	7	8
Solvent	7	8	12	7	8
5000	10	10	11		
10000	5	4	17		
20000	6	10	12		
100000	11	10	6		
Positive	160	144	154		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	8.40	5		2.07			
5000	10.33	3	1.23 [^]	0.58	0.53 NS	0.00	0.77 NS
10000	8.67	3	1.03	7.23	0.06 NS	0.00	-0.22 NS
20000	9.33	3	1.11	3.06	0.07 NS	0.00	0.33 NS
100000	9.00	3	1.07	2.65	0.00 NS	0.00	0.22 NS
Positive	152.67	3	18.17	8.08			
M Statistic = 1.479							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in H2O) Phase 2

Table 12

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	15	12	15	13	17
5000	14	18	16		
10000	20	13	19		
20000	23	12	7		
100000	8	10	8		
Positive	47	37	45		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	14.40	5		1.95			
5000	16.00	3	1.11	2.00	0.41 NS	0.00	0.54 NS
10000	17.33	3	1.20^	3.79	0.49 NS	0.00	0.94 NS
20000	14.00	3	0.97	8.19	0.01 NS	-0.00	-0.40 NS
100000	8.67	3	0.60	1.15	0.57 NS	-0.00	-2.23 NS
Positive	43.00	3	2.99	5.29			
M Statistic = 1.091							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

^ : Maximum increase over control

**Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H₂O): summary of mean revertant colonies (-S 9)
Phase 2**

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	99 ± 8	7 ± 3
SNUS-CDM	5000	94 ± 7	7 ± 2
	10000	103 ± 14	9 ± 0
	20000	105 ± 12	9 ± 3
	100000	133 ± 18	10 ± 2
Positive controls	Compound	NaN ₃	AAC
	Dose Level	2 µg	50 µg
	Mean ± SD	661 ± 6	100 ± 17

SD Standard deviation

NaN₃ Sodium azide
AAC 9-Aminoacridine

**Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H₂O): summary of mean revertant colonies (+S-9)
 Phase 2**

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	77 ± 3	15 ± 5
SNUS-CDM	5000	62 ± 8	20 ± 3
	10000	74 ± 11	15 ± 2
	20000	73 ± 11	18 ± 1
	100000	158 ± 30	12 ± 3
Positive controls	Compound	AAN	AAN
	Dose Level	5 µg	5 µg
	Mean ± SD	823 ± 50	38 ± 7

SD Standard deviation

AAN 2-Aminoanthracene

Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H₂O) Phase 2

Table 13

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	100	87	96	108	106
5000	101	87	94		
10000	96	119	94		
20000	93	117	106		
100000	145	112	142		
Positive	667	661	656		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	99.40	5		8.41			
5000	94.00	3	0.95	7.00	0.35 NS	-0.00	-0.65 NS
10000	103.00	3	1.04	13.89	0.12 NS	0.00	0.41 NS
20000	105.33	3	1.06	12.01	0.29 NS	0.00	0.70 NS
100000	133.00	3	1.34 [^]	18.25	0.77 ***	0.00	3.73 ***
Positive	661.33	3	6.65	5.51			
M Statistic = 1.282							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H₂O) Phase 2

Table 14

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	78	75	79	73	79
5000	83	M	66	67	
10000	86		71	64	
20000	74		62	83	
100000	7189		129	156	
Positive	838		864	768	

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	76.80	5		2.68			
5000	62.00	3	0.81	7.81	0.86 NS	-0.00	-1.89 NS
10000	73.67	3	0.96	11.24	0.24 NS	-0.00	-0.41 NS
20000	73.00	3	0.95	10.54	0.06 NS	-0.00	-0.49 NS
100000	158.00	3	2.06 [^]	30.05	0.91 ***	0.00	7.93 ***
Positive	823.33	3	10.72	49.65			
M Statistic = 1.687							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H₂O) Phase 2

Table 15

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate					
	7	6	3	11	7	M
Solvent	7	6	3	11	7	M
5000	5	9	8			
10000	9	9	9			
20000	6	11	11			
100000	8	10	12			
Positive	89	91	120			

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	6.80	5		2.86			
5000	7.33	3	1.08	2.08	0.11 NS	0.00	0.41 NS
10000	9.00	3	1.32	0.00	0.41 NS	0.00	1.38 NS
20000	9.33	3	1.37	2.89	0.44 NS	0.00	1.47 NS
100000	10.00	3	1.47^	2.00	0.41 NS	0.00	1.85 NS
Positive	100.00	3	14.71	17.35			
M Statistic = 0.716							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

^ : Maximum increase over control

Swedish Moist Snuff (Snus)-CDM (200 mg/ml in H2O) Phase 2

Table 16

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	14	12	9	18	21
5000	17	21	22		
10000	13	15	16		
20000	19	18	18		
100000	8	14	14		
Positive	46	M	32	35	

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	14.80	5		4.76			
5000	20.00	3	1.35 [^]	2.65	0.57 NS	0.00	2.02 NS
10000	14.67	3	0.99	1.53	0.07 NS	0.00	0.06 NS
20000	18.33	3	1.24	0.58	0.24 NS	0.00	1.46 NS
100000	12.00	3	0.81	3.46	0.41 NS	-0.00	-1.13 NS
Positive	37.67	3	2.55	7.37			
M Statistic = 0.766							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

M : Plate counted manually
^ : Maximum increase over control

Swedish Moist Snuff (Snus)-Kentucky (200 mg/ml in H₂O): summary of mean revertant colonies (-S-9) Phase 2

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	91 ± 10	7 ± 3
SNUS-Kentucky	5000	99 ± 8	8 ± 3
	10000	87 ± 17	8 ± 5
	20000	101 ± 7	9 ± 3
	100000	110 ± 7	10 ± 1
Positive controls	Compound	NaN ₃	AAC
	Dose Level	2 µg	50 µg
	Mean ± SD	630 ± 15	133 ± 51

SD Standard deviation

NaN₃ Sodium azide

AAC 9-Aminoacridine

Swedish Moist Snuff (Snus)-Kentucky (200 mg/ml in H₂O): summary of mean revertant colonies (+S-9) Phase 2

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	71 ± 9	14 ± 4
SNUS-Kentucky	5000	75 ± 7	12 ± 3
	10000	56 ± 7	14 ± 3
	20000	74 ± 14	15 ± 2
	100000	125 ± 19	11 ± 2
Positive controls	Compound	AAN	AAN
	Dose Level	5 µg	5 µg
	Mean ± SD	852 ± 52	95 ± 18

SD Standard deviation

AAN 2-Aminoanthracene

Swedish Moist Snuff (Snus)-Kentucky (200 mg/ml in H2O) Phase 2

Table 17

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	86	99	77	89	102
5000	107	98	92		
10000	74	80	106		
20000	107	101	94		
100000	108	105	118		
Positive	612	637	640		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	90.60	5		10.11			
5000	99.00	3	1.09	7.55	0.45 NS	0.00	1.12 NS
10000	86.67	3	0.96	17.01	0.09 NS	-0.00	-0.58 NS
20000	100.67	3	1.11	6.51	0.25 NS	0.00	1.34 NS
100000	110.33	3	1.22^	6.81	0.56 **	0.00	2.54 *
Positive	629.67	3	6.95	15.37			
M Statistic = 1.169							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

^ : Maximum increase over control

Swedish Moist Snuff (Snus)-Kentucky (200 mg/ml in H₂O) Phase 2

Table 18

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	63	60	80	72	79
5000	69	83	74		
10000	48	59	61		
20000	59	76	86		
100000	143	128	105		
Positive	820	823	912		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	70.80	5		9.09			
5000	75.33	3	1.06	7.09	0.29 NS	0.00	0.59 NS
10000	56.00	3	0.79	7.00	0.53 NS	-0.00	-2.01 NS
20000	73.67	3	1.04	13.65	0.03 NS	-0.00	0.34 NS
100000	125.33	3	1.77 [^]	19.14	0.86 ***	0.00	6.02 ***
Positive	851.67	3	12.03	52.27			
M Statistic = 1.555							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-Kentucky (200 mg/ml in H₂O) Phase 2

Table 19

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
	Solvent	9	4	8	4
5000	11	8	6		
10000	3	12	9		
20000	11	11	6		
100000	10	9	11		
Positive	176	77	146		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	6.80	5		2.59			
5000	8.33	3	1.23	2.52	0.32 NS	0.00	0.75 NS
10000	8.00	3	1.18	4.58	0.20 NS	0.00	0.41 NS
20000	9.33	3	1.37	2.89	0.31 NS	0.00	1.15 NS
100000	10.00	3	1.47 [^]	1.00	0.34 NS	0.00	1.48 NS
Positive	133.00	3	19.56	50.76			
M Statistic = 1.058							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-Kentucky (200 mg/ml in H₂O) Phase 2

Table 20

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate					
Solvent	12	8	15	M	14	20
5000	10	M	10		16	
10000	14		11		17	
20000	16		12		16	
100000	12		12		8	
Positive	108		103		75	

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	13.80	5		4.38			
5000	12.00	3	0.87	3.46	0.24 NS	-0.00	-0.68 NS
10000	14.00	3	1.01	3.00	0.01 NS	-0.00	0.15 NS
20000	14.67	3	1.06 [^]	2.31	0.14 NS	0.00	0.42 NS
100000	10.67	3	0.77	2.31	0.32 NS	-0.00	-1.22 NS
Positive	95.33	3	6.91	17.79			
M Statistic = 0.882							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

M : Plate counted manually
^ : Maximum increase over control

Phase 3.2 results

Swedish Moist Snuff (Snus)-CPS (400 mg/mL in H₂O): summary of mean revertant colonies (-S-9) Phase 3.2

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Water	500 µl	114 ± 10	15 ± 5
CPS(400 mg/mL in H ₂ O)	50000	122 ± 8	22 ± 6
	100000	123 ± 14	22 ± 2
	200000	127 ± 4	22 ± 6
Positive controls	Compound	NaN ₃	AAC
	Dose Level	2 µg	50 µg
	Mean ± SD	716 ± 7	186 ± 37

SD Standard deviation

NaN₃ Sodium azide

AAC 9-Aminoacridine

**Swedish Moist Snuff (Snus)-CPS (400 mg/mL in H₂O): summary of mean revertant colonies (+S-9)
 Phase 3.2**

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Water	500 µl	105 ± 4	21 ± 5
CPS(400 mg/mL in H ₂ O	50000	112 ± 15	16 ± 6
	100000	116 ± 3	25 ± 6
	200000	129 ± 12	18 ± 4
Positive controls	Compound	AAN	AAN
	Dose Level	10 µg	5 µg
	Mean ± SD	1282 ± 122	61 ± 8

SD Standard deviation

AAN 2-Aminoanthracene

Swedish Moist Snuff (Snus)-CPS (400 mg/mL in H₂O) Phase 3.2

Table 21

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	119	119	118	117	96
50000	119	117	131		
100000	109	136	125		
200000	128	123	130		
Positive	710	716	723		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	113.80	5		9.98			
50000	122.33	3	1.07	7.57	0.46 NS	0.00	1.23 NS
100000	123.33	3	1.08	13.58	0.42 NS	0.00	1.34 NS
200000	127.00	3	1.12 [^]	3.61	0.50 *	0.00	1.88 NS
Positive	716.33	3	6.29	6.51			
M Statistic = 0.764							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (400 mg/mL in H₂O) Phase 3.2

Table 22

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	101	105	109	108	100
50000	96	124	117		
100000	119	116	114		
200000	122	143	121		
Positive	1368	1143	1335		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	104.60	5		4.04			
50000	112.33	3	1.07	14.57	0.43 NS	0.00	1.17 NS
100000	116.33	3	1.11	2.52	0.59 *	0.00	1.83 NS
200000	128.67	3	1.23 [^]	12.42	0.76 ***	0.00	3.64 **
Positive	1282.00	3	12.26	121.50			
M Statistic = 0.691							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (400 mg/mL in H₂O) Phase 3.2

Table 23

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	24	10	15	12	16
50000	16	27	22		
100000	23	20	23		
200000	20	28	17		
Positive	152	226	179		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	15.40	5		5.37			
50000	21.67	3	1.41	5.51	0.54 NS	0.00	1.80 NS
100000	22.00	3	1.43 [^]	1.73	0.57 *	0.00	1.94 NS
200000	21.67	3	1.41	5.69	0.44 NS	0.00	1.80 NS
Positive	185.67	3	12.06	37.45			
M Statistic = 1.354							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (400 mg/mL in H₂O) Phase 3.2

Table 24

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	12	21	26	21	23
50000	10	22	16		
100000	27	30	19		
200000	20	20	13		
Positive	63	67	52		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	20.60	5		5.22			
50000	16.00	3	0.78	6.00	0.42 NS	-0.00	-1.22 NS
100000	25.33	3	1.23 [^]	5.69	0.27 NS	0.00	1.11 NS
200000	17.67	3	0.86	4.04	0.07 NS	-0.00	-0.71 NS
Positive	60.67	3	2.94	7.77			
M Statistic = 1.420							

Key to significance:

* p ≤ 0.05 ** p ≤ 0.01 *** p ≤ 0.005 NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in H₂O): summary of mean revertant colonies (-S-9) Phase 3.2

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	500 µl	125 ± 19	16 ± 3
SNUS-CPS Positive controls	40000	122 ± 14	15 ± 5
	100000	140 ± 27	25 ± 2
	250000	179 ± 9	24 ± 2
	Compound	NaN ₃	AAC
	Dose Level	2 µg	50 µg
	Mean ± SD	742 ± 40	86 ± 16

SD Standard deviation

NaN₃ Sodium azide

AAC 9-Aminoacridine

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in H₂O): summary of mean revertant colonies (+S-9) Phase 3.2

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	500 µl	103 ± 12	18 ± 2
SNUS-CPS Positive controls	40000	112 ± 10	19 ± 2
	100000	129 ± 14	17 ± 7
	250000	173 ± 7	25 ± 5
	Compound	AAN	AAN
	Dose Level	10 µg	5 µg
	Mean ± SD	1257 ± 217	92 ± 75

SD Standard deviation

AAN 2-Aminoanthracene

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in H₂O) Phase 3.2

Table 25

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	157	109	125	124	112
40000	109	121	136		
100000	136	115	168		
250000	181	170	187		
Positive	697	771	758		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	125.40	5		19.03			
40000	122.00	3	0.97	13.53	0.11 NS	-0.00	-0.24 NS
100000	139.67	3	1.11	26.69	0.31 NS	0.00	1.06 NS
250000	179.33	3	1.43 [^]	8.62	0.79 ***	0.00	3.85 ***
Positive	742.00	3	5.92	39.51			
M Statistic = 2.559							

Key to significance:

* p ≤ 0.05 ** p ≤ 0.01 *** p ≤ 0.005 NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in H₂O) Phase 3.2

Table 26

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	110	84	108	101	113
40000	101	119	117		
100000	132	141	114		
250000	181	167	170		
Positive	1095	1173	1503		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	103.20	5		11.61			
40000	112.33	3	1.09	9.87	0.42 NS	0.00	1.17 NS
100000	129.00	3	1.25	13.75	0.73 **	0.00	3.15 *
250000	172.67	3	1.67 [^]	7.37	0.94 ***	0.00	7.84 ***
Positive	1257.00	3	12.18	216.58			
M Statistic = 1.051							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in H2O) Phase 3.2

Table 27

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	14	14	20	17	15
40000	12	21	13		
100000	25	23	26		
250000	26	23	24		
Positive	71	103	84		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	16.00	5		2.55			
40000	15.33	3	0.96	4.93	0.11 NS	-0.00	-0.42 NS
100000	24.67	3	1.54^	1.53	0.74 ***	0.00	3.81 ***
250000	24.33	3	1.52	1.53	0.71 ***	0.00	3.68 **
Positive	86.00	3	5.38	16.09			
M Statistic = 0.518							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

^ : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in H₂O) Phase 3.2

Table 28

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	21	18	17	17	18
40000	21	19	17		
100000	9	23	19		
250000	29	27	20		
Positive	177	35	65		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	18.20	5		1.64			
40000	19.00	3	1.04	2.00	0.24 NS	0.00	0.25 NS
100000	17.00	3	0.93	7.21	0.14 NS	-0.00	-0.58 NS
250000	25.33	3	1.39 [^]	4.73	0.56 *	0.00	2.08 NS
Positive	92.33	3	5.07	74.84			
M Statistic = 0.890							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

M : Plate counted manually
^ : Maximum increase over control

Phase 3.3 results

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in DMSO): summary of mean revertant colonies (-S-9) Phase 3.3

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	101 ± 8	19 ± 7
CPS(200 mg/ml in DMSO)	5000	101 ± 4	14 ± 1
	10000	101 ± 21	17 ± 7
	20000	102 ± 20	19 ± 3
Positive controls	Compound	NaN ₃	AAC
	Dose Level	2 µg	50 µg
	Mean ± SD	624 ± 54	208 ± 41

SD Standard deviation

NaN₃ Sodium azide

AAC 9-Aminoacridine

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in DMSO): summary of mean revertant colonies (+S-9) Phase 3.3

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
Blank Extract	100 µl	98 ± 11	18 ± 2
CPS(200 mg/ml in DMSO)	5000	84 ± 2	20 ± 5
	10000	87 ± 8	22 ± 3
	20000	80 ± 8	24 ± 2
Positive controls	Compound	AAN	AAN
	Dose Level	10 µg	5 µg
	Mean ± SD	1051 ± 105	69 ± 3

SD Standard deviation

AAN 2-Aminoanthracene

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in DMSO) Phase 3.3

Table 29

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	102	107	87	103	105
5000	97	104	101		
10000	123	98	81		
20000	98	85	124		
Positive	679	621	572		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	100.80	5		7.95			
5000	100.67	3	1.00	3.51	0.01 NS	-0.00	-0.00 NS
10000	100.67	3	1.00	21.13	0.01 NS	-0.00	-0.07 NS
20000	102.33	3	1.02^	19.86	0.05 NS	0.00	0.10 NS
Positive	624.00	3	6.19	53.56			
M Statistic = 1.933							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

^ : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in DMSO) Phase 3.3

Table 30

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	108	87	86	104	106
5000	82	85	84		
10000	79	89	94		
20000	71	85	85		
Positive	1060	941	1151		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	98.20	5		10.78			
5000	83.67	3	0.85	1.53	0.68 NS	-0.00	-2.31 NS
10000	87.33	3	0.89	7.64	0.52 NS	-0.00	-1.72 NS
20000	80.33	3	0.82	8.08	0.60 NS	-0.00	-2.90 NS
Positive	1050.67	3	10.70	105.31			
M Statistic = 0.775							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in DMSO) Phase 3.3

Table 31

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	14	18	11	28	22
5000	13	14	14		
10000	14	11	25		
20000	21	15	20		
Positive	240	162	223		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	18.60	5		6.69			
5000	13.67	3	0.73	0.58	0.45 NS	-0.00	-1.18 NS
10000	16.67	3	0.90	7.37	0.19 NS	-0.00	-0.50 NS
20000	18.67	3	1.00^	3.21	0.05 NS	0.00	0.11 NS
Positive	208.33	3	11.20	41.02			
M Statistic = 1.731							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

^ : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (200 mg/ml in DMSO) Phase 3.3

Table 32

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	20	19	15	17	18
5000	24	14	21		
10000	20	25	22		
20000	27	23	23		
Positive	72	67	68		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	17.80	5		1.92			
5000	19.67	3	1.10	5.13	0.30 NS	0.00	0.77 NS
10000	22.33	3	1.25	2.52	0.57 *	0.00	2.02 NS
20000	24.33	3	1.37^	2.31	0.70 ***	0.00	2.86 *
Positive	69.00	3	3.88	2.65			
M Statistic = 0.451							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

^ : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in DMSO): summary of mean revertant colonies (-S-9) Phase 3.3

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
DMSO Extract	100 µl	98 ± 6	18 ± 4
CPS(500 mg/ml in DMSO)	3125	114 ± 12	18 ± 2
	12500	102 ± 9	20 ± 5
	50000	105 ± 8	22 ± 4
Positive controls	Compound	NaN ₃	AAC
	Dose Level	2 µg	50 µg
	Mean ± SD	624 ± 42	213 ± 53

SD Standard deviation

NaN₃ Sodium azide

AAC 9-Aminoacridine

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in DMSO): summary of mean revertant colonies (+S-9) Phase 3.3

Substance	Dose Level µg/plate	TA100	TA1537
		Mean ± SD	Mean ± SD
DMSO Extract	100 µl	94 ± 11	17 ± 3
CPS(500 mg/ml in DMSO)	3125	95 ± 11	22 ± 3
	12500	83 ± 15	21 ± 2
	50000	84 ± 12	22 ± 0
Positive controls	Compound	AAN	AAN
	Dose Level	10 µg	5 µg
	Mean ± SD	1054 ± 69	84 ± 13

SD Standard deviation

AAN 2-Aminoanthracene

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in DMSO) Phase 3.3

Table 33

Test strain: TA100 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	101	91	100	105	94
3125	120	121	100		
12500	94	111	100		
50000	109	96	110		
Positive	579	661	632		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	98.20	5		5.63			
3125	113.67	3	1.16 [^]	11.85	0.72 *	0.00	2.53 *
12500	101.67	3	1.04	8.62	0.06 NS	0.00	0.58 NS
50000	105.00	3	1.07	7.81	0.09 NS	0.00	1.14 NS
Positive	624.00	3	6.35	41.58			
M Statistic = 0.638							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

Swedish Moist Snuff (Snus) CPS (500 mg/ml in DMSO) Phase 3.3

Table 34

Test strain: TA100 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	91	77	104	101	96
3125	88	89	107		
12500	91	66	92		
50000	72	85	95		
Positive	1112	1071	978		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient		Slope of best fit		Dunnett's t value	
Solvent	93.80	5		10.62						
3125	94.67	3	1.01^	10.69	0.05	NS	0.00	0.10	NS	
12500	83.00	3	0.88	14.73	0.42	NS	-0.00	-1.27	NS	
50000	84.00	3	0.90	11.53	0.34	NS	-0.00	-1.12	NS	
Positive	1053.67	3	11.23	68.66						
M Statistic = 1.562										

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

^ : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in DMSO) Phase 3.3

Table 35

Test strain: TA1537 -S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	22	12	20	20	16
3125	20	17	18		
12500	15	22	24		
50000	25	24	18		
Positive	157	263	218		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	18.00	5		4.00			
3125	18.33	3	1.02	1.53	0.05 NS	0.00	0.18 NS
12500	20.33	3	1.13	4.73	0.29 NS	0.00	0.83 NS
50000	22.33	3	1.24^	3.79	0.45 NS	0.00	1.53 NS
Positive	212.67	3	11.81	53.20			
M Statistic = 0.729							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

^ : Maximum increase over control

Swedish Moist Snuff (Snus)-CPS (500 mg/ml in DMSO) Phase 3.3

Table 36

Test strain: TA1537 +S-9

Treatment (µg/plate)	Revertant numbers/plate				
Solvent	18	22	14	15	16
3125	25	22	19		
12500	24	20	20		
50000	22	22	22		
Positive	69	95	88		

Treatment (µg/plate)	Mean (of)	N	Fold Increase	Standard Deviation	Correlation Coefficient	Slope of best fit	Dunnett's t value
Solvent	17.00	5		3.16			
3125	22.00	3	1.29 [^]	3.00	0.67 *	0.00	2.64 *
12500	21.33	3	1.25	2.31	0.46 NS	0.00	2.32 NS
50000	22.00	3	1.29	0.00	0.41 NS	0.00	2.67 *
Positive	84.00	3	4.94	13.45			
M Statistic = 0.367							

Key to significance:

* $p \leq 0.05$ ** $p \leq 0.01$ *** $p \leq 0.005$ NS Not significant

Key to postfixes:

[^] : Maximum increase over control

MOUSE LYMPHOMA ASSAY

TOXICITY RESULTS

Toxicity assessments from treatments of each phase were performed using Relative Total Growth (RTG) evaluation. No mutagenicity endpoint evaluations were conducted in this assay system.

- Phase 1: 3 hour treatments performed in the absence and presence of S-9 using water extracts of Snus batches CPS and CDM at 200 mg/mL equivalent. No toxic effects approaching toxicity limit level of 10-20% relative total growth (RTG) occurred. Due to lack of toxicity, next phase to be performed using 24 hour extractions at 37°C, and also DMSO extracts toxicity investigated.
- Phase 2: 3 hour treatments performed in the absence and presence of S-9 using water extracts of Snus batches CPS and CDM and Kentucky 2S3 at 200 mg/mL equivalent. No toxic effects approaching toxicity limit level of 10-20% RTG occurred. Due to lack of toxicity, next phases performed using extractions at higher concentrations.
- Phase 3 3 and 24 hour treatments performed using water and DMSO extracts of Snus batch CPS at 200 and 500 mg/mL equivalent (24 hour treatments only with water extracts at 200 mg/mL). An RTG value of 12% occurred following 24 hour treatments with 500 mg/mL equivalent water extract (treatment concentration of 50,000 µg/mL equivalent). Extreme toxicity (<10% RTG) was observed following 3 hour treatments in the absence and presence of S-9 using DMSO extracts at greater than 3000 µg/mL equivalent (RTG values of 17% and 30% obtained at 3000 µg/mL equivalent in the absence and presence of S-9), but conflictingly no such toxicity was observed even at 5000 µg/mL equivalent with 24 hour treatments, which would be expected to demonstrate greater toxicity than corresponding 3 hour treatments. No dose limiting toxic effects were observed following any other treatments in this phase in this assay system.

Summary of results – Phase 1

Swedish Moist Snuff (Snus) CPS- water extract 3 hour treatment

Treatment (µg/mL)	-S-9 % RTG	+S-9 % RTG
0	100	100
5000	67	107
10000	54	113
20000	61	123

Swedish Moist Snuff (Snus) CDM- water extract 3 hour treatment

Treatment (µg/mL)	-S-9 % RTG	+S-9 % RTG
0	100	100
5000	108	113
10000	136	101
20000	91	74

%RTG Percent relative total growth

Summary of results – Phase 2

Swedish Moist Snuff (Snus) CPS - water extract 3 hour treatment

Treatment (µg/mL)	-S-9 % RTG	+S-9 % RTG
0	100	100
5000	92	87
10000	82	124
20000	90	164

Swedish Moist Snuff (Snus) CDM - water extract 3 hour treatment

Treatment (µg/mL)	-S-9 % RTG	+S-9 % RTG
0	100	100
5000	71	110
10000	89	91
20000	55	97

Kentucky Reference Moist Snuff, batch 2S3 3 hour treatment

Treatment (µg/mL)	-S-9 % RTG	+S-9 % RTG
0	100	100
5000	106	112
10000	86	94
20000	81	48

%RTG Percent relative total growth

Summary of results – Phase 3.1

Swedish Moist Snuff (Snus) CPS- water extract 24 hour treatment

Treatment ($\mu\text{g/mL}$)	-S-9 % RTG
0	100
2000	95
6500	81
20000	52

%RTG Percent relative total growth (adjusted by day 0 factor)

Summary of results –Phase 3.2

Swedish Moist Snuff (Snus) CPS- water extract 3 hour treatment

Treatment (µg/mL)	-S-9 % RTG	+S-9 % RTG
0	100	100
12500	119	117
25000	102	115
50000	60	95

Swedish Moist Snuff (Snus) CPS- water extract 24 hour treatment

Treatment (µg/mL)	-S-9 % RTG
0	100
12500	91
25000	89
50000	12

%RTG Percent relative total growth (adjusted by day 0 factor for 24 hour treatment)

Summary of results –Phase 3.3

Swedish Moist Snuff (Snus) CPS- DMSO 200 mg/mL extract 3 hour treatment

Treatment ($\mu\text{g/mL}$)	-S-9 % RTG	+S-9 % RTG
0	100	100
UTC	102	78
750	79	106
1500	86	115
3000	87	86

Swedish Moist Snuff (Snus) CPS- DMSO 200 mg/mL extract 24 hour treatment

Treatment ($\mu\text{g/mL}$)	-S-9 % RTG
0	100
500	164
1000	136
2000	138

%RTG Percent relative total growth (adjusted by day 0 factor for 24 hour treatment)
UTC Untreated control

Summary of results –Phase 3.3 (continued)

Swedish Moist Snuff (Snus) CPS- DMSO 500 mg/mL extract 3 hour treatment

Treatment (µg/mL)	-S-9 % RTG	+S-9 % RTG
0	100	100
750	72	53
1500	65	114
3000	17	30

Swedish Moist Snuff (Snus) CPS- DMSO 500 mg/mL extract 24 hour treatment

Treatment (µg/mL)	-S-9 % RTG
0	100
1250	86
2500	71
5000	64

%RTG Percent relative total growth (adjusted by day 0 factor for 24 hour treatment)

IN VITRO MICRONUCLEUS ASSAY

TOXICITY RESULTS

Toxicity assessments from treatments of each phase were performed based on cytotoxicity, calculated by replication index (RI) in treated cultures versus concurrent controls. An appropriate maximum level of cytotoxicity in this test system would be approximately 60% reduction in RI. No analysis of micronuclei were conducted in this study.

- Phase 1: Treatments for 3 hours performed in the absence and presence of S-9 using water extracts of Snus batches CPS and CDM at 200 mg/mL equivalent, but subsequently diluted to 25 mg/mL in water, such that the maximum final concentration was 2500 µg/mL equivalent. No toxic effects (approaching 60% cytotoxicity) were observed for Snus batches CPS and CDM. As these treatments were performed with extract diluted to a lower concentration, treatments in the next experimental phase were conducted using undiluted water extract at 200 mg/mL, up to the highest addition volumes (10% v/v) that are tolerated in this assay system.
- Phase 2: Treatments for 3 and 20 hours in the absence of S-9 and for 3 hours in the presence of S-9 performed using water extracts of Snus batches CPS and CDM and Kentucky 2S3 at 200 mg/mL equivalent, giving a maximum final concentration of 20000 µg/mL equivalent. For the 3 hour treatments in the absence and presence of S-9, no toxic effects (approaching 60% cytotoxicity) were observed for Snus batches CPS and CDM and Kentucky 2S3. For the 20 hour treatments in the absence of S-9, ≥59% cytotoxicity was observed at 5000 µg/mL and above with all three Snus batches. Due to lack of toxicity with 3 hour treatments, the next phase used higher extraction concentrations and both water and DMSO extracts.
- Phase 3.2 Treatments for 3 and 20 hours in the absence of S-9 and for 3 hours in the presence of S-9 performed using water extracts of Snus batch CPS at 400 and 500 mg/mL equivalent. Maximum final concentrations were 40000 and 50000 µg/mL equivalent respectively (10% v/v additions). For the 3 hour treatments in the absence and presence of S-9, no toxic effects (approaching 60% cytotoxicity) were observed. For the 20 hour treatments in the absence of S-9, a 48% reduction in RI was observed at 12500 µg/mL equivalent but extreme cytotoxicity (≥77% reduction in RI)

was observed at all other concentrations tested.

Phase 3.3 Treatments for 3 and 20 hours in the absence of S-9 and for 3 hours in the presence of S-9 performed using DMSO extracts of Snus batch CPS at 200 and 500 mg/mL equivalent. Maximum final concentrations were 4000 and 10000 µg/mL equivalents respectively (2% v/v additions). For the 3 hour treatments in the absence and presence of S-9, 48% cytotoxicity was observed at 10000 µg/mL equivalent following 3 hour treatment in the absence of S-9, otherwise no toxic effects (approaching 60% cytotoxicity) were observed. For the 20 hour treatments in the absence of S-9, extreme cytotoxicity ($\geq 69\%$ reduction in RI) was observed at all concentrations tested.

Results Summary

Phase 1 Swedish Moist Snuff (Snus) CPS and CDM- water extract

Concentration (µg/mL equivalent)	%Cytotoxicity					
	3+17-S9 CPS	3+17 +S9 CPS	20+0-S9 CPS	3+17-S9 CDM	3+17 +S9 CDM	20+0-S9 CDM
69.98	-	1	-	10	0	11
116.6	-	1	1	13	0	19
194.4	-	0	6	8	0	8
324	-	0	6	8	0	4
540	-	2	3	4	0	8
900	4	1	11	5	0	15
1500	4	0	15	5	5	21
2500	2	0	22	10	0	30

Phase 2 Swedish Moist Snuff (Snus) CPS, CDM and Kentucky - water extract

Concentration (µg/mL equivalent)	%Cytotoxicity					
	3+17-S9 Kentucky	3+17 +S9 Kentucky	20+0-S9 Kentucky	3+17-S9 CPS	3+17 +S9 CPS	20+0-S9 CPS
0	-	-	-	-	-	-
625	1	2	8	0	7	5
1250	3	6	31	1	3	16
2500	8	6	46	6	4	34
5000	-	8	64	12	6	59
10000	16	7	82	21	7	74
20000	18	15	94	32	8	92

Concentration (µg/mL equivalent)	%Cytotoxicity		
	3+17-S9 CDM	3+17 +S9 CDM	20+0-S9 CDM
625	5	6	17
1250	12	8	29
2500	15	8	43
5000	16	7	70
10000	23	12	76
20000	30	8	91

Phase 3.2 Swedish Moist Snuff (Snus) CPS- water extract

Concentration (µg/mL equivalent)	%Cytotoxicity					
	3+17-S9 aqueous 400 mg/mL	3+17 +S9 aqueous 400 mg/mL	20+0-S9 aqueous 400 mg/mL	3+17-S9 aqueous 500 mg/mL	3+17 +S9 aqueous 500 mg/mL	20+0-S9 aqueous 500 mg/mL
0						
10000	4	0	88	NT	NT	NT
12500	NT	NT	NT	10	15	48
20000	8	0	92	NT	NT	NT
25000	NT	NT	NT	27	28	77
40000	6	0	94	NT	NT	NT
50000	NT	NT	NT	19	16	93

Phase 3.3 Swedish Moist Snuff (Snus) CPS- DMSO extract

Concentration (µg/mL equivalent)	%Cytotoxicity					
	3+17-S9 DMSO 200 mg/mL	3+17 +S9 DMSO 200 mg/mL	20+0-S9 DMSO 200 mg/mL	3+17-S9 DMSO 500 mg/mL	3+17 +S9 DMSO 500 mg/mL	20+0-S9 DMSO 500 mg/mL
0	-	-	-	-	-	-
UTC	-	-	-	-	-	-
1000	6	14	69	NT	NT	NT
2000	8	10	80	NT	NT	NT
2500	NT	NT	NT	8	0	88
4000	15	11	98	NT	NT	NT
5000	NT	NT	NT	14	1	95
10000	NT	NT	NT	48	13	96

UTC Untreated control

NEUTRAL RED UPTAKE ASSAY

CYTOTOXICITY RESULTS

- Phase 1: Assay performed using water extracts of Snus batches CPS and CDM at 200 mg/mL equivalent. No cytotoxic effects approaching the toxicity limit level of 50% occurred. Due to lack of toxicity, next phase to be performed using 24 hour extractions at 37°C.
- Phase 2: Assay performed using water extracts of Snus batches CPS, CDM and Kentucky Reference 2S3 at 200 mg/mL equivalent. No cytotoxic effect approaching the toxicity limit level of 50% occurred for Snus CPS or Kentucky Reference 2S3. Snus CDM provided cytotoxicity of 37% at the highest concentration tested (200 mg/mL equivalent) however, as this did not occur in Phase 1 it is not a reproducible effect and the relevance may be questioned. Due to lack of toxicity, future phases are to include extractions at higher concentrations and DMSO extracts.
- Phase 3.1 Assay performed using three water extracts of Snus batch CPS at 200 mg/mL equivalent. No cytotoxic effect approaching the toxicity limit level of 50% occurred and consistency was noted between all three extracts.
- Phase 3.2 Assay performed using three water extracts of Snus batch CPS at 300 to 500 mg/mL equivalents. No cytotoxic effect approaching the toxicity limit level of 50% occurred for any extract and no marked differences were noted between extract concentrations.
- Phase 3.3 Assay performed using four DMSO extracts of Snus batch CPS at 200 to 500 mg/mL equivalents. No cytotoxic effect approaching the toxicity limit level of 50% occurred for any extract and no marked differences were noted between extract concentrations. It may be noted that Phase 3.3 was repeated as low survival in the negative control replicates caused an apparent increase in survival of the 400 and 500 mg/mL extracts. This repeat confirmed the lack of cytotoxicity observed in the initial experiment and both sets of data have been reported.

Summary of results: cytotoxicity at highest concentration

Batch	Percent survival	
	Phase 1 (2 hour extracts)	Phase 2 (24 hour extracts)
CPS	102%	64%
CDM	87%	37%
Kentucky	NE	83%

Phase	Extract solvent	Extract conc. (mg/mL)	Percent survival	
3.1	Water	200 (A)	85%	90%
		200 (B)	85%	88%
		200 (C)	86%	90%
3.2	Water	300	85%	84%
		400	88%	77%
		500	86%	83%
3.3	DMSO	200	87% (92%)	90% (100%)
		300	90% (81%)	90% (90%)
		400	136%* (88%)	89% (90%)
		500	156%* (89%)	83% (78%)

CPS Swedish Moist Snuff (Snus) CPS
 CDM Swedish Moist Snuff (Snus) CDM
 Kentucky Kentucky Reference Moist Snuff, Batch 2S3
 NE Not evaluated
 * Values above negative control due to low vehicle cell survival
 Data in parentheses indicates repeat data

Appendix 1

Extraction conditions

Extracts of moist snuff/Snus for use in this study 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.

- Appropriate numbers of pouches/sachets of moist snuff/Snus were cut approximately in half, and both the contents and the packaging (pouches and sachets) 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 (for phase 1 only, a 2 hour extraction at room temperature was employed).
- 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 then 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).
- Where extracts were prepared in two or more separate flasks for a Snus/moist snuff batch, extracts were pooled prior to use in the assay.
- Aliquots of extracts were stored at approximately -80°C, and used within 3 months of extraction..

1138/016

Analysis of nicotine content in snus extracts

Authors: M Greenwood, A Battle

SUMMARY

An analytical procedure for the determination of nicotine in polacrilex gum was identified in the literature (USP 27, 2004; p1318). The method was adapted and validated for use in determining the nicotine content in snus sample extracts using High Performance Liquid Chromatography (HPLC) and UV detection. The method was used to determine the nicotine content of snus extract samples generated in study 1138/016 by Covance Molecular Toxicology Department. These samples comprised extracts in both water and dimethylsulfoxide (DMSO).

The analytical procedure was validated in this study, and is presented in Appendix 3.

PROCEDURES

Validation of analytical method

The following criteria were evaluated during validation:

- Linearity of response
- Precision
- Recovery
- Specificity and Selectivity
- Sensitivity

Statements of intent, including acceptance criteria, were put into place prior the work being carried out.

Linearity of Response

The method was validated over the calibration range 0-2500 μ g/mL.

The exact concentrations of future extracts for analysis were unknown prior to testing but initial trials had suggested a concentration in the region of 2000 μ g/mL would be appropriate. The linearity of response was, therefore, evaluated at concentrations of 0, 400, 800, 1200, 1600, 2000 and 2500 μ g/mL.

Regression data were generated for the calibration standards to confirm that the method is suitable to determine nicotine content.

The linearity of any other compounds was not determined.

Precision

The precision was calculated using the response observed in spiked blank extraction fluid solutions and pure standard solutions.

Analytical Recovery

The analytical recovery was performed using pure standards against extracts of spiked blank extraction fluid. The spiked extracts covered the range of linearity determined above. Pure standards and extracts were prepared at concentrations of 400, 1200 and 2000µg/mL.

The precision was evaluated for nicotine only.

Sensitivity

The sensitivity was determined from the signal to noise ratio and the precision of replicates at concentrations of 80 and 200ng/mL, following initial trial sensitivity investigations.

Specificity and Selectivity

The specificity of the system for nicotine and the known degradants, cotinine, nicotine N-Oxide and mysomine was also determined. In addition blank control extraction solutions were analysed for interferences.

Stability of extract solutions

The extracts were analysed for nicotine content after 60 hours at room temperature.

Dilution into calibration range

Spiked control samples of water and DMSO were diluted five-fold into the calibration range and the results measured against water standards.

Sample analysis

Analytical procedure

The written analytical procedure Covance CLE1138/16A was used to determine concentrations.

Nicotine content

Determination of the nicotine content of sample extract solutions from the Covance Molecular Toxicology department was carried out using the validated method. The samples were extracts from five different snus types, namely 'CPS', 'CDM1', 'CDM2', 'Kentucky' and 'G'.

RESULTS

Validation

Linearity of response

For the aqueous extraction solvent the correlation coefficient (R) was 0.9990 and for the DMSO extraction solvent the correlation coefficient (R) was 0.9995.

Both these criteria are within acceptance limits and are, therefore, acceptable.

Precision

The precision varied between 0% and 0.5% for water extracts and between 0% and 0.2% for DMSO extracts. This is considered to be acceptable.

The results are presented in Tables 1 and 2

Recovery

The mean recovery for water extracts was 78.7%. Individual values varied between 71.9% and 89.1%. The mean recovery for DMSO extracts was 69.3%. Individual values varied between 67.6% and 70.7%.

The results are summarised in Tables 3 and 4.

The recoveries of any other compounds were not determined.

Sensitivity

The limit of quantification (LOQ) was adequate at 100 ng/mL.

Specificity and Selectivity

This was satisfactory with none of the peaks tested eluting in the same region of the chromatogram as nicotine.

There was no significant detector response from control solution thereby confirming selectivity of the method.

Stability of extract solutions

During the stability test the maximum deviation from the initial peak area result was -1.5%. The extract solutions are, therefore, considered to be stable for this amount of time at room temperature. The results are presented in Table 5.

Dilution into calibration range

The results for dilution into the calibration range were acceptable for the water extracts. The results for the DMSO extracts gave unacceptable results. When samples were subsequently analysed with and without dilution and the results compared this suggested that the original unacceptable results were anomalous and that dilution of DMSO extracts into range is acceptable.

Sample analysis

The sample analysis results are presented in Table 6. The recovery values derived from the validation exercise were used to calculate the results.

Table 1 Precision for aqueous extracts

Precision of nicotine standards (water as initial extraction solvent)

Conc. of standard (µg/mL)	Extracted std. peak area	Mean Std dev ⁿ CV (%)	Pure standard peak area	Mean Std dev ⁿ CV (%)
0	357303.7		NA	
0	356560.3	356932.2	NA	NA
0	356932.5	371.70	NA	
		0.1		
400	463065.0		119708.2	
400	462821.0	463122.1	118587.8	119169.9
400	463480.3	333.34	119213.7	561.48
		0.1		0.5
800	543387.3			
800	543357.7	543241.1		
800	542978.4	228.01		
		0.0		
1200	630033.0		364211.9	
1200	630423.0	630386.7	364583.1	364423.1
1200	630704.0	336.97	364474.3	190.82
		0.1		0.1
1600	705558.0			
1600	705004.9	705315.9		
1600	705384.9	282.93		
		0.0		
2000	790138.3		602032.8	
2000	790538.9	790077.8	602444.5	602162.3
2000	789556.2	494.14	602009.6	244.67
		0.1		0.0
2500	886958.1			
2500	886382.5	886986.8		
2500	887619.9	619.20		
		0.1		

NA = Not applicable

Table 2 Precision for DMSO extracts

Precision of nicotine standards (DMSO as initial extraction solvent)

Conc. of standard (µg/mL)	Extracted std. peak area	Mean Std dev ⁿ CV (%)	Pure standard peak area	Mean Std dev ⁿ CV (%)
0	0		NA	
0	0	NA	NA	NA
0	0	NA	NA	
		NA		
400	82090.1		116401.4	
400	82160.3	82110.6	116028.0	116105.2
400	82081.3	43.29	115886.1	266.18
		0.1		0.2
800	165912.2			
800	165838.1	165934.8		
800	166054.2	109.81		
		0.1		
1200	242976.1		359411.3	
1200	243087.8	243067.3	359526.5	359372.4
1200	243138.0	82.87	359179.3	176.84
		0.0		0.0
1600	330833.4			
1600	330716.1	331026.4		
1600	331529.8	439.86		
		0.1		
2000	415899.4		599353.4	
2000	416018.8	416258.7	599319.0	599394.8
2000	416857.9	522.35	599512.1	103.00
		0.1		0.0
2500	536678.1			
2500	537297.4	537077.2		
2500	537256.2	346.27		
		0.1		

NA = Not applicable

Table 3 Recovery for aqueous extracts

Recovery of spiked nicotine standards (water as initial extraction solvent)

Conc. of standard (µg/mL)	Extracted std. peak area	Extracted std. mean peak area	Mean peak area minus mean zero std. area	Pure standard area	Pure standard mean area	Recovery (%)	Mean recovery (%)
0	357303.7			NA			
0	356560.3	356932.2		NA	NA	NA	
0	356932.5			NA			
400	463065.0			119708.2			
400	462821.0	463122.1	106189.9	118587.8	119169.9	89.1	
400	463480.3			119213.7			
1200	630033.0			364211.9			
1200	630423.0	630386.7	273454.5	364583.1	364423.1	75.0	78.7
1200	630704.0			364474.3			
2000	790138.3			602032.8			
2000	790538.9	790077.8	433145.6	602444.5	602162.3	71.9	
2000	789556.2			602009.6			

NA = Not applicable

Table 4 Recovery for DMSO extracts

Recovery of spiked nicotine standards (DMSO as initial extraction solvent)

Conc. of standard (µg/mL)	Extracted std. peak area	Extracted std. mean peak area	Mean peak area minus mean zero std. area	Pure standard area	Pure standard mean area	Recovery (%)	Mean recovery (%)
0	0			NA			
0	0	0.0		NA	NA	NA	
0	0			NA			
400	82090.1			116401.4			
400	82160.3	82110.6	82110.6	116028.0	116105.2	70.7	
400	82081.3			115886.1			
1200	242976.1			359411.3			
1200	243087.8	243067.3	243067.3	359526.5	359372.4	67.6	69.3
1200	243138.0			359179.3			
2000	415899.4			599353.4			
2000	416018.8	416258.7	416258.7	599319.0	599394.8	69.4	
2000	416857.9			599512.1			

NA = Not applicable

Table 5 Stability of extracts

Stability of nicotine extracts at room temperature for 60 hours

Extract conc. (µg/mL)	Initial area (mean)	re-injection area	% difference*
400	82111	83287	1.4
1200	243067	243785	0.3
2000	416259	409998	-1.5

* (re-injection area / initial area) x 100

Table 6 Nicotine content of snus extracts

Sample	Peak area	dilution factor	nicotine conc. (mg/mL)
CPS A	157011.8	5	3.263
CPS B	176655.5	5	3.671
CPS C	166590.1	5	3.462
CPS D	172929.3	5	3.594
CPS E	171209.7	5	3.558
CPS Pool	175500.7	5	3.647
CDM1 A	363446.7	5	7.553
CDM1 B	372617.7	5	7.743
CDM1 C	366361.9	5	7.613
CDM1 D	369306.6	5	7.674
CDM1 E	369126.1	5	7.671
CDM1 Pool	374214.3	5	7.776
CDM2 A	389762.1	5	8.099
CDM2 B	387911.9	5	8.061
CDM2 C	398800.5	5	8.287
CDM2 D	405791.9	5	8.433
CDM2 E	393108.4	5	8.169
CDM2 Pool	393830.7	5	8.184
Kentucky A	222609.0	5	4.619
Kentucky B	205359.5	5	4.261
Kentucky C	240442.5	5	4.989
Kentucky D	252407.1	5	5.238
Kentucky E	218183.4	5	4.527
Kentucky Pool	219683.0	5	4.559
G A	153662.1	5	3.189
G B	147328.0	5	3.057
G C	157347.8	5	3.265
G D	154033.0	5	3.196
G E	159320.4	5	3.306
G Pool	129575.9	5	2.689
DMSO A	456522.8	5	9.722
DMSO B	461131.2	5	9.820
DMSO pool	443349.5	5	9.441

Appendix 2
Analytical method for Nicotine determinations



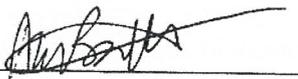
ANALYTICAL PROCEDURE

CLE 1138/016-A

**Analytical Method for the Determination of Nicotine in "Snus" Extracts using
Liquid/Liquid extraction and High Performance Liquid Chromatography.**

Written by 
Mike Greenwood, Scientist

Date 29 Sep 2005

Authorised by 
Andrew Battle, Responsible Analyst

Date 29 Sep 05

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Test Article: Nicotine
Issue Date: 29 September, 2005

**Analytical Method for the Determination of Nicotine in "Snuss" Extracts using
Liquid/Liquid extraction and High Performance Liquid Chromatography.**

1 INTRODUCTION

This method details the procedure for the analysis of nicotine content in extracts from snus samples where either water or dimethylsulfoxide (DMSO) have been used as the initial extraction solvent.

The method is based on the USP method for Nicotine Polacrilex Gum (USP 27, 2004; pg 1318).

Samples of nicotine extracts are assayed using liquid/liquid extraction for clean-up and High Performance Liquid Chromatography (HPLC). They are assayed against an external standard using a pre-determined recovery factor.

2 SAFETY AND HANDLING

All procedures in this method have handling control codes.

Laboratory coats and safety glasses [2a & 3a] must be worn at all times in the laboratory.

3 APPARATUS, MATERIALS & REAGENTS

3.1 Apparatus, glassware etc.

General laboratory glassware, including grade A pipettes, flasks, beakers, measuring cylinders, etc.

Calibrated electronic dispensing pipette (EDP) capable of dispensing up to 1mL.

AGILENT HP1100 HPLC or Equivalent.

6 place balance.

3.2 Materials

The materials listed below do not have any special hazards or toxicity under normal laboratory use. If the operator is unsure of any material, which might present potential hazards, they should consult a supervisor.

Acetonitrile (HPLC grade)	e.g. Rathburns
n-Hexane (HPLC grade)	e.g. Rathburns

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Sodium acetate	e.g. Aldrich	
Sodium -1-decanesulfonate		e.g. Sigma
Glacial Acetic acid (AnalaR Grade)		e.g. VWR
Purified water	e.g. Elga	

4 REAGENT PREPARATION PROCEDURE

Acetate buffer

To make 1L:- add 13.6g of sodium acetate to 57.2mL of glacial acetic acid in a 1L volumetric flask. Make to 1L with purified water.

(May be made up with varying amounts pro rata, as necessary).

(Expiry: 1 month)

1a/b, 4b]

Sodium 1-decanesulfonate solution (SDS solution)

To make 250mL:- add 15.27g of Sodium 1-decanesulfonate to a 250 mL volumetric flask. Add approximately 200mL of water. Sonicate to dissolve. Allow to cool to room temperature and make to volume with water. Mix well.

(May be made up with varying amounts pro rata, as necessary).

(Expiry: 3 months)

[1a/b, 4a/b]

Mobile Phase

To make 1L:-add together: Water. Acetonitrile, Acetate buffer and SDS solution in the ratio 685mL : 200mL : 75mL : 40mL

(Expiry: 1 month)

"Solvent"

To make 1L:-add together: Water. Acetonitrile, SDS solution and Acetate buffer in the ratio 785mL : 150mL : 40mL : 25mL

(Expiry: 1 month)

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5 STANDARD PREPARATION PROCEDURE

Accurately weigh approximately 156.25mg of nicotine hydrogen tartrate into a 10mL volumetric flask. Make to volume with water. This is the stock standard solution and contains approximately 5000 μ g/mL of nicotine.

(Expiry: 1 month).

[1a/b, 4a/b, 5a(ii), 8]

Accurately transfer 80 μ L of stock standard solution into a 10mL volumetric flask. Make to volume with "solvent". This is the working standard solution and contains approximately 40 μ g/mL of nicotine (equivalent to a snuss extract solution of approximately 2mg/mL of nicotine).

(Expiry: 1 month).

[1a/b, 4a/b, 8]

6 TEST SAMPLE PREPARATION PROCEDURE

An aliquot of sample (1mL) is taken and added to a 150mL volumetric flask. 50 mL of "solvent" is added followed by 50 mL of n-hexane. A stirrer bar is added and the mixture is stirred for 30 minutes at approximately 500 rpm. The flask and mixture are left to stand for 30 minutes to allow separation of the immiscible layers. A portion of the lower aqueous layer is taken and submitted for analysis by HPLC.

NB The volumes of sample, solvent and n-hexane may be scaled down, eg to 500 μ L : 25mL : 25mL

(Expiry: at least 60 hours at room temperature).

[1a/b, 2c&e, 4b, 8]

7 HPLC PARAMETERS

Column: Symmetry 5 μ m C₁₈ 150 x 3.9mm.

Mobile Phase : As section 4.

Flow Rate: 1.5mL/min.

Injection Volume: 50 μ L.

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Column Temp: 25°C.
Detector Wavelength: 254nm.

8 SYSTEM SUITABILITY - ACCEPTANCE CRITERIA

The nicotine response factors of the first 6 standard A injections at the beginning of the run should have an RSD \leq 2%.

The tailing factor of the nicotine peak should be 0.9 - 1.5.

The concordance of the two calibration standards (A and B) should be between 98 and 102%, where:-

$$\text{Concordance} = \frac{W_A}{A_A} \times \frac{A_B}{W_B} \times 100$$

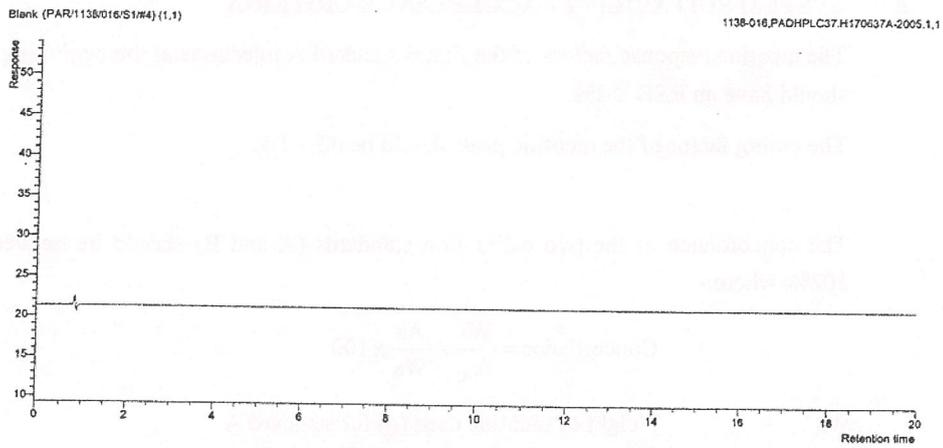
W_A	-	Weight of nicotine used (g) for standard A
W_B	-	Weight of nicotine used (g) for standard B
A_A	-	Area of nicotine peak in standard solution A
A_B	-	Area of nicotine peak in standard solution B

Data outside of these criteria may only be accepted at the discretion of the Responsible Analyst and Sponsor. The reasoning must be fully documented.

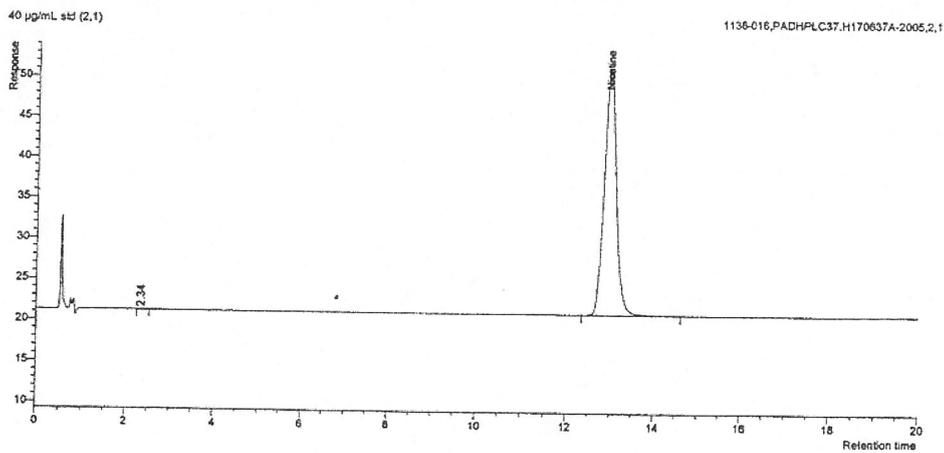
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9 EXAMPLE CHROMATOGRAMS

Blank Chromatogram

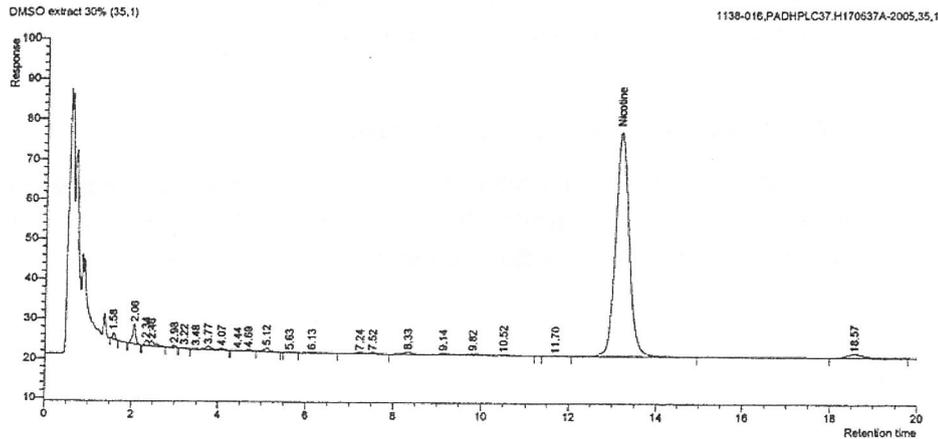


Standard Chromatogram



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Sample Chromatogram



10 CALCULATION OF NICOTINE CONTENT

Determine the peak area of the nicotine in the sample solutions and calculate the nicotine content of the solutions using the following equation: -

Nicotine content (mg/mL) = :-

$$[A_u \times W_S \times 80 \times P \times DF_{uA} \times DF_{uB} / A_S \times V_S \times 10,000 \times 100 \times 3.125] \times 100/R$$

- W_S - Weight of nicotine used for standard (mg)
 P - Purity of standard expressed as percent
 A_S - Area Std
 A_u - Area of nicotine peak in sample
 V_S - Volume of stock standard (mL) (ie.10mL in method)
 DF_{uA} - Sample dilution factor into validated calibration range (normally 5)
 DF_{uB} - Sample dilution factor with "solvent" S1 (S1)
 R - Recovery figure (%) for nicotine (derived from extracted and pure standards during method validation).

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11 METHOD DEVIATIONS

Deviations from this method may only be accepted at the discretion of the Responsible Analyst and/or the Sponsor and the reasoning must be fully documented.

12 COSHH ASSESSMENT OF THIS METHOD

The hazards and risks of the substances used in this method have been assessed. There should be no foreseeable hazards to health, provided that the method is accurately followed and the control measures specified in the method are correctly used.