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**CLINICAL PHARMACOLOGY STUDY REPORT**

STUDY PRODUCTS    “General Large” 1 g portion  
                          “Catch White Licorice Large” 1 g portion  
                          “Catch Licorice Mini” 0.5 g portion  
                          “Catch Licorice Dry Mini” 0.3 g portion

STUDY CODE         **SM WS 03**

DATE                2005-09-26

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DRAFT NO.         2

**The *in-vivo* extraction of cadmium, lead and Tobacco Specific Nitrosamines (TSNA) from four brands of Swedish ‘snus’ in regular snus users.**

2005-09-26

## Signature page

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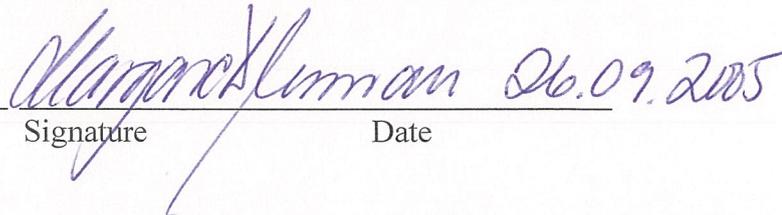
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**Appendices**

**Appendix 1.** Analytical results, individual residual amounts in used snus.  
Amounts in unused snus.

**Appendix 2.** Analytical results, individual extraction.

**STUDY SYNOPSIS**

<b>Study code:</b>	SM WS03
<b>Title:</b>	The <i>in-vivo</i> extraction of cadmium, lead and tobacco specific nitrosamines (TSNA) from four brands of Swedish 'snus' in regular snus users.
<b>Objectives:</b>	To estimate the <i>in-vivo</i> extraction of lead, cadmium, nicotine and tobacco specific nitrosamines (TSNA) from Swedish snus.
<b>Total sample size:</b>	32 male healthy regular snus users.
<b>Study design:</b>	Open label, randomized, four-way single dose study.
<b>Subject population criteria:</b>	18-50 years old, male non-smoking subjects, regularly using $\geq 7$ portions of snus daily since minimum 1 year.
<b>Test articles:</b>	<b>A=</b> "General Large" 1 g portion snus containing approximately 8 mg nicotine per portion. <b>B=</b> "Catch White Licorice Large" 1 g portion snus containing approximately 8 mg nicotine per portion. <b>C=</b> "Catch Licorice Mini" 0.5 g portion snus containing approximately 4 mg nicotine per portion. <b>D=</b> "Catch Licorice Dry Mini" 0.3 g portion snus containing approximately 4 mg nicotine per portion.
<b>Procedure:</b>	The "General Large", "Catch White Licorice Large", "Catch Licorice Mini" and "Catch Licorice Dry Mini" snus portions were administered once every hour (4 administrations/brand) and were kept between the upper lip and the gum for 30 minutes.
<b>Snus sampling:</b>	Each portion of used snus was collected and frozen (-20 °C) pending analysis of lead (Pb), cadmium (Cd), nicotine and tobacco specific nitrosamines (TSNA). Unused snus was also collected and deep frozen for analysis and calculation of extracted dose.
<b>Study parameters:</b>	Extracted amount of lead, cadmium, nicotine and TSNA, respectively, from each portion of snus.
<b>Analysis:</b>	One portion was collected for analysis of lead and cadmium, one for nicotine and 1 portion for analysis of TSNA. Mean extracted amount of lead, cadmium, nicotine and TSNA, respectively, are shown for each type of snus.
<b>Statistics:</b>	The analyses were carried out according to a randomized four-way cross-over design, i.e. as differences within in each subject between the four snus preparations.

**Results:**

Cadmium extraction was 10.5, 8.2, 5.7 and 3.0%, respectively, of the amount in unused snus for “General Large”, “Catch White Licorice Large”, “Catch Licorice Mini” and “Catch Licorice Dry Mini”.

Lead extraction was negligible for all brands.

The mean TSNA extraction from “General Large”, “Catch White Licorice Large”, “Catch Licorice Mini” and “Catch Licorice Dry Mini” was 0.4µg (39%), 0.4µg (41%), 0.09µg (20%) and 0.05µg/portion (14%), respectively.

The mean nicotine extraction was 2.4 (25%), 2.2 (26%), 1.4 (30%) and 0.7mg/portion (13%), respectively.

**Conclusion:**

The cadmium extraction was below 10% for all brands except “General Large”, where it was 10.5%. This is in contrast to the 40-50% of the inhaled Cd oxide that is absorbed into the lung tissues of smokers.

The lead extraction was negligible for all brands. Pb levels in blood and hair are significantly higher in smokers than non-smoker and Pb toxicity associated with cigarette smoking may, according to some research, increase the risk of chronic renal failure. The risk profile of snus apparently is considerably lower than that of cigarettes with respect to both metals.

Nicotine is the substance that the user of smokeless tobacco is seeking for. Adequate delivery of nicotine in relation to harmful substances is therefore a desirable feature. The percent extraction of TSNA seemed to largely parallel the nicotine extraction, which should favour use of smokeless tobacco containing low levels of TSNA, such as the Swedish brands tested in the present study.

## 1 SUMMARY

In an open label, randomized, four-way cross-over study, 32 male healthy regular snus users were given repeated doses of four different types of portion snus: "General Large", "Catch White Licorice Large", "Catch Licorice Mini" and "Catch Licorice Dry Mini". Each portion of used snus was collected and frozen (-20 °C) pending analysis of lead (Pb), cadmium (Cd), nicotine and tobacco specific nitrosamines (TSNA). Unused snus was collected and deep frozen for analysis and calculation of extracted dose. Calculations of extracted amount of lead, cadmium, nicotine and tobacco specific nitrosamines (TSNA) respectively, were done for each type of snus.

Cadmium extraction was below 10% for all brands except "General Large", where it was 10.5%. Cadmium extraction was 8.2, 5.7 and 3.0%, respectively, of the amount in unused snus for "Catch White Licorice Large", "Catch Licorice Mini" and "Catch Licorice Dry Mini". This is in sharp contrast to the 40-50% of the inhaled Cd oxide that is absorbed into the lung tissues of smokers.

Lead extraction was negligible for all brands. Pb levels in blood and hair are significantly higher in smokers than non-smokers and Pb toxicity associated with cigarette smoking may, according to some research, increase the risk of chronic renal failure. The risk profile of snus apparently is considerably lower than that of cigarettes with respect to both metals.

The mean TSNA extraction from "General Large", "Catch White Licorice Large", "Catch Licorice Mini" and "Catch Licorice Dry Mini" was 0.4µg (39%), 0.4µg (41%), 0.09µg (20%) and 0.05µg/portion (14%), respectively. The mean nicotine extraction from "General", "Catch", "Catch Mini" and "Catch Dry Mini" was 2.4 (25%), 2.2 (26%), 1.4 (30%) and 0.7mg/portion (13%), respectively. Nicotine is the substance that the user of smokeless tobacco is seeking for. Adequate delivery of nicotine in relation to harmful substances is therefore a desirable feature. Studies have demonstrated that steady-state nicotine plasma concentrations may be sustained at any chosen level with Swedish snus to replace the more hazardous cigarette smoking. A comparison of the extraction of harmful substances in relation to the nicotine extraction was therefore made in the present study. The percent extraction of TSNA seemed to largely parallel the nicotine extraction, which should favor use of smokeless tobacco containing low levels of TSNA, such as the Swedish brands tested in the present study.

## 2 INTRODUCTION

### 2.1 Background

*Cadmium.* The main threats to human health from heavy metals are associated with exposure to cadmium, lead, mercury and arsenic. The levels of Cd in organs such as liver and kidney cortex increase with age because of the lack of an active biochemical process for its elimination coupled with renal reabsorption. Median Blood Cd and Urinary Cd in one study both increased with age and were higher in ex-smokers, who had stopped smoking more than 5 years before the study, compared to never-smokers (Olsson IM et al 2002). Smoking is a major source of cadmium (Cd) exposure in cigarette smokers (CSMs) and food in non-

smokers. Recent data indicate that adverse health effects of Cd exposure may occur at lower exposure levels than previously anticipated, primarily in the form of kidney damage and osteomalacia (Järup L 2003). Such Cd-linked bone and kidney toxicities were observed in people whose dietary Cd intakes were well within the provisional tolerable weekly intake (PTWI) set by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives of 1 µg/kg body weight/day or 70 µg/day (Satarug et al 2005). Cd is an integral constituent of tobacco because of the propensity of the *Nicotiana* species to concentrate Cd independent of soil-Cd content. Tobacco Cd content varies widely, but a typical range is 1-2 µg/g dry weight, equivalent to 0.5-1 µg/cigarette. Cd oxide generated during the burning of cigarettes is highly bioavailable. Smokers have 4-5 times higher Cd levels in blood and 2-3 times greater amounts of Cd in their kidneys than do Non-smokers (Satarug & Moore 2004). The dietary Cd absorption rate in humans has been estimated at 5%, however, may increase to 20-30% in some individuals (Kikuchi et al. 2003; Satarug et al. 2004). There is no published information on the *in-vivo* Cd extraction from Swedish snus.

*Lead.* The general population is exposed to lead (Pb) from air and food in roughly equal proportions. During the last century, Pb emissions to ambient air have caused considerable pollution, mainly due to lead emissions from petrol. However, Pb in petrol has dramatically decreased over the last decades. Dietary exposure estimates for heavy metals in e.g. France appear to be reassuring, representing 28% of the provisional tolerable weekly intake (PTWI) of 0.025 mg/kg bodyweight (Leblanc JC et al 2000). Pb is a classical chronic or cumulative poison. Health effects are generally not observed after a single exposure. The rate of absorption of Pb after ingestion is heavily influenced by food intake, much higher rates of absorption occurring after fasting. Absorption is also affected by age, the typical absorption rates in adults and infants being 10% and 50%, respectively (O'Flaherty, 1995; Agency for Toxic Substances and Disease Registry, 1997). Recent data indicate that there may be neurotoxic effects of Pb at lower levels of exposure than previously anticipated (Järup L 2003). The *in-vivo* extraction of Pb for Swedish snus has not been found in the literature.

*Nitrosamines.* Tobacco contains nitrate that is microbially activated to nitrite, which may react with alkaloids to form cancer causing tobacco specific nitrosamines (TSNA) during curing and storage of the tobacco. NNK and *N'*-nitrosonornicotine are the most abundant strong carcinogens in unburned tobacco products and may, although controversial, play a role in the induction of oral cavity tumors in snuff-dippers (Andersson G et al 1994, Hecht 1998). Levels of NNK in smokeless tobacco products marketed in the US are typically ~1-2 µg/g tobacco (Hoffmann et al 1995). NNAL plus NNAL-Gluc, the main metabolites of NNK, are biomarkers of tobacco carcinogen uptake. A significant association between levels of NNAL plus NNAL-Gluc, in the urine of smokeless tobacco users and the presence of oral leukoplakia has been observed, supporting the potential role of NNK as a causative factor for this lesion (Kresty et al 1996). Levels of NNAL and NNAL-Gluc in the urine of snuff-dippers and tobacco chewers are similar to those found in smokers (Kresty et al 1996). A correlation between number of tins or portions of smokeless tobacco consumed per week and NNAL plus NNAL-Gluc in urine has been observed in urine of smokeless tobacco users (Hecht et al 2002).

## 2.2 Study rationale

The content of tobacco specific nitrosamines (TSNA) in moist snuff is well studied. Their extraction *in-vivo*, however, is less studied, as is the extraction of heavy metals such as lead (Pb) and cadmium (Cd). A documentation of the *in-vivo* extraction of lead (Pb), cadmium (Cd) and tobacco specific nitrosamines (TSNA) from various brands of snus therefore appeared well motivated. Estimation of the *in-vivo* uptake of harmful substances by assays of body fluids is technically difficult due to their long half-lives and long-term accumulation from various sources. In the present study *in-vivo* extraction of the above harmful substances was therefore estimated by assays of the content of the unused snus minus the content in snus used over a standardized time-period of 30 minutes.

## 3 STUDY OBJECTIVES

The objectives of the present study were to estimate the *in-vivo* extraction of lead, cadmium and tobacco specific nitrosamines (TSNA) and nicotine from four brands of Swedish snus. The primary objective was to compare the *in-vivo* extraction of cadmium from “General Large” and “Catch Licorice Mini” snus.

## 4 STUDY DESIGN

The study had a randomized, cross-over design, and was an open testing of 4 different types of portion snus. Thus the study comprises a total of four sessions for each subject. Statistic power calculation showed a necessary sample size of 29 subjects. Thirty two subjects participated in the study.

## 5 STUDY SITE AND TIMETABLE

The study was performed at CROel AB, Helsingborg, SWEDEN, during 2004. The analysis of cadmium and lead in snus samples before and after usage were performed by the AnalyCen AB laboratories, Lidköping, Sweden. The analysis of nicotine and tobacco specific nitrosamines (TSNA) were carried out by the Department of Research and Analysis, Swedish Match North Europe.

## 6 MATERIAL AND METHODS

### 6.1. Subjects

Thirty two male non-smoking healthy volunteers, regularly using  $\geq 7$  portions snus daily since minimum 1 year were selected for participation in the study. They should have no history of cardiac, kidney or hepatic disease, alcohol abuse or drug dependence. A health declaration and interview by the responsible physician should rule out any disease. The subjects do not have to abstain from nicotine prior to the experimental sessions.

### **6.1.1. Screening phase/procedures**

After giving informed consent subjects were interviewed and filled in a health declaration prior to the inclusion to the study. The health declaration included the following:

- personal data
- previous history of psychiatric, neurological or serious somatic disorders that may interfere with the study
- use of long-term medication
- previous history of allergic reactions
- a self-estimate of the subject's general physical fitness

### **6.1.2. Inclusion criteria**

1. Male non-smokers, 18 to 50 years of age.
2. Habitual use of  $\geq 7$  portions snus daily since minimum 1 year.
3. Healthy according to the health declaration and interview.
4. Written informed consent given.

### **6.1.3 Exclusion criteria**

- 2.2.1.1 Concurrent participation in another clinical trial.
- 2.2.1.2 History of allergy.

### **6.1.4 Admission to study**

A subject was eligible for admission to study if inclusion criteria were fulfilled and if no exclusion criteria were present as verified by the investigator.

### **6.1.5 Subject identification**

As subjects were included they got a number between 1 and 32. Each subject included in the study was uniquely identified by this number and the subject's initials, which appear on all study documents.

### **6.1.6 Subject recruitment**

The subjects were mainly students recruited from the University, Lund, Sweden.

## **6.2 Study products**

### **6.2.1 Study products**

The products studied are found in Table 1.

**Table 1**

A.	“General Large” 1g containing approximately 9.6 mg nicotine per portion. Extracted nicotine dose approximately 2.4 mg/portion.
B.	“Catch White Licorice Large” 1g containing approximately 8.5 mg nicotine per portion. Extracted dose approximately 2.2 mg/portion.
C.	“Catch Licorice Mini” 0.5g containing approximately 4.7mg nicotine per portion. Extracted dose approximately 1.4 mg/portion.
D.	“Catch Licorice Dry Mini” 0.3g containing approximately 5 mg nicotine per portion. Extracted dose approximately 0.7 mg/portion.

**6.2.2 Randomization procedure**

The snus treatments were given according to a computer generated randomization list.

**6.2.3 Packaging, labeling and storage**

The various types of snus were delivered in their original packs as delivered from Swedish Match, Stockholm, Sweden. Individual packaging according to a computer generated randomization list was made. Labeling was in Swedish. The snus was stored in a refrigerator (+2 - +8 °C).

Each pack was labeled (in Swedish):

Snus, containing approximately 8mg, 8 mg, 4 mg and 4 mg nicotine, respectively.

For clinical trial.

CTN: SM WS03

Subject No.: 1 (32)

Treatment.: A (B, C, D)

Expiry date:

Dosage: according to physician's instruction

Responsible investigator: Erik Lunell, M.D.

Keep out of reach of children.

Packed on the: Initials:

**6.2.4 Product accountability**

The selected test articles were ordered from CROel AB, Helsingborg, Sweden. After selection, packaging and labeling the articles were delivered to the Investigator in due time before start of the study.

The snus was delivered in its original container. A "confirmation of receipt note" was completed. All unused test articles were returned at study termination to Swedish Match, Stockholm, Sweden.

### 6.3 Treatments

#### 6.3.1 Snus

The treatments were given as single doses. A total of four portions per session were given. Only non-smoking personnel were allowed to perform practical functions in this study. Snus was used under standardized conditions and executed as follows: One portion was placed and kept in the same place between the upper lip and the gum for 30 minutes. Nicotine is a potent local vasoconstrictor. The subjects were therefore requested to administer one portion of their regular brand of snuff before leaving home each study day (Preload), in order to eliminate any discrepancies between the first dose and subsequent doses of snuff.

#### 6.3.2 Concomitant therapy

There were no restrictions as to the use of OTC drugs, however the participants were requested to report such use, which was recorded on the CRF. No other drug under investigation was allowed concomitantly with the study drug. The subjects were not allowed to participate concurrently in any other study.

### 6.4 Analytical Methods

#### 6.4.1 Residual content of lead (Pb) and cadmium (Cd)

Each used sachet of snus from each brand was placed in a sealed glass container, labelled with a unique number, frozen and stored at  $-20^{\circ}\text{C}$  until analysed for Pb and Cd content. Eight sachets of unused snus were also analysed. Mean Pb respectively Cd content of these sachets was used for calculation of the extraction of Pb respectively Cd.

Lead and cadmium were analysed at AnalyCen AB, Lidköping, Sweden with ICP-MS. LOQ for Pb is 0.04 mg/kg and for Cd is 0.01 mg/kg. Measurement uncertainty for Pb =  $\pm 20\%$  and for Cd =  $\pm 25\%$ .

#### 6.4.2 Residual content of tobacco specific nitrosamines (TSNA) in used snus

Each used portion of snus of each brand/subject was placed in a sealed glass container, labelled with a unique number, frozen and stored at  $-20^{\circ}\text{C}$  until analysed for TSNA. Ten portions of unused snus were also analysed. Mean content of TSNA in these portions was used for the calculation of the extraction of TSNA.

The 4 analysed compounds are N-Nitrosornicotine (NNN), N-Nitrosoanatabine (NAT), N-Nitrosoanabasine (NAB) and 4-(N-Methyl-N-nitrosoamino)-1-(3-pyridyl)-1-butanone (NNK).

The following method was used to analyse TSNA in “General Large” and “Catch White Licorice Large”.

Analyses: Gas Chromatography, Research & Analysis, Stockholm, Swedish Match North Europe.

LOQ for the respective TSNA are 0.1 mg/kg

Measurement uncertainty for NNN=  $\pm 10$  % and for NAT, NAB and NNK=  $\pm 12$  %.

The following method was used to analyse TSNA in “Catch Licorice Mini” and “Catch Licorice Dry Mini”.

Analyses: LC-MS/MS, Research & Analysis, Stockholm, Swedish Match North Europe.

LOQ for the respective TSNA are 0.1  $\mu$ g/kg

Measurement uncertainty has not yet been determined but the accuracy is for NNN= 4.8%, NAT= 5.3%, NAB= 2.8% and NNK= 1.2%.

#### **6.4.3 Residual nicotine in used snus**

Each used portion of snus of each brand/subject was placed in a sealed container, labelled with a unique number, frozen and stored at -20°C until analysed for nicotine content. Ten portions of unused snus were also analysed. Mean nicotine content of these portions was used for the calculation of extracted dose of nicotine.

Nicotine was analysed at Swedish Match, Research and Analysis, Stockholm, Sweden with GC-FID.

LOQ for nicotine is 0.5 mg/g.

Measurement uncertainty for nicotine is  $\pm 6$  %.

### **6.5 Calculation**

Extracted amount of the various contents analysed was calculated for each individual and each analytical result as follows:

$$\text{Extracted amount} = \text{mean of 10 unused portions} - \text{residual amount}$$

Extracted amount of the various contents is given as absolute amount. Mean extracted amount is presented in relation to the mean extraction of nicotine.

## **7 ASSESSMENT OF SAFETY**

### **7.1 Definition**

An adverse event (AE) is any untoward medical occurrence in a patient or trial subject administered a drug or biologic (medicinal product) or using a medical device; the event does not necessarily have a causal relationship with that treatment or usage.

## 7.2 Description of and Recording Instructions for Adverse Events (AE)

Adverse events were to be recorded in the case report forms as specified. If required on the adverse event case report forms, the investigator uses the adjectives MILD, MODERATE, or SEVERE to describe the maximum intensity of the adverse event.

For purposes of consistency, these intensity grades are defined as follows:

MILD	Does not interfere with subject's usual function.
MODERATE	Interferes to some extent with subject's usual function.
SEVERE	Interferes significantly with subject's usual function. Note the distinction between the gravity and the intensity of an adverse event. <b>Severe</b> is a measure of intensity; thus, a <b>severe</b> reaction is not necessarily a <b>serious</b> reaction. For example, a headache may be severe in intensity, but would not be classified as serious unless it met one of the criteria for serious events listed above.

### Follow-Up of Adverse Events

All adverse events should be followed until they were resolved or the subject's participation in the trial ends. Instructions for reporting changes in an ongoing adverse event during a subject's participation in the trial were provided in the instructions that accompany the adverse event case report forms.

In addition, all serious adverse events should continue to be followed even after the subject's participation in the trial is over. Such events should be followed until they resolve or until the investigator assesses them as "chronic" or "stable." Resolution of such events is to be documented on the appropriate follow-up CRF.

## 8 STATISTICAL CONSIDERATIONS

The snus preparations were given randomly according to a computer generated list. The study was carried out according to a randomized four-way cross-over design, i.e. as differences within in each patient for the four snus preparations. The primary endpoint was to assess if A and C are equivalent regarding the Cadmium extraction. Former studies have indicated that both A and C would have a 30% extraction of the baseline Cadmium level. In order to declare A and C as equivalent the extraction should not differ by more than 30% with a 90% confidence interval and a power of 80%. The number of patients needed to show this equivalence was 29. Thirty two subjects were included to compensate for possible withdrawals.

## 9 QUALITY CONTROL (QC) and QUALITY ASSURANCE(QA)

Monitoring visits to the trial site were made, to ensure that all aspects of the protocol were followed. The subject chart was reviewed for verification of agreement with data on Case Report Forms. The trial site was subject to quality assurance audit by monitor and QC auditor.

## 10 ETHICS

The trial was performed in accordance with the recommendations guiding physicians in biomedical research involving human subjects adopted by the 18th World Medical Association Assembly, Helsinki, Finland, 1964 (Declaration of Helsinki) and later revisions. Approval of the trial protocol/amendments from the regional Ethics Committee (EC) was obtained prior to the study start. Since the study products fall under the Swedish food legislation and no medicinal drug was under investigation in the present study no application or notification of the Swedish Medical Agency (MPA) was done. The trial was consistent with Good Clinical Practice (GCP). A signed informed consent was obtained from each subject prior to inclusion in the trial.

## 11 RESULTS

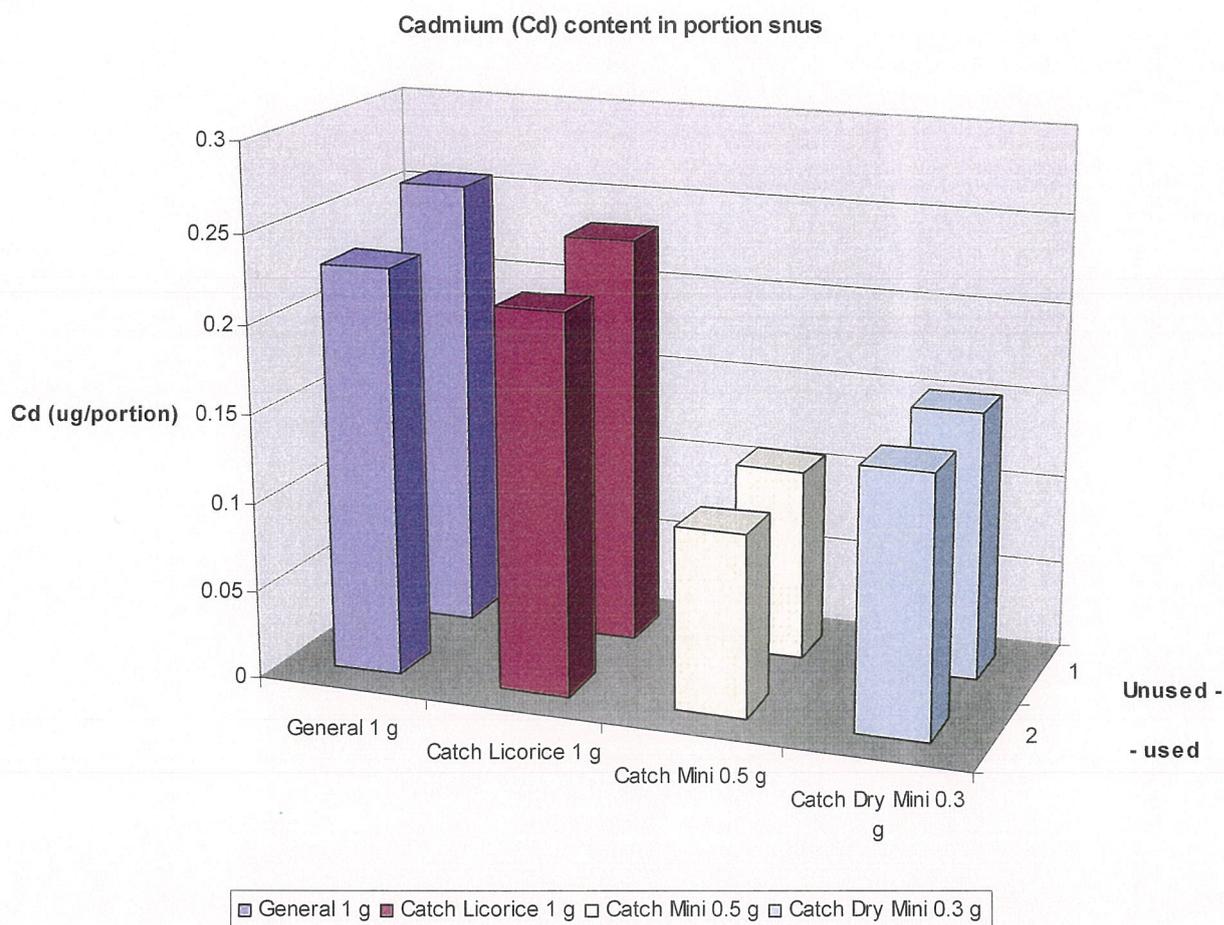
### 11.1 Subjects

In agreement with the plan of the study, 32 male healthy regular snus users, aged 18-32 years participated. They all took the scheduled four doses of each of the four different types of portion snus.

### 11.2 Extracted amount of Cadmium (Cd) from used snus

Eight portions of unused snus of "General Large", "Catch White Licorice Large", "Catch Licorice Mini" and "Catch Licorice Dry Mini" contained a mean ( $\pm$ SD) amount of  $0.257 \pm 0.025 \mu\text{g}$ ,  $0.233 \pm 0.009 \mu\text{g}$ ,  $0.109 \pm 0.010 \mu\text{g}$  and  $0.152 \pm 0.008 \mu\text{g}$  Cd/portion, respectively. The used portions of snus of each preparation analysed for Cd content showed a mean ( $\pm$ SD) residual amount of  $0.230 \pm 0.026 \mu\text{g}$ ,  $0.214 \pm 0.019 \mu\text{g}$ ,  $0.103 \pm 0.012 \mu\text{g}$  and  $0.147 \pm 0.012 \mu\text{g}$  Cd/portion, respectively. The mean ( $\pm$ SD) extracted amount of Cd was estimated at  $0.027 \pm 0.026 \mu\text{g}$ ,  $0.019 \pm 0.010 \mu\text{g}$ ,  $0.006 \pm 0.012 \mu\text{g}$  and  $0.005 \pm 0.012 \mu\text{g}$  Cd/portion, respectively. The mean results are demonstrated in figure 1 and Tables 2-5. Individual values are given in Appendices 1-2.

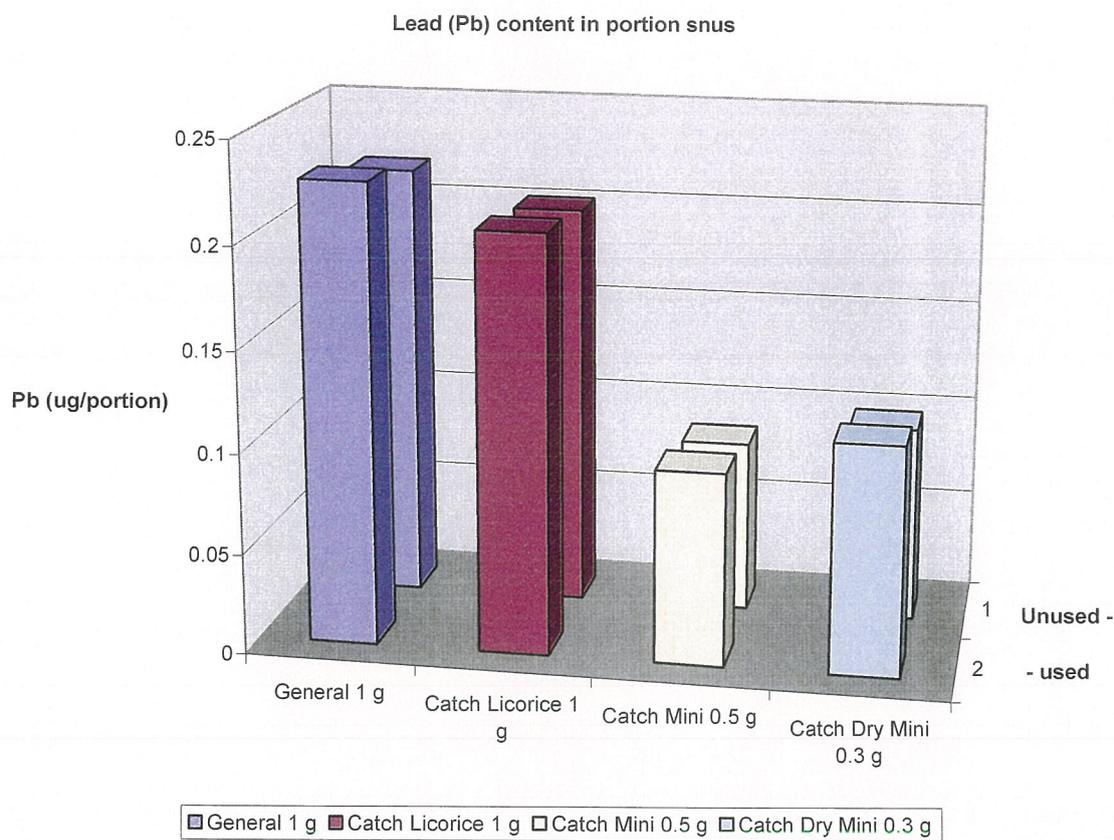
Figure 1.



### 11.3 Extracted amount of lead (Pb) from used snus

Eight portions of unused snus of “General Large”, “Catch White Licorice Large”, “Catch Licorice Mini” and “Catch Licorice Dry Mini” contained a mean ( $\pm$ SD) amount of  $0.218 \pm 0.028 \mu\text{g}$ ,  $0.201 \pm 0.017 \mu\text{g}$ ,  $0.086 \pm 0.010 \mu\text{g}$  and  $0.098 \pm 0.010 \mu\text{g}$  Pb/portion, respectively. The used portions of snus of each preparation analysed for Pb content showed a mean ( $\pm$ SD) residual amount of  $0.228 \pm 0.066 \mu\text{g}$ ,  $0.207 \pm 0.051 \mu\text{g}$ ,  $0.096 \pm 0.033 \mu\text{g}$  and  $0.114 \pm 0.029 \mu\text{g}$  Pb/portion, respectively. The mean ( $\pm$ SD) extracted amount of Pb was estimated at  $-0.014 \pm 0.067 \mu\text{g}$ ,  $-0.006 \pm 0.051 \mu\text{g}$ ,  $-0.007 \pm 0.035 \mu\text{g}$  and  $-0.016 \pm 0.030 \mu\text{g}$  Pb/portion, respectively. The mean results are demonstrated in figure 2 and Tables 2-5. Individual values are given in Appendices 1-2.

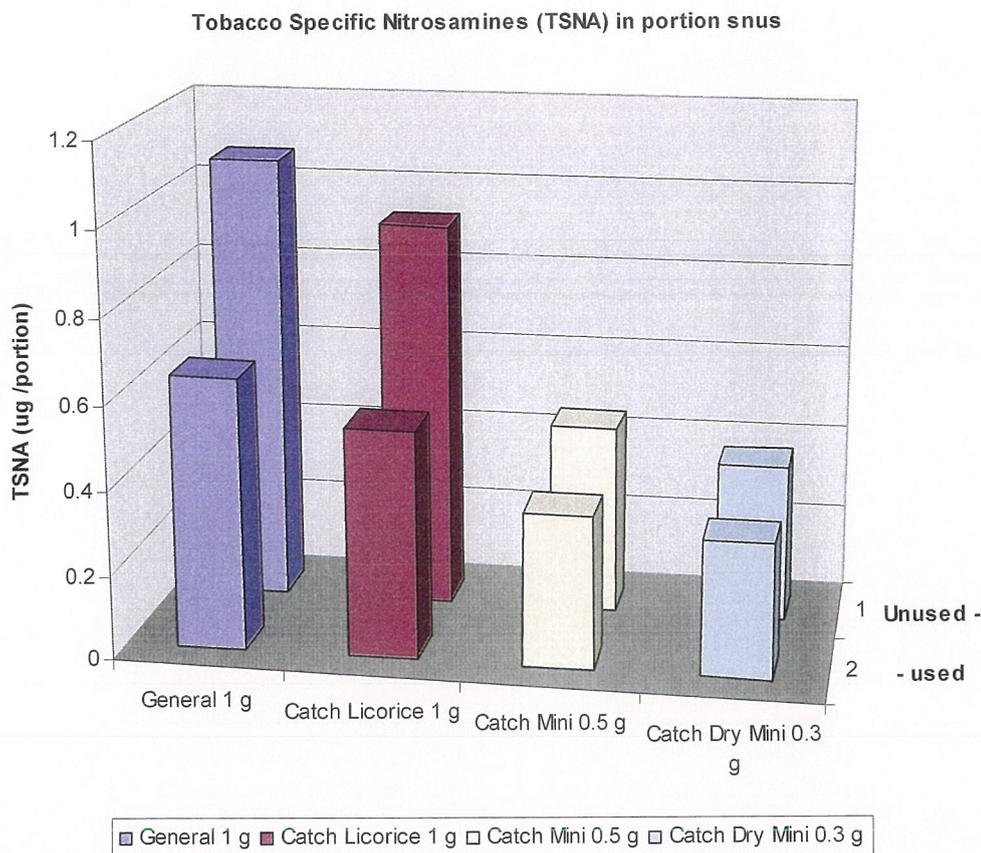
Figure 2.



#### 11.4 Extracted amount of tobacco specific nitrosamines (TSNA) from used snus

Ten portions of unused snus of “General Large”, “Catch White Licorice Large”, “Catch Licorice Mini” and “Catch Licorice Dry Mini” contained a mean ( $\pm$ SD) amount of  $1.070 \pm 0.138 \mu\text{g}$ ,  $0.925 \pm 0.195 \mu\text{g}$ ,  $0.452 \pm 0.019 \mu\text{g}$  and  $0.373 \pm 0.019 \mu\text{g}$  TSNA/portion, respectively. The used portions of snus of each preparation analysed for TSNA content showed a mean ( $\pm$ SD) residual amount of  $0.647 \pm 0.165 \mu\text{g}$ ,  $0.541 \pm 0.122 \mu\text{g}$ ,  $0.362 \pm 0.040 \mu\text{g}$  and  $0.320 \pm 0.024 \mu\text{g}$  TSNA/portion, respectively. The mean ( $\pm$ SD) extracted amount of TSNA was estimated at  $0.436 \pm 0.176 \mu\text{g}$ ,  $0.389 \pm 0.122 \mu\text{g}$ ,  $0.088 \pm 0.040 \mu\text{g}$  and  $0.050 \pm 0.024 \mu\text{g}$  TSNA/portion, respectively. The mean results are demonstrated in figure 3 and Tables 2-5. Individual values are given in Appendices 1-2.

Figure 3.



### 11.5 Extracted amount of nicotine from used snus

Ten portions of unused snus of “General Large”, “Catch White Licorice Large”, “Catch Licorice Mini” and “Catch Licorice Dry Mini” contained a mean ( $\pm$ SD) amount of  $9.656 \pm 0.410$ mg,  $8.505 \pm 0.233$ mg,  $4.751 \pm 0.121$ mg and  $5.191 \pm 0.129$ mg nicotine/portion, respectively. The used portions of snus of each preparation analysed for nicotine content showed a mean ( $\pm$ SD) residual amount of  $7.213 \pm 0.810$ mg,  $6.305 \pm 0.522$ mg,  $3.312 \pm 0.455$ mg and  $4.502 \pm 0.268$ mg nicotine/portion, respectively. The mean ( $\pm$ SD) extracted amount of nicotine was estimated at  $2.447 \pm 0.810$ mg,  $2.205 \pm 0.522$ mg,  $1.438 \pm 0.455$ mg and  $0.688 \pm 0.273$ mg nicotine/portion, respectively. The mean results are demonstrated in figure 4 and Tables 2-5. Individual values are given in Appendices 1-2.

Figure 4.

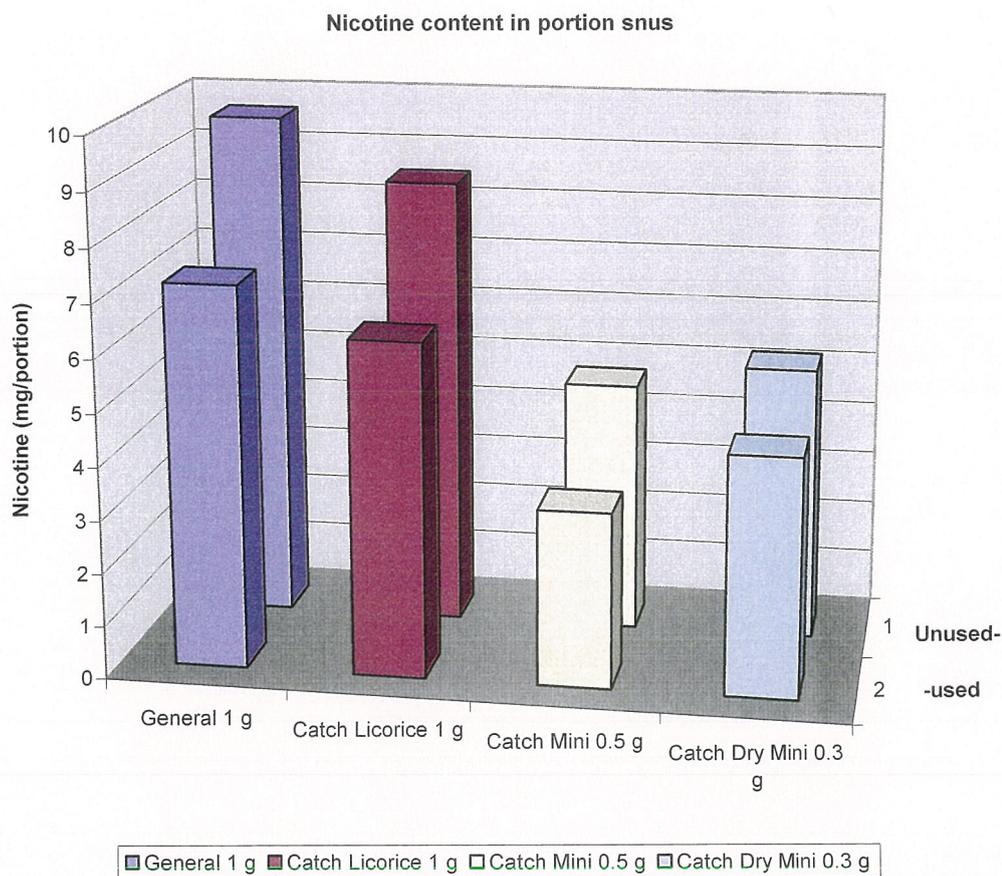


Table 2

<b>A: General LargePS 1 g, G 880</b>		<b>Pb µg/portion</b>	<b>Cd µg/portion</b>	<b>Nicotine mg/portion</b>	<b>TSNA µg/portion</b>
<b>Moisture ca: 50 %</b>	<b>pH=8.4</b>				
<b>Unused snus</b>	<b>Mean</b>	0.218	0.257	9.656	1.070
	<b>SD</b>	0.028	0.025	0.410	0.138
<b>Used snus</b>	<b>Mean</b>	0.228	0.230	7.213	0.647
	<b>SD</b>	0.066	0.026	0.810	0.165
<b>Extracted amount</b>	<b>Mean</b>	<b>-0.014</b>	<b>0.027</b>	<b>2.447</b>	<b>0.436</b>
	<b>SD</b>	<b>0.067</b>	<b>0.026</b>	<b>0.810</b>	<b>0.176</b>

Table 3

<b>B Catch Licorice PS 1 g, CPS 887</b>		Pb µg/portion	Cd µg/portion	Nicotine mg/portion	TSNA µg/portion
Moisture ca: 50 %	pH=8.5				
Unused snus	Mean	0.201	0.233	8.505	0.925
	SD	0.017	0.009	0.233	0.195
Used snus	Mean	0.207	0.214	6.305	0.541
	SD	0.051	0.019	0.522	0.122
Extracted amount	Mean	-0.006	0.043	2.205	0.389
	SD	0.051	0.101	0.522	0.122

Table 4

<b>C: Catch Licorice PS Mini 0.5g, CM 888</b>					
Moisture ca: 45%	pH=8.4	Pb µg/portion	Cd µg/portion	Nicotine mg/portion	TSNA µg/portion
Unused snus	Mean	0.086	0.109	4.751	0.452
	SD	0.010	0.010	0.121	0.019
Used snus	Mean	0.096	0.103	3.312	0.362
	SD	0.033	0.012	0.455	0.040
Extracted amount	Mean	-0.007	0.006	1.438	0.088
	SD	0.035	0.012	0.455	0.040

Table 5

<b>D: Catch Licorice PS Mini 0.3 g, CDM 861</b>					
Moisture ca 22 %	pH=7.3	Pb µg/portion	Cd µg/portion	Nicotine mg/portion	TSNA µg/portion
Unused snus	Mean	0.098	0.152	5.191	0.373
	SD	0.010	0.008	0.129	0.019
Used snus	Mean	0.114	0.147	4.502	0.320
	SD	0.029	0.012	0.268	0.024
Extracted amount	Mean	-0.016	0.005	0.688	0.050
	SD	0.030	0.012	0.273	0.024

## 10. DISCUSSION

*Cadmium.* Cd-induced renal osteomalacia (itai-itai disease) was characterized, in Japan after World War II, by a decrease in Bone Mineral Density (BMD), an increased prevalence of fractures with severe pain and proteinuria with content of markers of renal dysfunction (beta2-microglobulin and NAG, N-acetyl-beta-d-glucosaminidase) among persons with long term exposure to Cd. WHO, 1992, identified tubular damage and renal dysfunction as the critical effect (Nordberg 2004). Further, an association between Cd-related nephropathy and high blood pressure was evidenced by a 20% increase in the prevalence of high blood pressure in people with NAG-uria (Satarug et al 2005). Such Cd-linked bone and kidney toxicities were observed in people whose dietary Cd intakes were well within the provisional tolerable weekly intake (PTWI) set by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives of 1 µg/kg body weight/day or 70 µg/day (Satarug & Moore 2004). Median blood Cd and urinary Cd in one study both increased with age and were higher in ex-smokers, who had stopped smoking more than 5 years before the study, compared to never-smokers (Olsson IM et al 2002).

Metallothionein (MT) is an inducible protein that binds and detoxifies cellular Cd. Variation in MT gene expression has been shown to be related to development of renal dysfunction. In one study in pregnant women smokers showed higher placental MT and cadmium levels, as compared to non-smokers. Cigarette smoking increases Cd accumulation and stimulates MT production in placental tissue (Ronco AM et al 2005).

Evidence for the carcinogenic risk of chronic Cd exposure is accumulating and Cd effects on reproductive outcomes have begun to emerge.

Cd-induced renal osteomalacia (itai-itai disease, identified in Japan after World War II) is characterized by a decrease in Bone Mineral Density (BMD), an increased prevalence of fractures with severe pain and an increased urinary content of marker proteins of renal dysfunction (proteinuria) among persons with long term occupational exposure to Cd, in particular with nutritional deficiency. WHO, 1992, identified tubular damage and renal dysfunction, evidenced by increases in urinary excretion of beta2-microglobulin and NAG, N-acetyl-beta-d-glucosaminidase, as the critical effect (Nordberg 2004). Cd is a cumulative nephrotoxicant. Recent research has linked Cd exposure to kidney dysfunction and decrease in bone mineral density in non-occupationally exposed populations who showed no signs of nutritional deficiency. Such Cd-linked bone and kidney toxicities were observed in people whose dietary Cd intakes were well within the provisional tolerable weekly intake (PTWI) set by the Joint Food and Agriculture Organization/World Health Organization Expert Committee on Food Additives of 1 µg/kg body weight/day or 70 µg/day. Further, an association between Cd-related nephropathy and high blood pressure was evidenced by a 20% increase in the prevalence of high blood pressure in people with NAG-uria (Satarug et al 2005). This evidence points to a much-needed revision of the current PTWI for Cd (Satarug S & Moore MR 2004).

Cd is an integral constituent of tobacco because of the propensity of the *Nicotiana* species to concentrate Cd independent of soil-Cd content. Tobacco Cd content varies widely, but a typical range is 1-2 µg/g dry weight, equivalent to 0.5-1 µg/cigarette. Cd oxide generated during the burning of cigarettes is highly bioavailable. Approximately 10% of the inhaled Cd oxide is deposited in lung tissues, and another 30-40% is absorbed into systemic blood circulation of smokers. Smokers have 4-5 times higher Cd levels in blood and 2-3 times greater amounts of Cd in their kidneys than do Non-smokers (Satarug & Moore 2004). Cd is associated with lung emphysema and lung cancer. Metallothionein (MT) is an inducible

protein that binds and detoxifies cellular Cd. Variation in metallothionein gene expression has been shown to be related to development of renal dysfunction. Alveolar macrophages of cigarette smokers accumulate significant amounts of Cd without a concurrent increase in MT content, indicating greater saturation of MT (Grasseschi RM et al 2003). Increased Cd burden in alveolar cells could contribute to the development of lung diseases in CSMs (Grasseschi RM et al 2003). In one study in pregnant women smokers showed higher placental MT and Cd levels, together with decreased newborn birth weights, as compared to non-smokers. Cigarette smoking increases Cd accumulation and stimulates MT production in placental tissue (Ronco AM et al 2005).

The gastrointestinal Cd absorption rate in humans has been estimated at 5% (IPCS 1992;WHO 1989). However, Cd absorption rates may increase to 20-30% in some individuals (Kikuchi et al. 2003; Satarug et al. 2004). The metal transporter protein Nramp2, known also as DMT1, has been shown to be involved in Cd absorption (Tallkvist et al. 2001). Increased expression of the intestinal DMT1 was found in iron deficiency (Zoller et al. 2001). Increased expression of the metal transporter protein DMT1, in general, would provide individuals with a greater capacity to absorb Fe and possibly Cd. This provides a likely explanation for a 3.4 fold increase in Cd body burden in women with low Fe stores (Satarug et al. 2004). Olsson et al. (2002) also observed an increased rate of Cd absorption in individuals with low body Fe stores.

Approximately 0.001% of Cd in the body is excreted per day, mostly in urine. Such extremely slow excretion rate of Cd is due to a lack of an active biochemical mechanism for elimination coupled with renal reabsorption. Cd accumulation occurs in various tissues and organs, with the most extensive accumulation in kidney cortex (Jarup et al. 1998). Cd persists in the kidneys of humans for many years (half-life of 30 years). This provides an opportunity for Cd toxicity to occur with no additional exposure, when the previously bound (nontoxic) Cd is displaced and released (Satarug et al. 2000b). There is a lack of therapeutically effective chelating agents to reduce Cd burden, and this factor makes exposure minimization pivotal.

The mean ( $\pm$ SD) extracted amount of Cadmium in the present study was estimated at 0.027, 0.043, 0.006 and 0.005 $\mu$ g /portion. This corresponds to a cadmium extraction of 10.5, 8.2, 5.7 and 3.0%, respectively, of the amount in unused snus for “General Large”, “Catch White Licorice Large”, “Catch Licorice Mini” and “Catch Licorice Dry Mini”. This is in agreement with previous research, showing a dietary Cd absorption rate in humans of 5% (IPCS 1992;WHO 1989) and in contrast to the highly bioavailable (40-50%) Cd oxide generated during the burning of cigarettes (Grasseschi RM et al 2003).

*Lead.* The General Large population is exposed to lead from air and food in roughly equal proportions. During the last century, lead emissions to ambient air have caused considerable pollution, mainly due to lead emissions from petrol. However, lead in petrol has dramatically decreased over the last decades. In France ( dietary exposure estimates appeared reassuring, in that Estimated Daily Intake (EDI) estimates were generally low, representing at maximum 28% of the Provisional Tolerable Weekly Intake (PTWI) for heavy metals (for lead 0.025 mg/kg bw). Children are particularly susceptible to lead exposure due to high gastrointestinal uptake and the permeable blood-brain barrier. Blood levels in children should be reduced below the levels so far considered acceptable, recent data indicating that there may be neurotoxic effects of lead at lower levels of exposure than previously anticipated. Lead

extraction in the present study was negligible for all studied brands of snus, not measurable with the methods employed.

Children are particularly susceptible to Pb exposure due to high gastrointestinal uptake and the permeable blood-brain barrier. Recent data indicate that there may be neurotoxic effects of Pb at lower levels of exposure than previously anticipated (Järup L 2003). The provisional tolerable weekly intake (PTWI) of 0.025 mg/kg bw was maintained at the fifty-third meeting of the Joint FAO/WHO Expert Committee on Food Additives (Report TRS 896-JECFA 53/81, 1999). However, Pb in petrol has dramatically decreased over the last decades. Dietary exposure estimates for heavy metals in e.g. France appear to be reassuring, representing 28% of the provisional tolerable weekly intake (PTWI) of 0.025 mg/kg bw. These dietary exposures for heavy metals are lower than those from previous French studies and similar to those from other countries (Leblanc JC et al 2000).

Lead is a classical chronic or cumulative poison. Health effects are generally not observed after a single exposure. The rate of absorption of lead after ingestion is heavily influenced by food intake, much higher rates of absorption occurring after fasting. Absorption is also affected by age, the typical absorption rates in adults and infants being 10% and 50%, respectively (O'Flaherty, 1995; Agency for Toxic Substances and Disease Registry, 1997).

The steady state kinetics of lead follows a three compartmental model for lead metabolism. The first compartment encompasses blood and is 1.5-2.2 times larger than the blood mass. It contains approximately 1.7-2.0 mg of lead and has a mean life of 35 days. This pool is in direct communication with ingested lead, urinary lead, and pools two and three. The second compartment is largely composed of soft tissue, contains about 0.3-0.9 mg of lead, and has a mean life of approximately 40 days. This pool gives rise to lead in hair, nails, sweat, and salivary, gastric, pancreatic, and biliary secretions. Pool three resides primarily in the skeleton, contains the vast quantity of body lead (Rabinowitz et al 1976). This pool displays a 5- to 19-year elimination half-life, which reflects the slow mobilization of skeletal lead. The bulk turnover rates for compact bone are about 2% per year and 8% for spine (Rabinowitz 1991).

Median blood lead elimination half-life was 619 days in patients with occupational chronic lead intoxication who were removed from exposure with normal renal function and 1,907 days in patients with renal impairment. Slow-phase elimination half-lives in patients followed for longer than 5 years ranged from 1,658 to 7,189 days. When treated with chelation with intravenous EDTA, blood lead concentrations declined with a mean half-life of 7 days, but rebounded to prechelation concentrations following termination of chelation (Hryhorczuk et al 1985).

Several large epidemiological studies have found that older people have higher blood and bone lead levels than younger adults. Lead can hibernate within bone for decades. Conditions of bone resorption, such as osteoporosis, can cause bone lead to reenter the bloodstream where it can then re-expose the soft tissue, and, potentially, exert delayed deleterious effects, such as hypertension, renal insufficiency, and cognitive impairment. In the future, treatment of osteoporosis may be undertaken not only to improve bone health but also to prevent mobilization of bone lead stores and subsequent toxicity (Vig & Hu 2000).

Chronic occupational exposure to lead, or consumption of illicit alcohol ("moonshine") adulterated with lead, has been linked to a high incidence of renal dysfunction, which is characterized by glomerular and tubulointerstitial changes resulting in chronic renal failure (CRF), hypertension, hyperuricemia, and gout. Blood lead levels are a poor indicator of body lead burden and evidence for increased body lead burden shown by in vivo X-ray

fluorescence for determination of bone lead content is a prerequisite for the diagnosis of lead nephropathy (Loghman-Adham 1997).

Smokers are thus exposed to both Cd and Pb. The Cd level in blood and Pb levels in blood and hair are significantly higher in smokers than non-smokers (Mortada et al 2004). One Swedish case-control study (Ejerblad et al 2004) suggests that heavy cigarette smoking increases the risk of chronic renal failure (CRF) for both men and women, at least CRF classified as nephrosclerosis and glomerulonephritis. Smoking increased risk most strongly for CRF classified as nephrosclerosis (odds ratio, OR, among smokers with >20 pack-years, 2.2; 95% confidence interval, CI, 1.3 to 3.8). In contrast to their results, the study by Mortada et al (2004), did not find exposure high enough to produce nephrotoxicity. Markers of kidney damage were neither elevated among the smokers nor correlated with the exposure indices of these metals. Nevertheless, according to the authors, the exposure may incite signs of nephrotoxicity in the presence of other risk factors for kidney diseases.

*Nitrosamines.* Tobacco contains nitrate that is microbially activated to nitrite, which may react with alkaloids to form cancer causing tobacco specific nitrosamines (TSNA) during curing and storage of the tobacco. NNK and *N'*-nitrosonornicotine are the most abundant strong carcinogens in unburned tobacco products and likely play a role in the induction of oral cavity tumors in snuff-dippers (Hecht 1998). Levels of NNK in smokeless tobacco products marketed in the US are typically ~1–2 µg/g tobacco (Hoffmann et al 1995). NNAL plus NNAL-Gluc, the main metabolites of NNK, are biomarkers in urine of tobacco carcinogen uptake. A significant association between levels of NNAL plus NNAL-Gluc, in the urine of smokeless tobacco users and the presence of oral leukoplakia has been observed, supporting the potential role of NNK as a causative factor for this lesion (Kresty et al 1996). Levels of NNAL and NNAL-Gluc in the urine of snuff-dippers and tobacco chewers are similar to those found in smokers (Kresty et al 1996). A correlation between number of tins or portions of smokeless tobacco consumed per week and NNAL plus NNAL-Gluc in urine has been observed, as well as a correlation between salivary cotinine and NNAL plus NNAL-Gluc in urine of smokeless tobacco users (Hecht et al 2002). The mean ratio ± standard deviation of NNAL-Gluc to NNAL is  $3.7 \pm 2.2$  in the urine of adult smokers (Carmella et al 1995). NNAL-Gluc has been detected in the urine of newborns of women who smoked, indicating that NNK, a transplacental carcinogen, crosses the placental barrier and is taken up by the fetus (Lackmann et al 1999). Consistent with these results, NNAL was detected in amniotic fluid of pregnant smokers (Milunsky A et al 2000).

After cessation of smokeless tobacco use, NNAL and NNAL-Gluc disappear slowly from the body, as in smokers (Hecht et al 2002). The distribution half-lives  $t_{1/2\alpha}$  (days) of NNAL ( $1.32 \pm 0.85$  versus  $3.35 \pm 1.86$ ) and NNAL-Gluc ( $1.53 \pm 1.22$  versus  $3.89 \pm 2.43$ ) were significantly shorter in smokeless tobacco users than in smokers. There were no significant differences in the terminal half-lives  $t_{1/2\beta}$  (days) of NNAL ( $26.3 \pm 16.7$  versus  $45.2 \pm 26.9$ ) and NNAL-Gluc ( $26.1 \pm 15.1$  versus  $39.6 \pm 26.0$ ) in smokeless tobacco users and smokers (Hecht et al 2002).

Murphy et al (2004) recently completed a study in which smokers were required to reduce the number of cigarettes smoked by 75% over a 6-week period. Urine samples were collected at four time points and analyzed for 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL), and its glucuronide. Total NNAL levels were statistically significantly lower in users of smokeless tobacco after they switched to snus ( $P < .001$ ) than they were before the switch. It was concluded that switching to reduced-exposure tobacco products or pharmaceutical nicotine products can decrease carcinogen uptake.

Because of the differences in selection, curing, manufacturing and storage, Swedish snus has been claimed to contain lower levels of some harmful substances than many of the brands available in North America (Wahlberg et al 1999, Österdahl et al 1983, Nilsson R 1998). The total TSNA concentration varied greatly among the US brands, from 4.1 for to 128 ( $\mu\text{g/g}$  dry tobacco). Snus brands selected in Sweden were generally lower in TSNA content and was approximately 2.8  $\mu\text{g/g}$  in 2000. The Swedish manufacturing includes a heating process to approximately 100°C, producing a nearly sterile product. This is in contrast to the one used in the USA, in which the moist snuff product is fermented, allowing a continued formation of TSNA. In addition, North American smokeless tobacco, in contrast to Swedish snus, is usually not stored in refrigerators. One study found that nitrite and TSNA levels increased significantly in US snuff stored at 37°C over four weeks (Djordjevic 1993).

Studies show that cigarette smoking produces more negative health effects, is likely to have a higher addiction potential and more severe withdrawal, and leads to a higher rate of relapse compared to snus use (Holm et al 1992). A survey of 985 current daily Swedish smokers and 1000 ex-smokers showed that there was an increased probability of being a former rather than a current smoker with ever use (OR 1.72, 95% CI = 1.30–2.28) or current use (OR 1.81, 95% CI = 1.31–2.53) of snus. Having used snus at the latest quit attempt increased the probability of being abstinent by about 50% (OR 1.54, 95% CI = 1.09–2.20). Current smokers who made use of snus smoked on average fewer cigarettes per day than non-users of snus (Gilljam & Galanti 2003).

Swedish snus analyzed for tobacco-specific N-nitrosamines (TSNA) by Österdahl et al (2004), was found to contain detectable levels of N'-nitrosonornicotine (NNN), N'-nitrosoanatabine (NAT), N'-nitrosoanabasine (NAB), and 4-(N-methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK). Total TSNA levels of between 0.15 and 3.0 microg/g wet weight were found. The mean content of total TSNA was 1.0-1.1 microg/g. The result showed that the level of TSNA in Swedish snus has been greatly reduced since the 1980s. Clearly, efforts made by the manufacturers to reduce the level of TSNA in snus have been successful.

In the present study the mean TSNA content in “General Large”, “Catch White Licorice Large”, “Catch Licorice Mini” and “Catch Licorice Dry Mini” was 1.1, 0.9, 0.5 and 0.4  $\mu\text{g/portion}$ , respectively. These figures are comparatively low and in agreement with the results of Österdahl et al (2004). The mean TSNA extraction in the present study was 0.4 $\mu\text{g}$  (39%), 0.4 $\mu\text{g}$  (41%), 0.09 $\mu\text{g}$  (20%) and 0.05 $\mu\text{g/portion}$  (14%), respectively. The mean nicotine extraction from “General”, “Catch”, “Catch Mini” and “Catch Dry Mini” was 2.4 (25%), 2.2 (26%), 1.4 (30%) and 0.7mg/portion (13%), respectively.

## 11. CONCLUSION

Cadmium extraction was below 10% for all brands. This is in contrast to the 40-50% of the inhaled Cd oxide that is absorbed into the lung tissues of smokers. Lead extraction was negligible for all brands. Pb levels in blood and hair are significantly higher in smokers than non-smokers and Pb toxicity associated with cigarette smoking may, according to some research, increase the risk of chronic renal failure. The risk profile of snus apparently is considerably lower than that of cigarettes with respect to both metals.

A recent study demonstrated that steady-state nicotine plasma concentrations may be sustained at any chosen level with Swedish snus to replace the more hazardous cigarette smoking (Lunell & Lunell 2005). Nicotine is the substance that the user of smokeless tobacco is seeking for. Adequate delivery of nicotine in relation to harmful substances is therefore a desirable feature. A comparison of the extraction of harmful substances in relation to the nicotine extraction was therefore made in the present study. The percent extraction of TSNA seemed to largely parallel the nicotine extraction, which should favour use of smokeless tobacco containing low levels of TSNA, such as the Swedish brands tested in the present study.

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