AP 4 - de Roton - Potential changes of TSNA composition in stored tobacco powder : consequences for sample preparation and ground tobacco storage

Authors: DE ROTON C.; GIRARD C.; JACQUET L.; POISSON C.

Potential changes of TSNA composition in stored tobacco powder : consequences for sample preparation and ground tobacco storage.

Christian de Roton, Christian Girard, Laëtitia Jacquet and Catherine Poisson
Altadis/Institut du Tabac, Domaine de la Tour, 24100 Bergerac – France

Introduction

During the past 2 years, several trials aiming at monitoring the changes of TSNA concentrations in cured leaves or strips of Burley tobacco during storage were carried out at the "Institut du Tabac de Bergerac" (ITB). Some of the trials produced inconsistent and incomprehensible results, and it was suspected that the control samples of ground tobacco taken at the beginning of the trials and stored at ambient temperature had formed TSNAs with time.

More generally, we needed to find out how to prepare (= to dry) tobacco samples, which have sometimes high moisture content, before grinding, and how to keep the powder when chemical analyses cannot be performed immediatly, while avoiding the formation of TSNAs during preparation and storage. To answer this question, a trial was undertaken in May 2003 at ITB.

Objectives

- To compare a method of sample drying and powder storage, which will be considered *a priori* without effect on TSNA concentrations, and taken as reference, with 3 methods of sample drying, combined with 3 methods of powder storage, easily feasible in routine operations.
- To define which combined method of drying and storage gives the TSNA concentrations that are the closest to those in the reference sample.

Materials and Methods

Plants of the Burley variety BB16NN were grown at ITB in 2002, according to the usual cultural practices. Stalk-cut plants were cured under a plastic shed. After curing, upper middle leaves were stripped and stored in dry conditions.

In May 2003, whole leaves were chopped to obtain 6 kg of 0,8mm wide cut-rag. The cut-rag was homogeneized and conditionned to reach 34% moisture.

Reference samples ("*Reference Lyophilisée Ambiante*": RLA): a batch of 900 g of moist cut-rag was dipped into liquid N, stored at -18 °C and sent by refrigerator transport (-18 °C)

to the plant where it was freeze-dried.
The freeze-dried batch of cut-rag was ground to 500 µm. 3 samples of about 100 g of powder were stored at -70 °C until they were sent to Global Laboratory Services ("GLS" -Wilson NC, USA).
6 other samples of about 100 g were stored in plastic jars at ambient temperature.

Modes of sample preparation

Before grinding the moist cut-rag to obtain powders for chemical analyses, 3 modes of sample preparation were implemented:

- Drying method TA: 1,7 kg of moist cut-rag were dried in wire mesh baskets at ambient temperature (20-25 °C) for 48h.
- Drying method TE: 1,7 kg of moist cut-rag were force air dried at 30 °C in a ventilated oven for 48h.
- Freezing method TN: 1,7 kg of moist cut-rag were dipped into liquid N.

Just after drying (TA, TE) or freezing (TN), all the batches of cut-rag were stored in an ice-box for 24h, to simulate a transportation from the place of sample taking to the laboratory.

The cut-rags that were already dry (TA, TE) or unfrozen (TN), were then force air dried to 6% moisture for 48h in a ventilated oven at 30 °C. Finally, they were ground to 500 µm.

For each mode of preparation (TA, TE, TN), 2 samples of powder were freeze-dried and stored at -70 °C until they were shipped to Global Laboratory Services (Wilson, USA) for TSNA analyses: this allows one to measure the effect of the sample preparation in itself, by comparison with an immediate freeze-drying (RLA), regardless of the subsequent storage.

Conditions of powder storage

Powder samples of each mode of preparation TA, TE and TN were stored in closed plastic jars containing 40g of powder according to 3 modes of storage:

- at ambient temperature (20-25 °C) in the laboratory (TAA, TEA, TNA)
- in a refrigerator (+ 6 °C) (TAF, TEF, TNF)
- in a freezer (-18 °C) (TAC, TEC, TNC) (1)

In each of the 9 resulting treatments (3 modes of preparation*3 modes of storage), 8 plastic jars were filled with the tobacco powder.
Two replications of the powder from each of the 9 treatments were freeze-dried and analysed after 4 lengths of storage (41 days, 133 days, 276 days and 424 days). These lengths include the transportation from Bergerac to Wilson, by train, plane etc...at various and uncontrolled temperatures, and the waiting time before analysis by Global Laboratory Services (10-16 days in total).
At the same dates, 2 samples of the reference RLA, which had been stored at ambient temperature, were freeze-dried and sent to Global Laboratory Services, where the analyses of TSNAs and moisture content were performed.

(1) T= Treatment; A= Ambient; E= Oven (*Etuve)*; N= Liquid N; F= "Fridge"; C= Freezer

(*Congelator*)

Example: TEC = oven dried and stored in a freezer ..... etc..

Results

All the powders had a residual moisture content of 1,3% – 2,8%, whatever the length and the mode of storage. Results are given on a dry matter basis.

Alkaloids

Alkaloid concentrations were analysed at ITB by HPLC.

Individual alkaloid concentrations in reference samples RLA were, in % d.m.:

nicotine:	3.71
anatabine:	0.18
anabasine:	0.02
myosmine:	below detection limit
nornicotine:	0.46
Total alkaloids:	4.37

The concentrations of individual alkaloïds did not change significantly with time.

TSNAs

(GC-TEA Method. Detection limit: 0,1 µg/g. Accuracy: ± 5%)

The reference samples RLA which were immediatly analysed after freeze-drying had a low TSNA concentration of **1,07 µg/g** (mean of the 3 replications).

The modes of preparation themselves slightly increased the concentrations of TSNAs in tobacco powder, regardless of the conditions of storage. Just after freeze-drying and before storage, the TSNA concentrations in the samples that had been prepared by drying or freezing and drying were (means of 2 replications):

- 1,30 µg/g in TA
- 1,39 µg/g in TE
- 1,42 µg/g in TN

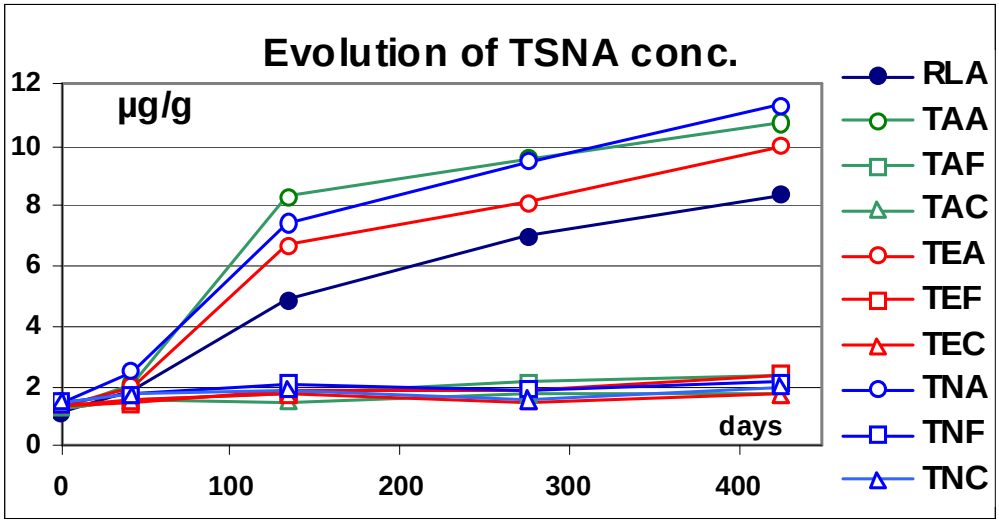
TSNA concentrations increased in all the stored powders, more or less sharply, according to the length and the temperature of storage.

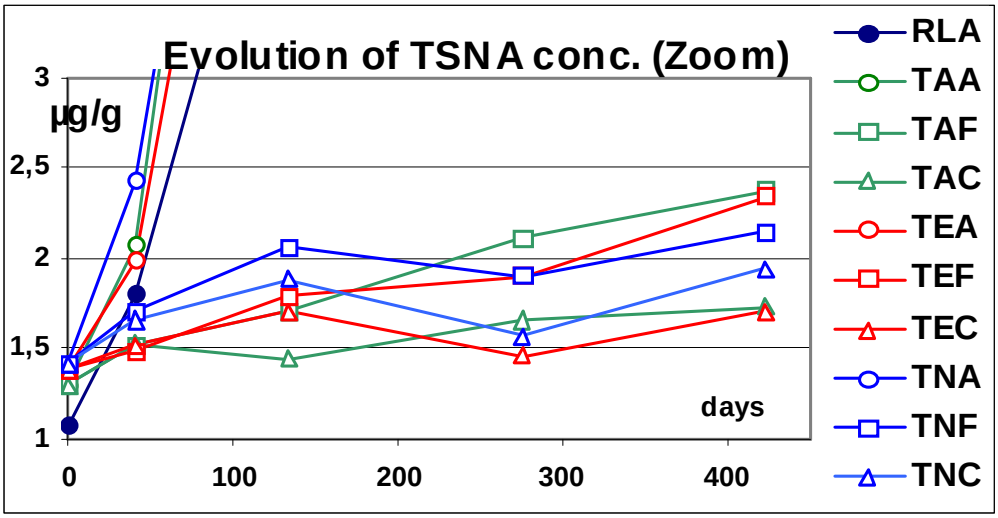
Results of TSNA concentrations in tobacco powders (µg/g d.m., means of 2 replications) after storage and final freeze-drying are shown in table 1 and in Figures 1 and 2.

Table 1: TSNA concentrations (µg/g) in stored tobacco powders.

	PREPARATION							STORAGE		1st analysis
	14/05/03	15/05/03	16/05/03	17/05/03	18/05/03	19/05/03	21/05/03	04/06/03	12/06/03	24/06/03
days	0	1	2	3	4	5	7	21	29	41
RLA	Liquid N	- 18℃ then freeze-drying at day 21 then - 70℃ unt il shipment at day 29							ambient	1,07
TAA	drying at ambient T℃			icebox 24h	oven drying 30℃ - grinding		powder dispatching	ambient		2,07
TAF								6℃		1,51
TAC								-18℃		1,53
TEA	oven drying 30℃			icebox 24h	oven drying 30℃ - grinding		powder dispatching	ambient		1,99
TEF								6℃		1,48
TEC								-18℃		1,51
TNA	Liquid N	icebox 24h	unfreezing		oven drying 30℃ - grinding		powder dispatching	ambient		2,43
TNF								6℃		1,70
TNC								-18℃		1,66

Figures 1 and 2: Evolution of TSNA concentrations in stored tobacco powders.





TSNA concentrations increased only slightly, though significantly, in the powders stored at low temperature: after a storage of 424 days, they reached 2,29 µg/g in powders stored at + 6 °C and 1,79 µg/g in powders stored at -18 °C (means of 2 replications and 3 modes of preparation).

The difference of TSNA concentrations between the 2 temperatures of storage is significant.

TSNA concentrations increased dramatically in the powders stored at ambient temperature, especially during the first 3 months, and reached 10,69 µg/g after 424 days of storage (means of 2 replications and 3 modes of preparation). One year later, TSNA concentrations were multiplied by about 10. A parallel increase was observed in reference samples RLA stored at ambient temperature.

The mode of preparation had no significant nor consistent effect on this evolution.

Individual nitrosamines were not affected the same way by the conditions of storage, as shown in tables 2, 3 and 4.

Table 2: Powders stored at ambient T °C
(µg/g - means of 2 reps and 3 modes of preparation)

days	0	41	133	276	424
NNN	0,53	1,39	5,42	6,67	7,90
NAT	0,50	0,76	1,70	1,83	2,48
NAB	0,00	0,00	0,00	0,03	0,06
NNK	0,04	0,05	0,30	0,48	0,25
TSNA	1,07	2,20	7,42	9,01	10,69

Table 3: Powders stored at 6 °C
(µg/g - means of 2 reps and 3 modes of preparation)

days	0	41	133	276	424

NNN	0,53	0,95	1,28	1,34	1,67
NAT	0,50	0,62	0,39	0,19	0,49
NAB	0,00	0,00	0,00	0,00	0,00
NNK	0,04	0,00	0,18	0,44	0,13
TSNA	1,07	1,56	1,85	1,97	2,29

Table 4: Powders stored at -18 °C
(µg/g - means of 2 reps and 3 modes of preparation)

days	0	41	133	276	424
NNN	0,53	0,94	1,16	1,05	1,29
NAT	0,50	0,63	0,35	0,13	0,39
NAB	0,00	0,00	0,00	0,00	0,00
NNK	0,04	0,00	0,17	0,43	0,11
TSNA	1,07	1,57	1,68	1,61	1,79

With time, NNN conc. increased in all the samples, much more at ambient than at low temperatures. After 424 days of storage, they were multiplied by 15 at ambient temperature, by 3,2 at 6 °C and by 2,4 at -18 °C.

NAT conc. increased continuously in samples stored at ambient temperature, and were multiplied by 5 after 424 days.
NAT conc. remained low at low temperatures and did not evolve evenly, with a negative trend until day 276, and a positive trend afterwards.
NNK conc. increased with time until day 276, then decreased in all the samples, but remained low and, at each date of analysis, were similar whatever the temperature of storage.
NAB was detected only in samples stored at ambient temperature, after 276 days, at a very low concentration.

As a result, the % distribution of individual nitrosamines in the samples evolved with slight differences depending on the temperature of storage, as shown in Table 5.
NNN became predominant in all the samples, to the detriment of NAT.
The proportion of NNK in total TSNAs increased as the temperature of storage decreased, to the detriment of NNN and NAT.

Table 5: % distribution of individual nitrosamines in stored tobacco powder.

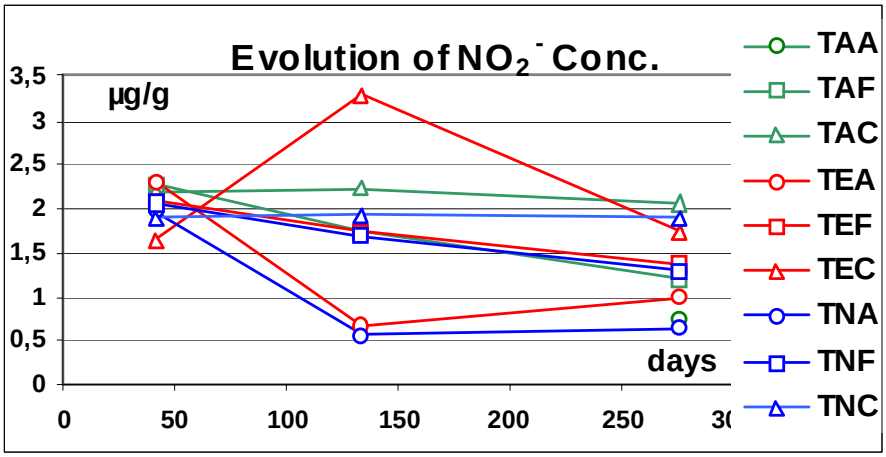
	Days	**0**	**41**	**133**	**276**	**424**
Ambient	NNN	49.6	63.2	73.1	74.1	73.9
	NAT	46.7	34.5	22.9	20.3	23.2
	NAB	0.0	0.0	0.0	0.3	0.6
	NNK	3.7	2.3	4.0	5.3	2.3
	TSNA	100	100	100	100	100

6 °C	NNN	49.6	60.5	69.2	68.0	72.9
	NAT	46.7	39.5	21.1	9.7	21.4
	NAB	0.0	0.0	0.0	0.0	0.0
	NNK	3.7	0.0	9.7	22.3	5.7
	TSNA	100	100	100	100	100
- 18 °C	NNN	49.6	59.9	69.1	65.2	72.1
	NAT	46.7	40.1	20.8	8.1	21.8
	NAB	0.0	0.0	0.0	0.0	0.0
	NNK	3.7	0.0	10.1	26.7	6.1
	TSNA	100	100	100	100	100

Nitrite

Nitrite concentrations were analysed by colorimetry in the laboratory of Altadis on powder samples stored at ambient temperature, at 6 °C and at -18 °C, after 41, 133 and 276 days of storage, in 1 replication. Results are shown in Figure 3.

Figure 3: Changes of nitrite concentrations in stored tobacco powder.



Nitrite concentrations were low in all the samples, from 0,5 to 3,3 µg/g. The concentration of 3,29 µg/g observed in the sample TEC at day 133 is an abnormal value.

Nitrite concentrations remained stable, around 2 µg/g during storage at -18 °C.
They decreased slightly and steadily, from 2 µg/g to 1,3 µg/g during storage at + 6 °C.
They decreased sharply, from 2 µg/g to 0,5 µg/g, between days 41 and 133, then levelled off in samples stored at ambient temperature.

The changes of nitrite concentrations were, therefore, exactly the reverse of the changes of TSNA concentrations with time and temperature.
Taking into account the molecular weights of nitrite (46 g/mole) and TSNAs (177 to 192 g/mole), it seems likely that the 2 µg/g of nitrite disappeared in the samples stored at ambient temperature (TAA, TEA, TNA) account for the 6-10 µg/g of TSNAs formed in these

samples.

Conclusion

The results of this study show that even a low concentration of nitrite may result in a significant formation of TSNAs in tobacco with time, if the tobacco is kept in a finely divided form, such as powder, at ambient temperature.
The increase of TSNA concentrations occured mainly during the first 3 months of storage, but went on afterwards, for at least one year.

The method of sample preparation for chemical analyses resulting in the lowest TSNA concentrations consists in dipping the sample in liquid N, freeze-drying and grinding. The powder should be kept at low temperature (ideally -70 °C/ -80 °C) until analysis.

If this method cannot be carried out, it is preferable:
- to dry the sample in a ventilated oven at 30-35 °C, or failing that, at ambient temperature, in a warm and aired environment
- to grind the sample **at the very last moment**, just before shipping to the laboratory
- if the powder has to be kept for a time, to store it at the low temperatures of 6 °C or, **better, -18 °C**.