PROTEIC COMPOUNDS REDUCTION IN SMOKE TOBACCO BY SUPERCRITICAL ANTISOLVENT TECHNIQUE (SAS)

Scrugli S*., Frongia M., Sfacteria G., Loi G., Pinna M.B., Crobu V. and Carta D.

Consorzio Ricerche Associate (C.R.A.) Macchiareddu, Assemini (CA), Italy

e-mail: stescrugli@yahoo.it,
fax: +39 070 2464253

INTRODUCTION

In the last years cigarette manufacturers have devised systems to reduce the nicotine and tar content of the burning tobacco for health reasons. Most of these systems have resulted in a safer smoking product, but they do not remove all of the undesirable constituents in the tobacco smoke and, because of a reduction in taste, has been criticized by smokers. Therefore, it is still necessary to develop a tobacco product which is inherently low in nicotine and tars but that retains important flavor ingredients [1].

Supercritical fluids based techniques have sometimes been applied to the extraction and fractionation of vegetable compounds. Among the various possible processes, the Supercritical Antisolvent (SAS) precipitation is the one that can allow the effective fractionation of liquid solutions containing solid solutes [2]. In this process, the solute to be precipitated is dissolved in an organic solvent and the solution is concurrently sprayed into a compressed or supercritical fluid, which serves as an antisolvent [3]. CO_2 soluble compounds at process conditions remain in the fluid phase; whereas, insoluble compounds precipitate and a very effective fractionation can be obtained [4-7].

Therefore, the aim of this work is to perform a new process, using the Supercritical Antisolvent (SAS) precipitation technique, that allows to reduce precursor compounds of toxic molecules from cured tobacco (proteins, peptides, amino acids and lipids) without loosing desirable compounds such as nicotine, flavours, sugar, etc.,

In order to restore starting desirable characteristics in ethanol extracted tobacco we have performed a recharging with ethanolic residues recovered from the two separators of SAS apparatus.

MATERIALS AND METHODS

Plant material

Kentucky cured tobacco has been purchased from Comatab (Avellino, Italy). Burley cured tobacco has been purchased from Dimon (Italy). Cured tobacco leaves have been grinded and blended, to obtain the same granulometry as the one used by tobacco manufactures. Particle size distribution is shown in Table 1.

Particle size (mm)	Weight percentage (% wt)
<2000, >1700	**45**
<1700, >1000	**27**
<1000, >710	**20**
<710	**9**

Table 1: particle size distribution of cured tobacco blend

Extraction

All solvents used for the extraction procedures and in the dilution steps for the preparation of analysable samples, were RPE grade from Baker J.T. (Deventer, Netherlands) and Carlo Erba (Milano, Italy). Water has been purified by a Milli-Q plus system from Millipore (Milford, MA, USA).

The extraction was performed for 3 hours at 40°C, using a KOH ethanol solution (1%, w/v) and a tobacco/solution ratio of 1 to 10 (w/v).

At the end of the extraction the biomass was removed by filtration and dried at 40°C.

Nitrogen and protein determination

The amount of organic nitrogen in the cured tobacco (**CR**), in the ethanol extracted tobacco (**EET**) and in the reconstituted tobacco (**RT**) was measured following the Kjeldhal nitrogen determination method using the Büchi-Digestion-System and the Büchi-Kjeldhal-System [8,11].

The amount of protein in the precipitated samples was performed using the Bradford reagent for protein determination (product number B6916) and a spectophotometer UV-VIS Helios Alpha (Spectronic Unicam).

Amino acid determination

We used the Waters AccQTag method for the amino acids determination, which is a precolumn derivatization technique for peptide and protein hydrolysate amino acids, using Waters AccQFluor Reagent to derivitize the amino acids.

Waters AccQFluor Reagent is a highly reactive compound, 6-aminoquinolyl-N-hydroxy-succinimidyl carbamate, which readily forms stable derivatives with primary and secondary amino acids. The derivatives are easily separated by reverse phase HPLC using Waters AccQTag Amino Acid Analysis System.

Alkaloids determination

The alkaloid concentration in the CR, EET and in RT was determined by the AOAC official method [12].

SAS apparatus

SAS apparatus consists of a piston pump used to deliver the liquid solution and a diaphragm pump used to deliver the supercritical carbon dioxide (SC-CO_2). A cylindrical vessel of 500 cm^3 I.V. (I. D. = 5 cm) is used as precipitation chamber. The liquid mixture is delivered to the precipitator as a rule through a stainless steel nozzle that have a diameter of 120 μm. Supercritical CO_2 is delivered through another inlet port located on the top of the chamber. Before entering the precipitator, CO_2 is heated at the process temperature. The precipitation chamber is electrically heated using thin band heaters. The pressure in the chamber is measured using a test gauge manometer and regulated by a micrometering valve located at the exit (bottom) of the chamber. This valve is heated by a cable heater connected to a controller. A stainless steel frit located at the bottom of the chamber is used to collect the produced powder; but it allows the CO_2-organic solvent solution to pass through. Other two collection chambers are located downstream the micrometering

valve and are used to recover the liquid solvent. A backpressure valve regulates the pressure in this chamber. At the exit of the second vessel a rotameter and a dry test meter are used to measure the CO_2 flow rate and the total quantity of antisolvent delivered, respectively.
SAS experiments were performed at the conditions indicated in Table 2.

Liquid solvent	EtOH/1% KOH
Antisolvent	CO_2
Precipitator Pressure (bar)	100
Precipitator Temperature (°C)	40
Separator Pressure (bar)	35
Separator Temperature (°C)	10

Table 2: conditions of SAS experiments

The residue recovered from the two separators has been added up to the EET, previous acidification witl HCl (1% V/V) to neutralize tobacco. The solvent has been evaporated under vacuum and the tobacco drie at 40°C.

II - RESULTS AND DISCUSSION

The selection of the process parameters was based on the expected solubilities in SC-CO_2 of the various compounds in the extract. Ethanol is readily soluble in SC-CO_2; therefore, the couple solvent-antisolvent should not deserve problems in SAS processing. Proteins and their derivative aminoacids should be virtually insoluble in SC-CO_2 due to their composition and their molecular weight. Moreover, in the scientific literature [4,5], examples are reported of successful precipitation of proteic compounds using SC-CO_2 as the antisolvent. Alkaloids like nicotine are soluble in SC-CO_2 especially at high densities, cuticular waxes and flavouring compounds can also been readily extracted [2]. Therefore, based on these considerations, a fractionation of the ethanolic extract is in principle possible, if processing conditions are selected to induce proteic compounds precipitation and the transfer in the fluid phase of the liquid solvent together with nicotine, waxes and flavouring components.

The ethanolic extract fed to SAS apparatus was efficiently fractionated obtaining a solid precitate and an ethanolic residue. As we reported in previews works, the major part of the precipitate is potassium bicarbonate ($KHCO_3$), coming from the following reaction: CO_2 + KOH ?$KHCO_3$, plus proteic compounds. Nicotine is totally present, probably with flavours and pigments, in the ethanolic residues that we have added up to smoke tobacco to avoid changes in taste and fragrance for smokers [13,14]

After residue addition we have performed chemical analysis on CT, EET and RT as describe before, results shown below express the percentage of each compound in tobacco:

Burley	Total N	Proteins	Amino acids	Nicotine	Lipids	pH	Weight (g)
CT	4.77	9.36	4.23	1.70	5.31	5.02	18.79
EET	3.49	5.53	3.32	0.50	1.98	9.25	17.17
RT	3.84	8.76	3.48	1.41	4.98	4.90	19.00
Reduction RTvsCT	**19.41**	**6.41**	**17.63**	**17.06**	**6.21**		

Kentucky	Total N	Proteins	Amino acids	Nicotine	Lipids	pH	Weight (g)
CT	3.54	10.27	1.33	2.80	6.81	5.33	15.00
EET	2.76	9.00	1.20	0.63	1.25	9.24	14.04
RT	2.90	9.48	1.15	2.53	4.93	5.16	15.19
Reduction RTvsCT	**18.08**	**7.69**	**13.53**	**9.64**	**27.61**		

Table 3: tobacco composition (%) in different process steps

Results show that the amount of macromolecules as proteic compounds and lipids, probably precursor of toxic molecules during burning, has been decreased at the end of the hole process, while the total amount of nicotine, as well as the weight and pH, has been re-established in RT.

Future work will be performed to investigate the smoke quality of the RT.

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