(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
30 May 2014 (30.05.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/080229 A1

(51) International Patent Classification:
A24B 15/24 (2006.01)

(21) International Application Number:
PCT/GB2013/053108

(22) International Filing Date:
25 November 2013 (25.11.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
1221199.1 26 November 2012 (26.11.2012) GB

(71) Applicant: **BRITISH AMERICAN TOBACCO (INVESTMENTS) LIMITED** [GB/GB]; Globe House, 1 Water Street, London WC2R 3LA (GB).

(72) Inventors: **HU, Jin**; British American Tobacco (Investments) Limited, Globe House, 1 Water Street, London WC2R 3LA (GB). **SAUNDERS, Robert**; Neem Biotech Limited, 9 Bowham Avenue, London CF31 3PD (GB).

(74) Agent: **RUSSELL, Tim**; Venner Shipley LLP, 200 Aldersgate, London EC1A 4HD (GB).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

(54) Title: TREATMENT OF TOBACCO MATERIAL

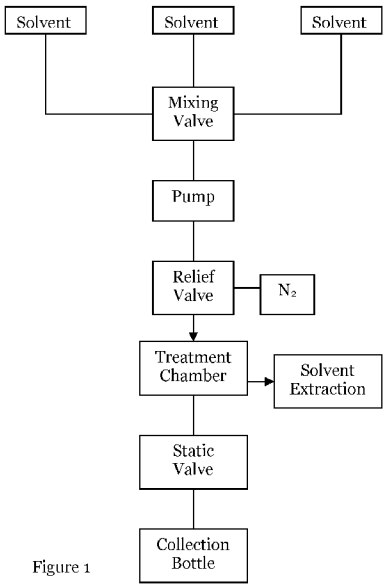


Figure 1

(57) Abstract: A method is provided for treating a tobacco material, wherein the method comprises treating the tobacco material with subcritical water. Also provided is a tobacco material which has been treated by such a method, or a derivative thereof, and a smoking article which comprises a tobacco material treated by such a method.

WO 2014/080229 A1

Treatment of Tobacco Material

Field of the Invention

The present invention relates to a method for the treatment of tobacco material.

Background

In some circumstances, it may be desirable to reduce the content of certain constituents from tobacco material before incorporating the tobacco material into a smoking article, such as a cigarette. For example, it may be desirable to reduce the protein content of tobacco material. Methods attempting to remove proteins have been proposed, although they have tended to be expensive, lengthy, and/or detrimental to the physical structure of the tobacco material, and/or not reduce the protein content to a desired level.

Summary

According to a first aspect, there is provided a method for treating a tobacco material, wherein the method comprises treating the tobacco material with subcritical water.

According to a second aspect, there is provided a tobacco material which has been treated by a method according to the first aspect, or a derivative thereof.

According to a third aspect, there is provided a smoking article comprising a tobacco material according to the second aspect.

According to a fourth aspect of the present invention, there is provided the use of subcritical water for removing one or more polyphenols or proteins from a tobacco material.

Brief Description of the Drawings

Embodiments of the invention will now be described, by way of example only, with reference to the accompanying drawings, in which:

Figure 1 shows a schematic of the Dionex ASE 350 pressurised hot water extraction equipment.

Figures 2 and 3 show the physical properties of tobacco material treated in accordance with a method of the invention with a static time of 30 minutes.

Figure 4 shows the physical properties of tobacco material treated in accordance with the invention at different static times.

Figure 5 shows an HPLC trace of an untreated tobacco material.

Figure 6 shows the concentration of polyphenols in a tobacco material treated in accordance with the invention with a static time of 45 minutes.

Figure 7 shows concentration of protein in a tobacco material treated in accordance with the invention.

Figure 8 is a schematic side view of a smoking article including treated tobacco material according to embodiments of the invention.

Figure 9 shows the chemical structure of the four reference polyphenol compounds detected and measured in experiments using HPLC: scopoletin, caffeic acid, chlorogenic acid, and rutin.

Detailed Description

There is provided a method for treating a tobacco material, wherein the method comprises treating the tobacco material with subcritical water. Subcritical water is liquid water under pressure at a temperature between its conventional boiling point and its critical temperature, i.e. between 100°C and 374°C. Subcritical water may also be referred to as "superheated water" or "pressurized hot water".

Treating the tobacco material with subcritical water may be used for the purpose of modifying the tobacco material in any suitable way. In some embodiments, treatment with subcritical water leads to the removal of one or more chemical substances. In particular, in some embodiments, treatment with subcritical water leads to the removal of one or more undesirable substances. In some embodiments, treatment with subcritical water leads to the removal of one or more polyphenols. In some embodiments, the treatment with subcritical water leads to the removal of one or more polyphenols selected from the group consisting of chlorogenic acid, caffeic acid, scopoletin, quercetin, and rutin. In particular, in some embodiments the treatment with subcritical water leads to the removal of chlorogenic acid and/or rutin. In some embodiments, the treatment with subcritical water may lead to the removal of one or more proteins. In some embodiments, the treatment with subcritical water may lead to the removal of one or more polyphenols and one or more proteins.

In some embodiments, treatment of the tobacco material with subcritical water results in a reduction in the content of one or more polyphenols of at least 5%, 10%, 15%, 20%,

25%, 30%, 35%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or a reduction in the content of one or more polyphenols of 100%, based upon the polyphenol content of the untreated tobacco material.

In some embodiments, the treatment of the tobacco material with subcritical water results in the extraction of one or more polyphenols in an amount of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or 100%, based upon the polyphenol content of the untreated tobacco material.

Alternatively or in addition, the treatment with subcritical water results in a reduction in the protein content of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95% or a reduction in the protein content of 100%, based upon the protein content of the untreated tobacco material.

In some embodiments, the treatment of the tobacco material with subcritical water results in the extraction of protein in an amount of at least 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 50%, 60%, 70%, 75%, 80%, 85%, 90% or at least 95% or 100%, based upon the protein content of the untreated tobacco material.

The method of the invention comprises at least one step in which the tobacco material is treated with subcritical water ("subcritical water treatment step"). In some embodiments, where the method comprises more than one subcritical water treatment step, the same or different conditions may be employed in each subcritical water treatment step.

The subcritical water treatment step employed in the method of the present invention may involve contacting the tobacco material with subcritical water. In some embodiments, the method may involve submerging the tobacco material in subcritical water. In some embodiments, the method involves submerging the tobacco material in water at ambient temperature and subsequently increasing the pressure and temperature. This increase in pressure and temperature provides subcritical water in which the tobacco material is submerged. For example, the method may involve first increasing the pressure and then increasing the temperature of the water so as to provide the tobacco material submerged in subcritical water.

Subcritical water exists under pressure, i.e. at an increased pressure in comparison to atmospheric pressure. The pressure employed in the method of the present invention may be any pressure suitable for providing subcritical water. That is, a pressure at which liquid water exists at a temperature between 100°C and 374°C. In some embodiments, the method of the present invention comprises treating the tobacco material with subcritical water at a pressure of from about 1500psi to about 1700psi (from about 100 bar to about 120 bar). In some embodiments, the pressure is about 1500psi (about 100 bar).

Subcritical water exists at temperatures between 100°C and 374°C. The temperature employed in the method of the present invention may be a temperature that provides liquid water at a temperature between 100°C and 374°C. In some embodiments, the treatment step is carried out at a temperature of at least 125°C. In some embodiments, the treatment step is carried out at a temperature between about 100°C and about 220°C or between about 120°C and about 200°C or between about 125°C and about 175°C. In some embodiments, the treatment step is carried out at a temperature between about 125°C and about 150°C.

In some embodiments, the water employed in the method may be de-oxygenated water. For example, the water may be degassed using a sonicated bath to remove dissolved oxygen. In some embodiments, the water employed is HPLC grade water.

In some embodiments, treating the tobacco material with subcritical water is a static treatment. For example, the tobacco material may be submerged in subcritical water for a period of time; referred to as the "static period".

The static period may be any length of time that allows the tobacco material to be modified in the required way. In some embodiments, the method of the present invention involves a static period of up to 2 hours. In some embodiments, the method involves a static period of up to 1 hour. In some embodiments, the method involves a static period of between about 5 minutes and about 55 minutes or between about 15 minutes and about 45 minutes.

After treatment with subcritical water, the tobacco material may be separated from the water (also referred to as the liquid extract). This separation may involve any suitable filtration method, any suitable filtering medium pore size, and any suitable number of

filtration steps. For example, the tobacco material may be filtered by paper filtration, nanofiltration, microfiltration, and/or ultrafiltration. Alternatively or in addition, the tobacco material may be separated from the liquid extract by centrifugation using any suitable centrifuge system, any suitable angular velocity, and any suitable number of centrifugation steps.

In some embodiments, the methods of the present invention therefore further comprise the step of separating the tobacco material from the water. The pressure at which the step of separating (whether by filtration or by any other means) is carried out is independent of the pressure employed in the subcritical water treatment step. In some embodiments, the step of separating (whether by filtration or by any other means) is carried out at the same pressure as the subcritical water treatment step.

The method of the present invention may involve one or more subcritical water treatment steps. In some embodiments, the method comprises two or more (multiple) subcritical water treatment steps.

In some embodiments involving a static process, the methods of the present invention may involve one or more static subcritical water treatment steps. In some embodiments involving a static process, the method comprises two or more (multiple) static subcritical water treatment steps. For example, the method of the present invention may comprise: a first subcritical water treatment step comprising treating a tobacco material with subcritical water by submerging it in subcritical water; a first subsequent separation step comprising separating the tobacco material from the subcritical water (e.g. by filtration); a second subcritical water treatment step comprising treating a tobacco material with subcritical water by submerging it in subcritical water; and a second subsequent separation step comprising separating the tobacco material from the subcritical water (e.g. by filtration). In some embodiments, such a method of the present invention further comprises a third subcritical water treatment step comprising treating a tobacco material with subcritical water by submerging it in subcritical water; and a third subsequent separation step comprising separating the tobacco material from the subcritical water (e.g. by filtration).

In some embodiments of the above multiple treatment/separation embodiments of the invention, each treatment step and each separation step is carried out at the same

pressure, e.g. from about 1500psi to about 1700psi (from about 100 bar to about 120 bar).

Once separated from the water/liquid extract, the tobacco material (also referred to as tobacco residue) may be washed any suitable number of times using any suitable liquid or liquids, such as water. In some embodiments, the methods of the invention further comprise a washing step comprising washing the treated tobacco material with water.

In some embodiments, the method of the present invention further comprises a step of drying the treated, separated tobacco material. For example, the tobacco material may be dried using a centrifuge and/or in an oven.

Tobacco material comprises dead plant cells, and dead plant cells have many functional groups. In some embodiments, the functional groups are reactive towards water under conditions provided by the invention. As a result, exposing tobacco material to water under favourable conditions is likely to result in the breakdown of different cellular structures, and the consequent release of different chemical substances. Most significantly, cellulose in the plant cell walls comprises O-glycosidic bonds, which may be broken under favourable conditions to cause the cell wall to rupture, and the cell membrane to rupture — without the cell wall to balance the positive pressure potential of the water (Ψ_p), and many intracellular substances to escape.

The method of the invention may be applied to any suitable tobacco material. The tobacco material may be derived from any suitable part of any suitable tobacco plant of the plant genus *Nicotiana*. The tobacco material may then be treated in any suitable way, and may be cured using any suitable method of curing, before being treated according to the method of the invention. In some embodiments, however, the tobacco material treated by the method of the invention has already been cured and may be cured cut rag and/or cured whole leaf tobacco. Examples of tobaccos which may be used in the method of the invention include, but are not limited to: Virginia, Burley, Maryland, Oriental, and Rustica.

In some embodiments, the method of the invention — in particular the step of treating tobacco material with subcritical water — reduces or minimises the removal of at least some of the chemical substances whose removal would be undesirable, which could be the case for a variety of different reasons. One reason, for example, could be that the

substance makes a positive contribution to the experience of smoking a smoking article which contains the treated tobacco material.

Nicotine may be an example of such a substance, and for this reason in some embodiments it is undesirable to remove this molecule. In some embodiments, the method of the invention removes less than 50%, 40%, 30%, 20%, 10%, or 5% of the nicotine from the tobacco material; in further embodiments, the method of the invention removes less than 2%, 1%, 0.5%, or 0.1% of nicotine from the tobacco material; and, in further embodiments still, the method of the invention removes essentially no nicotine from the tobacco material.

In embodiments wherein treating the tobacco material with subcritical water leads to the removal of one or more chemical substances from the tobacco material, one or more of these may be re-introduced into the material following treatment, and one or more of these may be substances whose removal would be undesirable, such as nicotine.

In addition to one or more subcritical water treatment steps, the method of the invention may comprise one or more further treatment steps. Further treatment steps may be particularly useful in the method of the invention for the purpose of removing large quantities of protein. This is because treatment with subcritical water is likely to rupture the plant cell walls in the tobacco material, thereby providing easier access to the intracellular components of the plant cells and the proteins found therein. Suitable additional treatment steps include, but are not limited to: treating the tobacco material with one or more suitable non-ionic liquids, such as water; treating the tobacco material with one or more enzymes, which may be enzymes which catalyse the modification of polyphenols or proteins, such as phenol-oxidising and proteolytic enzymes; treating the tobacco material with one or more suitable surfactants, such as sodium dodecylsulfate (SDS), in any suitable solvent; treating the tobacco material with one or more suitable adsorbent materials, such as polyvinyl polypyrrolidone (PVPP), hydroxylapatite, bentonite, activated carbon or attapulgite, in any suitable solvent if appropriate; and treating the tobacco material with one or more suitable non-aqueous liquids, such as ionic liquids.

Additionally or alternatively, the tobacco material subjected to subcritical water treatment may be subsequently subjected to further extraction processes.

Having undergone any of the previously-described treatment steps in accordance with the method of the invention, the tobacco material may be dried and further modified in any suitable way before being incorporated into a smoking article. For example, certain chemical substances may be added to the tobacco material, such as flavourants where local regulations permit, and the tobacco material may be cut and/or shredded before being incorporated into a smoking article using any suitable method of incorporation.

As used herein, the term "smoking article" includes smokeable products such as cigarettes, cigars and cigarillos whether based on tobacco, tobacco derivatives, expanded tobacco, reconstituted tobacco or tobacco substitutes and also heat-not-burn products. The smoking article may be provided with a filter for the gaseous flow drawn by the smoker.

As used herein, the terms "flavour" and "flavourant" refer to materials which, where local regulations permit, may be used to create a desired taste or aroma in a product for adult consumers. They may include extracts (e.g., licorice, hydrangea, Japanese white bark magnolia leaf, chamomile, fenugreek, clove, menthol, Japanese mint, aniseed, cinnamon, herb, wintergreen, cherry, berry, peach, apple, Drambuie, bourbon, scotch, whiskey, spearmint, peppermint, lavender, cardamon, celery, cascarilla, nutmeg, sandalwood, bergamot, geranium, honey essence, rose oil, vanilla, lemon oil, orange oil, cassia, caraway, cognac, jasmine, ylang-ylang, sage, fennel, piment, ginger, anise, coriander, coffee, or a mint oil from any species of the genus Mentha), flavour enhancers, bitterness receptor site blockers, sensorial receptor site activators or stimulators, sugars and/or sugar substitutes (e.g., sucralose, acesulfame potassium, aspartame, saccharine, cyclamates, lactose, sucrose, glucose, fructose, sorbitol, or mannitol), and other additives such as charcoal, chlorophyll, minerals, botanicals, or breath freshening agents. They may be imitation, synthetic or natural ingredients or blends thereof. They may be in any suitable form, for example, oil, liquid, or powder.

Referring to Figure 8, for purpose of illustration and not limitation, a smoking article **1** according to an exemplary embodiment of the invention comprises a filter **2** and a cylindrical rod of smokeable material **3**, such as tobacco treated in accordance with the invention described herein, aligned with the filter **2** such that one end of the smokeable material rod **3** abuts the end of the filter **2**. The filter **2** is wrapped in a plug wrap (not

shown) and the smokeable material rod **3** is joined to the filter **2** by tipping paper (not shown) in a conventional manner.

Figure 10 shows, for illustration only, a flow chart setting out the steps involved in one embodiment of the invention. The steps shown in Figure 10 should not be viewed as limiting the disclosure of the present application as a whole.

The methods of the invention may comprise any suitable steps, and any suitable number of steps, in order to reduce the polyphenol and/or protein content of the tobacco material. The method of the invention may also further modify the tobacco material in any suitable way, for example by modifying the flavour it generates upon combustion, and/or removing other types of chemical substances.

In some embodiments, the methods described herein may comprise one or more further steps to modify the tobacco material in any suitable way. For example, the tobacco material may be modified to provide it with one or more characteristics desirable for a tobacco material. For example, where the treated tobacco material is to be incorporated into a smoking article such as a cigarette, the tobacco material may be treated in order to modify the flavour it generates upon combustion, and/or may be treated in order to remove one or more of its chemical substances.

Experimental Work

A series of experiments were carried out in order to investigate how the treatment of a tobacco material with subcritical water can affect the protein and polyphenol content of the tobacco material. The disclosed experimental work is not intended to limit the scope of the invention.

Procedure

The experiments were conducted using a commercially available Dionex ASE 350 system. The Dionex uses a static extraction method and is capable of heating water to a temperature of 200°C while still remaining in a liquid state. Other systems can of course be used.

Merely for illustration, Figure 1 shows a schematic of the Dionex ASE 350 pressurised hot water extraction (PHWE) equipment, which is one type of equipment that can be used to perform the method of the present invention. Of course, other equipment may

also be used. Sample material is packed into a 100 ml stainless steel sample cell fitted with a paper filter at the base. The cell is then transferred to a preheated oven by a robotic arm. The cell and ASE equipment is capable of withstanding pressure of up to 3000psi.

Once the sample is loaded into the pre heated oven a pump delivers solvent at ambient temperature to the sample cell. The static release valve seals the cell automatically once solvent has travelled through the system to the collection vessel. The pump continues to pump solvent until the pressure reaches 1500psi. If the pressure reaches 1700psi at any time during operation the static valve opens briefly to relieve the pressure. The pump also delivers fresh solvent to the cell to maintain high pressure. Once the cell is loaded the oven undergoes two heating stages the first is an internally defined time for the cell and its contents to reach thermal equilibrium with the oven, the second is a static time where the cell is maintained at the required temperature. The static time is defined by the operator.

After the static period is complete the static valve is opened and the solvent drained to the collection bottle. Fresh solvent is then pumped through the cell to remove extracted materials. The amount of solvent used here in this purging stage is defined by the operator. Finally the solvent is purged out of the cell with nitrogen (150 psi). The cell is removed from the oven by a mechanical arm and the residual pressure vented to atmosphere. With the cell removed from the circuit the entire system is purged with clean solvent to prevent contamination.

Materials and Methods

15g of tobacco was loaded into a 100ml ASE Cell, the cell was fitted with a paper filter at the base. The water (HPLC grade purchased from Rathburn UK) was degassed for 25 mins using a sonicated bath to remove dissolved oxygen that may cause oxidation at elevated temperature.

In the initial batch of experiments a total of eighteen 15g samples were extracted under the following conditions.

Table 1: Extraction Conditions

Mass of Tobacco (g)	Temperature (°C)	Static Time (min)
15	75	15
15	75	30
15	75	45
15	100	15
15	100	30
15	100	45
15	125	15
15	125	30
15	125	45
15	150	15
15	150	30
15	150	45
15	175	15
15	175	30
15	175	45
15	200	15
15	200	30
15	200	45

In the second batch of extracts 2 x 15g samples of tobacco were extracted at 125°C and 150°C. The samples were heated for a total of 45 min, during this period the static valve was opened at 15 min intervals and the cell drained of water. The water was collected and the cell was then refilled with fresh solvent.

After extraction the cells were allowed to cool briefly and the extracted fibre was dried using a centrifuge at 1000 rpm for 15 mins. The filtrate was combined with the primary water extract. The dried fibre was then dried in an oven at 75°C for 12 hrs then at room temperature for 48 hrs.

The liquid extracts were transferred to weighed vessels, frozen then dried to a constant weight using a freeze drier. This took approximately 3 days. After freeze drying the samples were weighed placed in airtight containers and stored at -4°C.

Fibre Sample Pre-Treatment

Prior to treatment in any of the following analytical techniques the fibre was ground to a fine powder using an in house modified small domestic blender. The material was continually ground until it would pass through a 40 mesh screen.

Identification and Quantification of Individual Phenolics via High Performance Liquid Chromatography (HPLC)

The dried tobacco residue and freeze-dried extracts were analysed using HPLC. This was performed using an Agilent 1100 series HPLC fitted with a Diode array. Standards were supplied by Sigma Aldrich UK. HPLC solvents were supplied by Rathburn Chemicals UK.

Samples were diluted but shaken on an orbital shaker for 10 mins then sonicated using a sonic bath for a period of 20 mins. Following this the samples were centrifuged using a desk centrifuge for a period of 15 mins.

HPLC was used to measure the concentration of four reference polyphenol compounds, namely scopoletin, caffeic acid, chlorogenic acid, rutin in the aqueous filtrate following filtration. The chemical structures of these four reference polyphenol compounds are provided in Figure 9.

Protein Nitrogen Determination

The dried tobacco residues were analysed using a combustion method. The analysis was performed using a LECO TruMac.

The amount of sample used was 1g. Only the fibre could be analysed with this technique as the extracts were highly soluble in water and washes to remove non-protein nitrogen were not possible.

While it is known that tobacco would contain non protein nitrogen from alkaloids a pre-treatment with hot acetic acid washes is assumed to be capable of removing these.

Quantification of Total Phenolic Content – via Assay

Polyphenol content was determined for the freeze dried extracts and the tobacco residues using a Folin Ciocalteu (FC) method.

The FC assay is a colorimetric assay used to provide a measure of total polyphenol content in solution. In an FC assay, the magnitude of absorption at a particular radiation frequency - which polyphenols absorb - is measured for a sample. Following this, the measured magnitude of absorption is compared to the magnitude of absorption at the same radiation frequency for a solution of the polyphenol, Gallic Acid. The measured absorption of light may then be expressed in units of GAE (Gallic Acid Equivalents).

1 mg per ml samples of the freeze-dried extracts and a 10 mg per ml samples of the dried fibre were prepared in a 0.14 M NaCl solution. The samples were shaken on an orbital shaker for 10 mins then sonicated for a period of 60 mins. Following this the samples were centrifuged using a desk centrifuge for a period of 15 mins. Following shaking and prior to centrifugation the fibre samples were placed in an oven at 60°C for 18 hours to attempt to remove any phenolics that may still be bound to the cells of the fibre.

50µl and 30µl samples of the freeze-dried and fibre solutions respectively were added to 1 ml of 7% sodium carbonate in 5 ml test tubes, 100µl of Folin Ciocalteu phenol reagent (Sigma UK) was added. The tubes were then stirred with a vortex mixer. The mixtures were then incubated for 60 Mins at 40°C. The absorbance was measured at 688 nm using a Pharmacia Novaspec 2 Spectrophotometer. Different concentrations of gallic acid were used for the calibration curve.

Quantification of Total Protein content Via Assay

Total protein content was determined for the freeze dried extracts and the tobacco residues using a bichinchoninic acid assay.

1 mg per ml sample of the freeze-dried extracts and a 10 mg per ml sample of the dried fibre were prepared in a 0.14 M NaCl solution. The samples were shaken on an orbital shaker for 10 mins then sonicated for a period of 20 mins. Following this the samples were centrifuged for a period of 15 mins. Following shaking the fibre samples were placed in an oven at 60°C for 18 hours to attempt to remove any protein that may still be bound to the cells of the fibre, additionally 0.1% trifluoroacetic acid was added to the mixture to assist extraction of the protein.

30µl samples of the freeze dried extract and fibre solutions were added to 1 ml of solution containing Bichinchoninic acid solution and Copper (II) pentahydrate 4 % mixed in a 50.1 ratio (Sigma UK). The mixtures were then stirred with a vortex mixer and sealed with parafilm. The mixtures were incubated for 20 mins at 40°C. The absorbance was measured at 562 nm using a Novaspec 2 Spectrophotometer. Different concentrations of Bovine serum albumin (BSA) were used for the calibration curve.

Results & Discussion

Physical properties of extracts:

The masses of the dried tobacco residues are given in Table 2.

The temperatures 150 III and 125 III refer to the extractions where three extracts are produced from the same tobacco.

Table 2: Masses of Tobacco Residues

Dried Fibre Mass g		Static time Mins		
		15	30	45
Temperature °C	75	7.52	7.52	7.61
	100	7.55	7.18	7.23
	125	6.74	6.07	6.14
	150	6.11	4.91	4.65
	175	5.05	4.62	4.91
	200	3.76	4.12	4.14
	125III			5.5
	150III			4.6

A clear trend is seen in the dried fibre mass, as the temperature is increased the amount of material left as a fibre decreases.

As observed in Figures 2 and 3, the physical properties of the tobacco residue change dramatically as the temperature is increased. Up to 100°C the material remains mostly unchanged and un-compacted. Between 125°C and 150°C the material begins to compact slightly although individual strands/fibres can still be seen and broken up by crumbling, the material begins to take on a slightly darker colour. At 175°C and above the material begins to compact heavily, and the material takes on a dark brown black colour.

The effect of static time is not as significant as temperature on the material. Figure 4 shows the material extracted at 125°C for different periods. Some compacting and discolouration are observed, although the effects are far less pronounced.

HPLC Quantification of Select Polyphenol

HPLC was employed to quantify polyphenols in the treated tobacco material. An example trace of unextracted tobacco is shown in Figure 5 for comparison. The peak symmetry is good and the isomer of chlorogenic acid (cryptochlorogenic acid) is sufficiently separated from chlorogenic acid to allow accurate integration.

The HPLC traces for the extracts all show very similar trends. As the temperature increases the amount of polyphenol in the fibre decreased. Plots of phenol content (Figure 6) show the chlorogenic acid and rutin decrease at a very similar rate. The intensity of the caffeic acid peak is so low in virtually all traces that integration of the peak may not be reliable.

Changing the static time does not greatly affect the amount of polyphenol in the samples.

For the experiments at 125°C and 150 °C where the sample was extracted three times no polyphenol is detected in the fibre. This is seen to be more effective than one extraction for 45 min.

The total amount of each polyphenol present in the start sample is shown in Table 3. To allow a direct comparison of this to the extracted fibre the total amount of chlorogenic acid in the fibre is shown in Table 4.

Table 3: Amount of each selected polyphenol in the untreated tobacco

	mg per G	total mass of sample g	total mg of selected polyphenol
Chlorogenic acid	12.97	15	194.6
Caffeic acid	0.14	15	2.1
Scopoletin	0.71	15	10.6
Rutin	11.87	15	178.1

Table 4: Total amount of chlorogenic acid in the treated fibre for the material

Temp °C	Time	mg/g Chlorogenic acid	Total Mass g	Total mg chlorogenic acid
75	15	3.006	7.52	22.607
100	15	3.115	7.55	23.519

125	15	2.244	6.74	15.126
150	15	3.605	6.11	22.029
175	15	1.252	5.05	6.323
200	15	0.910	3.76	3.420
75	30	3.779	7.52	28.418
100	30	2.864	7.18	20.560
125	30	1.823	6.07	11.064
150	30	1.140	4.91	5.598
175	30	1.137	4.62	5.254
200	30	0.642	4.12	2.646
75	45	3.305	7.61	25.152
100	45	2.682	7.23	19.391
125	45	1.734	6.14	10.649
150	45	1.138	5.2	5.920
175	45	0.861	4.91	4.227
200	45	0.617	4.14	2.555
125	Triple	0.000	5.5	0.000
150	Triple	0.000	4.6	0.000

The table shows clearly that the total amount of chlorogenic acid in the fibre drops rapidly even when extracted with water at 75°C for 15 mins. The amount of chlorogenic acid decreases steadily as the temperature is increased. The effect of time is seen to be less influential. When the fibre was extracted with three volumes of fresh water no chlorogenic acid could be detected in the treated tobacco material.

Full data for the other polyphenols is provided for the fibre in Table 5. The trends shown by chlorogenic acid are mirrored by the other polyphenols. Some deviation was seen in results for scopoletin and caffeic acid where the amount present in the fibre tends to fluctuate. This could be attributed to the low concentration being at the limits to which the machine employed is accurate; some peaks were of such low intensity manual integration was required.

Table 5: Total mg of selected phenol in fibre

		Total Amount of Polyphenol in extract mg			
	Mass g	Chlorogenic Acid	Caffeic Acid	Scopoletin	Rutin
Unextracted start material	15	194.556	2.150	10.639	178.071

Temp °C	Time mins	Mass of fibre extract g	Chlorogenic Acid mg	Caffeic Acid mg	Scopoletin mg	Rutin mg
75	15	7.52	22.61	0.61	1.85	27.55
100	15	7.55	23.52	0.37	1.38	30.31
125	15	6.74	15.13	0.00	0.82	18.39
150	15	6.11	22.03	0.23	2.60	22.53
175	15	5.05	6.32	0.13	1.43	5.24
200	15	3.76	3.42	0.00	1.17	1.52
75	30	7.52	28.42	0.41	1.71	36.02
100	30	7.18	20.56	0.00	1.06	24.52
125	30	6.07	11.06	0.20	0.74	14.02
150	30	4.91	5.60	0.14	0.54	6.84
175	30	4.62	5.25	0.10	1.28	2.75
200	30	4.12	2.65	0.00	1.19	0.00
75	45	7.61	25.15	0.59	1.74	32.57
100	45	7.23	19.39	0.42	1.14	22.99
125	45	6.14	10.65	0.22	0.70	13.65
150	45	5.2	5.92	0.15	0.67	6.10
175	45	4.91	4.23	0.00	1.35	1.88
200	45	4.14	2.56	0.00	1.09	0.00
125	Triple	5.5	0.00	0.00	0.00	0.00
150	Triple	4.6	0.00	0.00	0.00	0.00

To show how the level of polyphenols is reduced in a typical treatment step, the polyphenol content of the untreated tobacco, and the treated tobacco material at 75°C for 15 mins are shown in Table 6.

Table 6: Phenolic content of untreated tobacco and tobacco treated at 75°C for 15 mins

	Chlorogenic acid		**Caffeic acid**		**Scopoletin**		**Rutin**	
	mg/g	Total mass present mg	mg/g	Total mass present mg	mg/g	Total mass present mg	mg/g	Total mass present mg
Untreated tobacco	12.97	194.56	0.14	2.15	0.71	10.64	11.87	178.07
Fibre	3.01	22.61	0.08	0.61	0.25	1.85	3.66	27.55

Total Phenolic Content

The total phenolic content of the treated tobacco material as expressed in Gallic acid equivalence (GAE) is shown in Table 8 below. The data shows a significant reduction in total phenolic content compared to the untreated tobacco.

When the fibres extracted three times at 125 and 150°C were analysed very low concentrations of phenolics were detected. These values along with the untreated tobacco are also shown in Table 7. The data shows clearly three extractions remove a far greater amount of phenolics from the tobacco.

Table 7: Total GAE of untreated tobacco and fibre

	GAE equivalent mg/G	Mass of sample g	Total GAE mg in Fibre
Start Material	33.8	15	507.1
Temp time			
75-15	20.7	7.52	155.5
100-15	14.7	7.55	111.0
125-15	12.4	6.74	83.5
150-15	19.7	6.11	120.5
175-15	19.3	5.05	97.6

200-15	20.5	3.76	77.0
75-30	28.4	7.52	213.6
100-30	15.3	7.18	109.7
125-30	8.5	6.07	51.8
150-30	7.9	4.91	39.0
175-30	18.2	4.62	84.0
200-30	18.8	4.12	77.3
75-45	28.8	7.61	219.0
100-45	18.0	7.23	130.0
125-45	8.3	6.14	51.2
150-45	14.7	5.2	76.4
175-45	16.1	4.91	78.8
200-45	26.3	4.14	108.8

125°C Triple extract	3.1	5.5	17.2
150 °C Triple extract	1.8	4.6	8.2

Similar to the HPLC results for the selected polyphenols a large reduction in polyphenol is seen even at 75°C for 15 mins. Increasing the temperature to 125°C causes the amount of polyphenol to drop further.

Protein Determination By Assay

As observed in Figure 7, the amount of protein present in the fibre follows a rapid descent from 75°C to 125°C after this point the amount of protein stabilises and increasing temperature has little effect. This could be due to the maximum amount of protein being removed at 125°C.

By comparison the amount of protein extracted with a static time of 45 mins is shown in Table 8. For comparison the untreated tobacco is included. The concentration (mg/g) of protein in some of the extracts is higher than that of the untreated material, although when the mass of the extract is take into account it is seen that the total amount of protein is less. Without being bound by theory, we attribute these results to low temperature water selectively removing non-protein materials.

Table 8: Amount of protein present in fibre – static time 45 mins

	mg/g protein	Mass g	mg protein present in sample
Untreated	90.0	15.0	1350.0
75	130.2	7.6	990.4
100	99.3	7.2	717.8
125	76.9	6.1	471.9
150	79.9	5.2	415.2
175	64.9	4.9	318.7
200	83.3	4.1	345.0

In order to address various issues and advance the art, the entirety of this disclosure shows by way of illustration various embodiments in which the claimed invention(s) may be practiced and provide for superior tobacco treatment, tobacco material, and products incorporating tobacco material. The advantages and features of the disclosure are of a representative sample of embodiments only, and are not exhaustive and/or exclusive. They are presented only to assist in understanding and teach the claimed features. It is to be understood that advantages, embodiments, examples, functions, features, structures, and/or other aspects of the disclosure are not to be considered limitations on the disclosure as defined by the claims or limitations on equivalents to the claims, and that other embodiments may be utilised and modifications may be made without departing from the scope and/or spirit of the disclosure. Various embodiments may suitably comprise, consist of, or consist essentially of, various combinations of the disclosed elements, components, features, parts, steps, means, etc. In addition, the disclosure includes other inventions not presently claimed, but which may be claimed in future.

Claims

1. A method for treating a tobacco material, wherein the method comprises treating the tobacco material with subcritical water.

2. A method according to claim 1, wherein the method removes one or more polyphenols from the tobacco material.

3. A method according to any one of the preceding claims, wherein the method removes one or more proteins from the tobacco material.

4. A method according to any one of the preceding claims, wherein the method is carried out at a temperature from about 125°C to about 175°C.

5. A method according to any one of the preceding claims, wherein the method is carried out at a pressure from about 1500psi to about 1700psi (from about 100 bar to about 120 bar).

6. A method according to any one of the preceding claims, wherein the method comprises submerging the tobacco material in subcritical water.

7. A method according to claim 6, wherein treating the tobacco material with subcritical water is a static treatment.

8. A method according to claim 7, wherein the method comprises submerging the tobacco material in subcritical water and subsequently separating the tobacco material from the subcritical water.

9. A method according to claim 8, further comprising a second treatment step comprising submerging the tobacco material with subcritical water and a second subsequent separation step comprising separating the tobacco material from the subcritical water.

10. A method according to claim 9, further comprising a third treatment step comprising submerging the tobacco material with subcritical water and a third

subsequent separation step comprising separating the tobacco material from the subcritical water.

11. A method according to any one of claims 8 to 10, wherein in each treatment step the tobacco material is separated from the subcritical water by filtration.

12. A method according to any one of the preceding claims, wherein the method further comprises the subsequent step of drying the treated tobacco material.

13. A tobacco material which has been treated by a method according to any one of the preceding claims, or a derivative thereof.

14. A smoking article which comprises a tobacco material according to claim 13.

15. Use of subcritical water for removing one or more polyphenols and/or one or more proteins from a tobacco material.

Figure 1

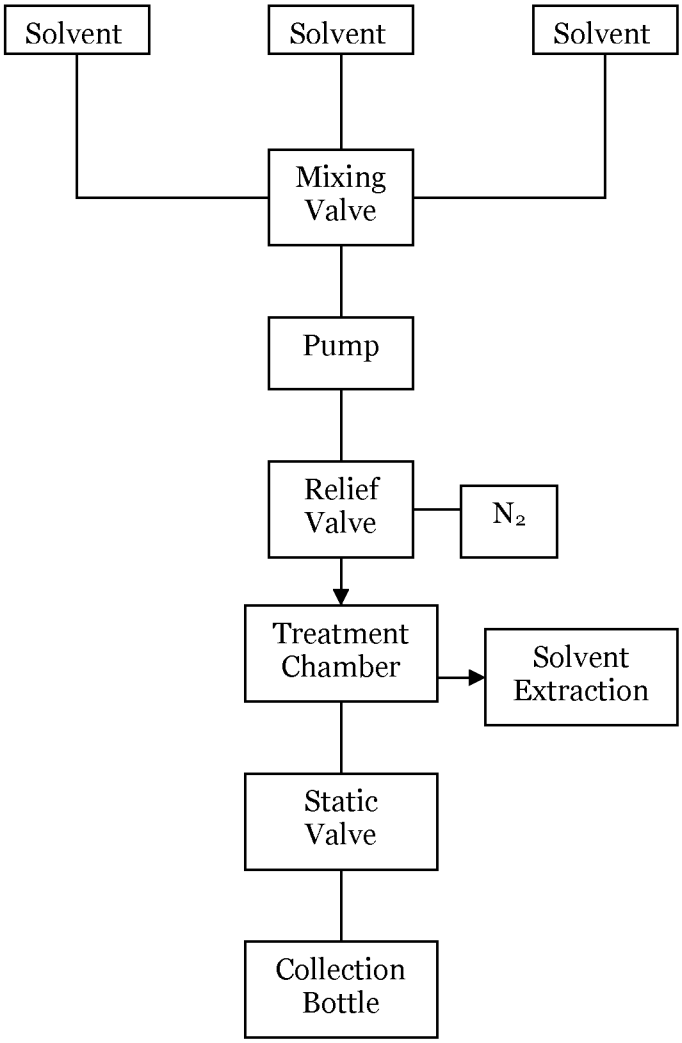

Solvent
Solvent
Solvent
Mixing Valve
Pump
Relief Valve
N_2
Treatment Chamber
Solvent Extraction
Static Valve
Collection Bottle

Figure 2: Oven dried tobacco residues, extracted with a static time of 30 mins

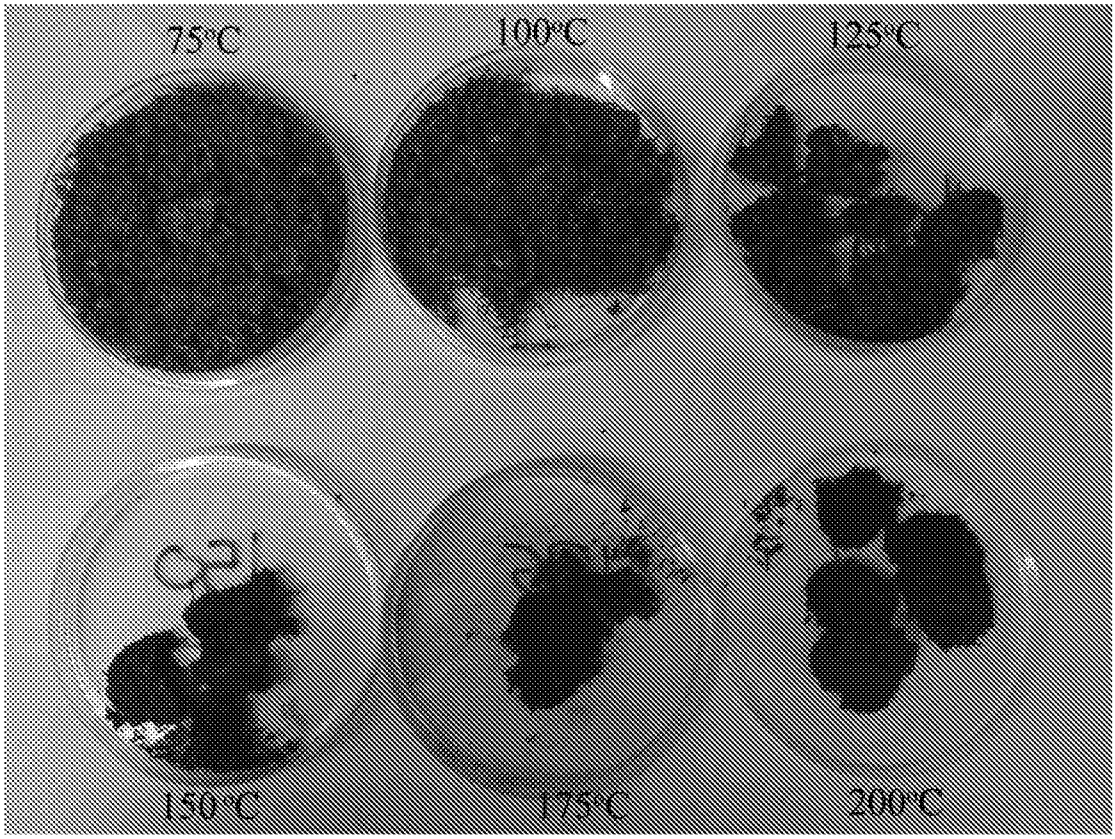


Figure 3: Oven dried fibre extracted with a static time of 30 minutes

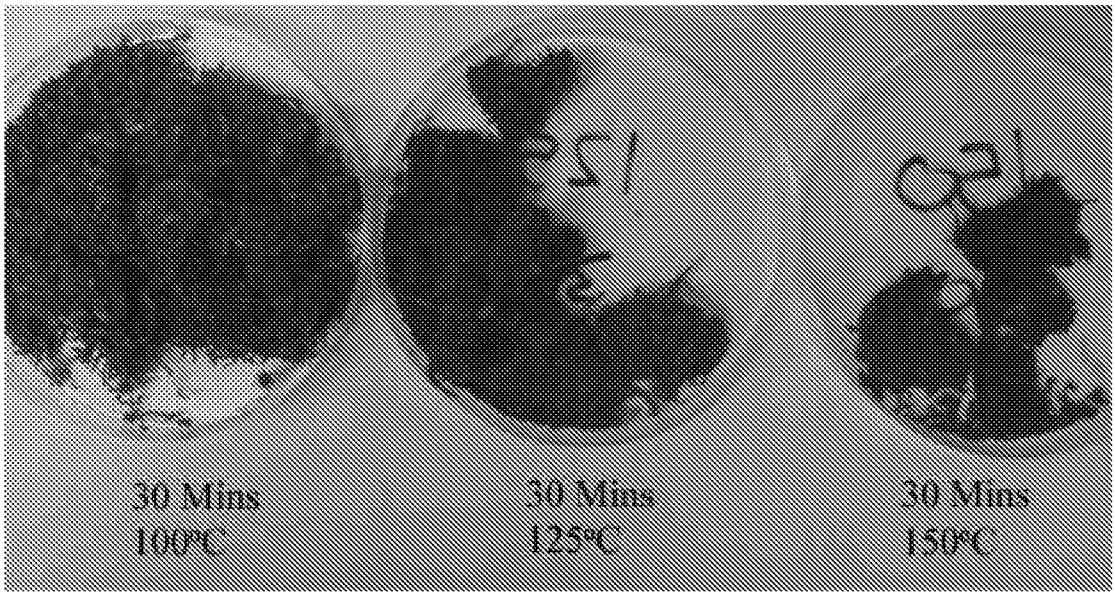


Figure 4: Oven dried fibre extracted at 125°C at different static times

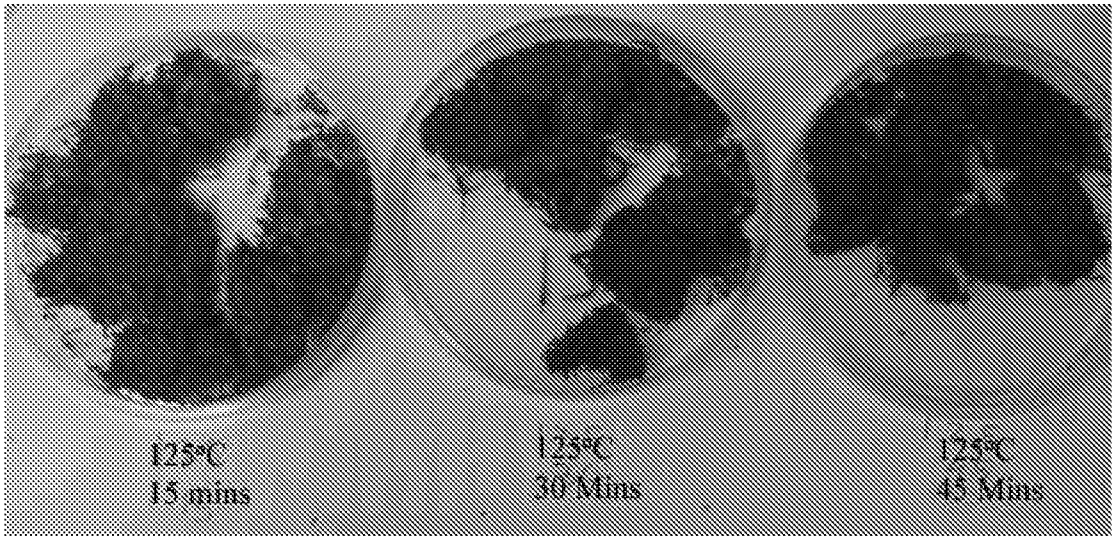


Figure 5: HPLC Trace of Unextracted Tobacco

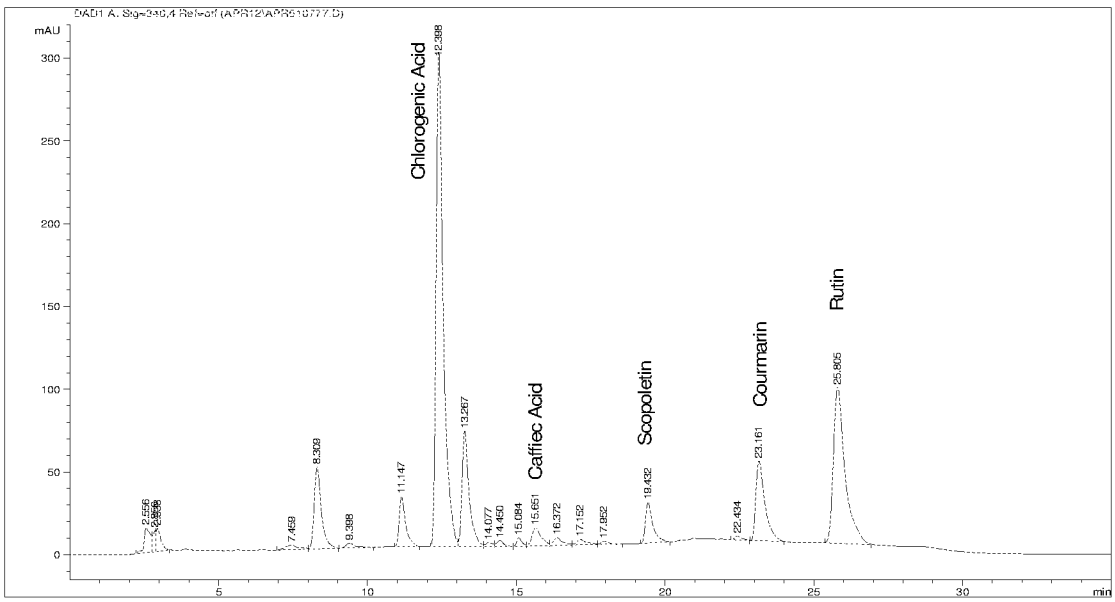


Figure 6:

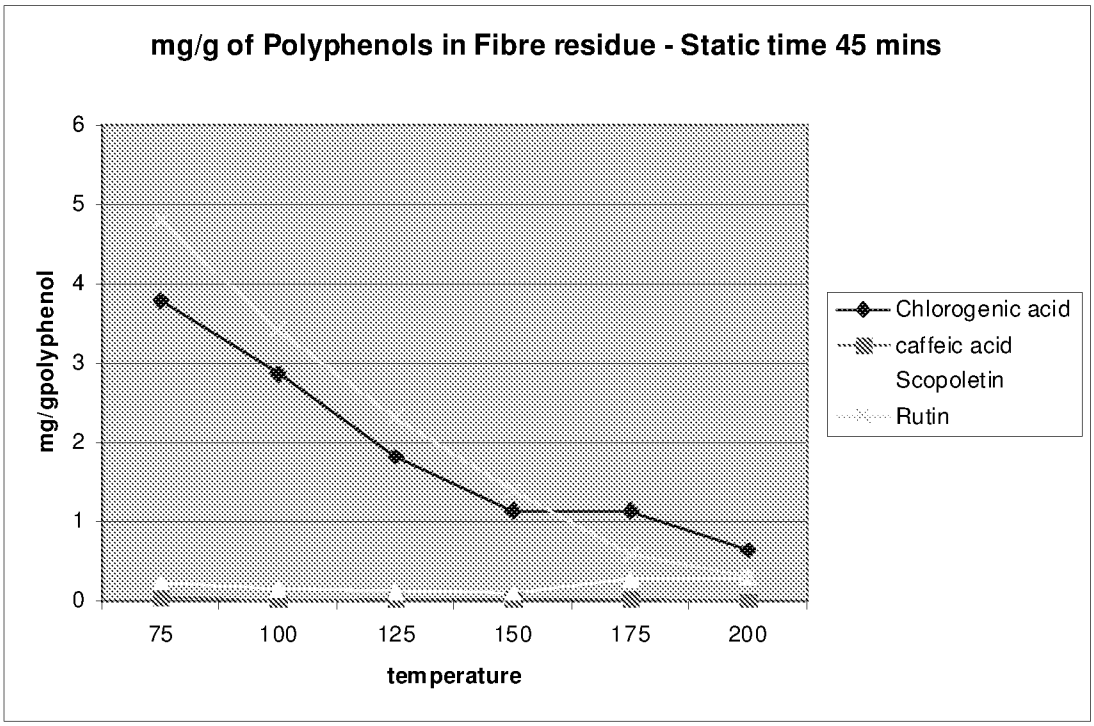


Figure 7:

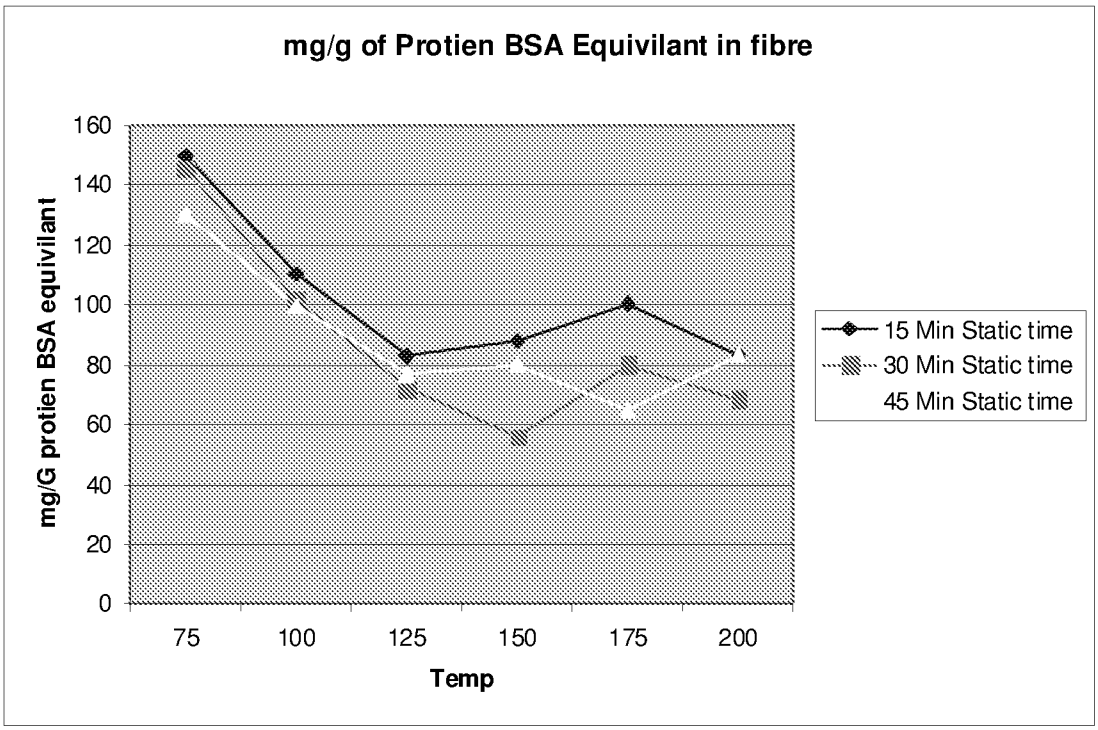


Figure 8:

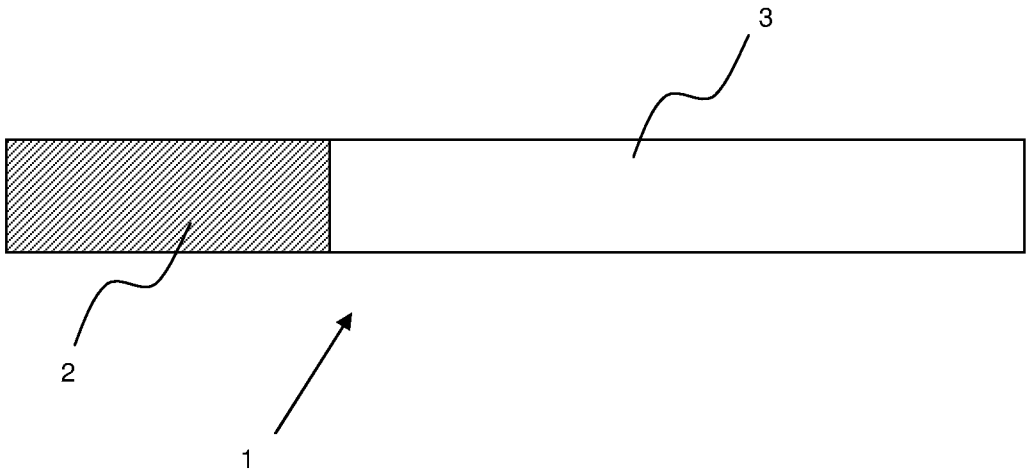


Figure 9:

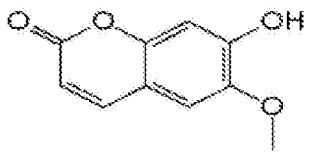
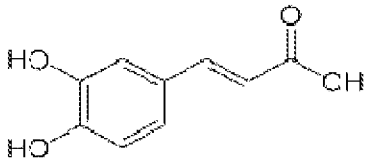
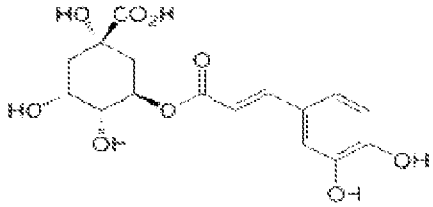
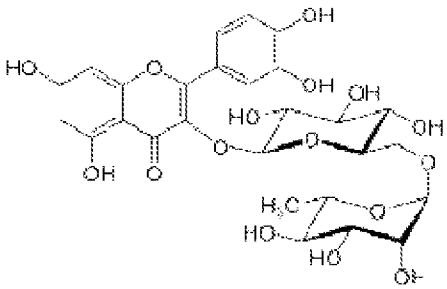
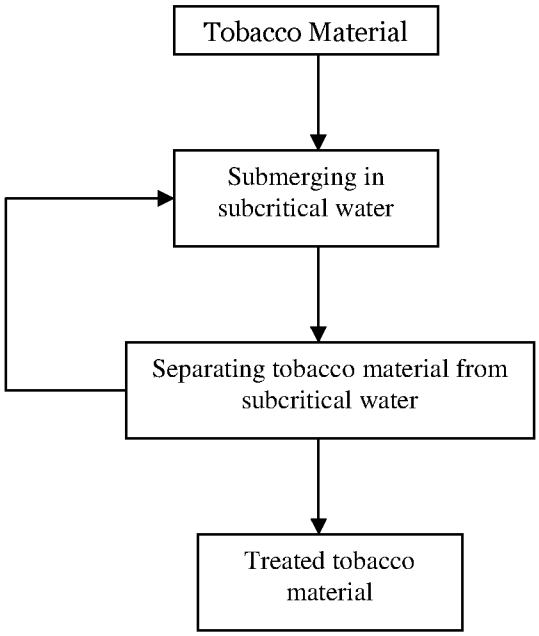
Polyphenol	Chemical Structure
Scopoletin	
Caffeic acid	
Chlorogenic acid	
Rutin	

Figure 10


Tobacco Material
Submerging in subcritical water
Separating tobacco material from subcritical water
Treated tobacco material

5

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2013/053108

A. CLASSIFICATION OF SUBJECT MATTER
INV. A24B15/24
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A24B

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 99/29190 A1 (BROWN & WILLIAMSON TOBACCO [US]) 17 June 1999 (1999-06-17) page 2, paragraph 3 - page 3, paragraph 1 page 4, paragraph 2 - page 5, paragraph 3	1,2
X	US 2007/014912 A1 (MAZZA GIUSEPPE [CA] ET AL) 18 January 2007 (2007-01-18)	1-3, 13-15
Y	paragraph [0001] paragraph [0005] - paragraph [0006] paragraph [0046] paragraph [0051] examples 1, 4, 5	1-12,15
X	WO 2012/110819 A1 (BRITISH AMERICAN TOBACCO CO [GB]; DITTRICH DAVID [GB]; BRANTON PETER []) 23 August 2012 (2012-08-23)	12,13
Y	page 10, line 7 - line 16	1-12,15

☐ Further documents are listed in the continuation of Box C.

☒ See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
14 February 2014	21/02/2014

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Dimoula, Kerasina

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/GB2013/053108

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9929190 A1	17-06-1999	BR 9813459 A	10-10-2000
		CA 2313130 A1	17-06-1999
		CN 1281341 A	24-01-2001
		EP 1039811 A1	04-10-2000
		JP 2001525191 A	11-12-2001
		US 5947128 A	07-09-1999
		WO 9929190 A1	17-06-1999
US 2007014912 A1	18-01-2007	CA 2546138 A1	13-11-2006
		US 2007014912 A1	18-01-2007
WO 2012110819 A1	23-08-2012	AR 085295 A1	18-09-2013
		AU 2012219223 A1	01-08-2013
		CA 2824731 A1	23-08-2012
		CN 103347408 A	09-10-2013
		EP 2675302 A1	25-12-2013
		TW 201247115 A	01-12-2012
		US 2014020699 A1	23-01-2014
		WO 2012110819 A1	23-08-2012