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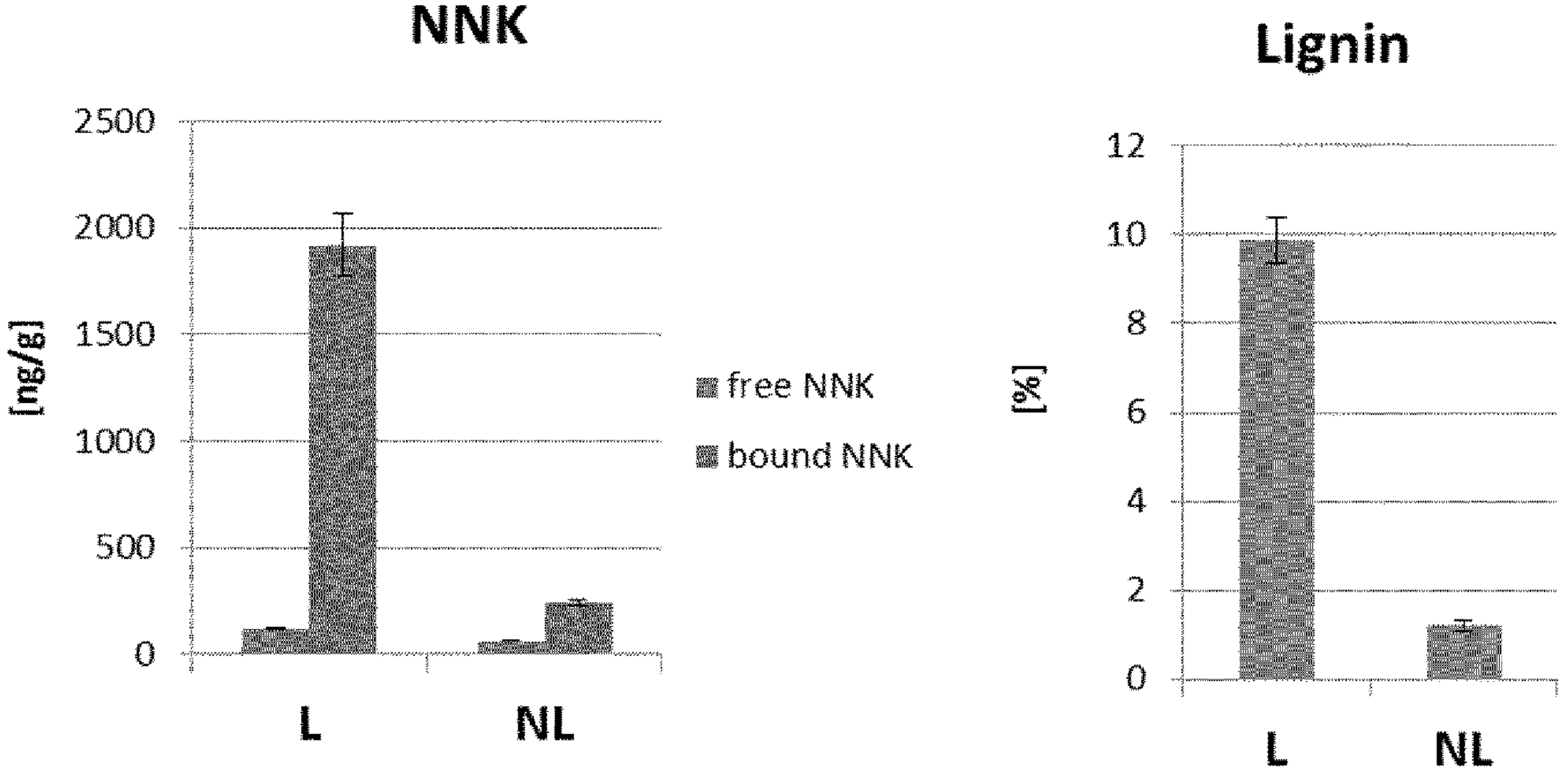
(54) **Titre : PROCEDES DE REDUCTION DE NITROSAMINE-CETONE DERIVEE DE NICOTINE (NNK) LIEE A UNE MATRICE DANS UN MATERIAU DE PLANTE DE TABAC**

(54) **Title: METHODS FOR REDUCING MATRIX-BOUND NICOTINE-DERIVED NITROSAMINE KETONE IN TOBACCO PLANT MATERIAL**

FIGURE 1



(57) **Abrégé/Abstract:**
A method of reducing the amount of matrix-bound NNK in cured tobacco plant material is provided comprising reducing the amount of lignin in the cured tobacco plant material. A further method of reducing the formation of matrix-bound NNK during the curing of tobacco plant material is described comprising reducing the amount of lignin therein prior to curing.

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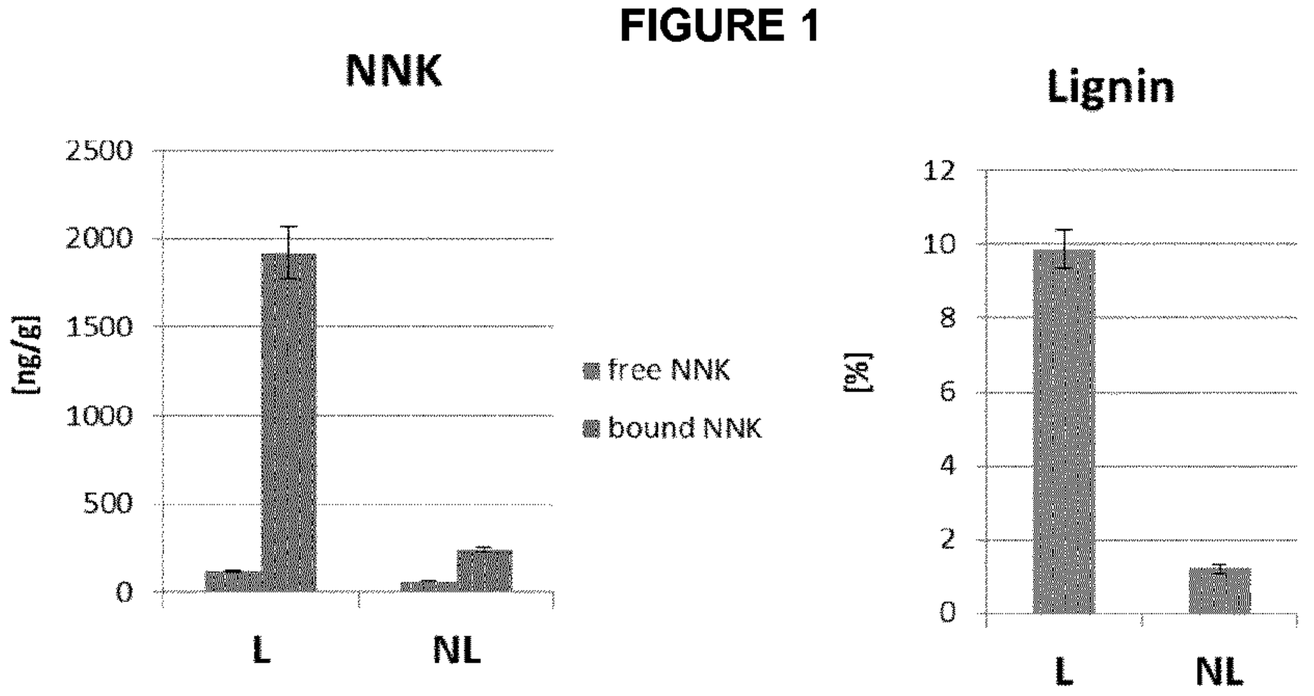
(54) Title: METHODS FOR REDUCING MATRIX-BOUND NICOTINE-DERIVED NITROSAMINE KETONE IN TOBACCO PLANT MATERIAL

FIGURE 1



(57) Abstract: A method of reducing the amount of matrix-bound NNK in cured tobacco plant material is provided comprising reducing the amount of lignin in the cured tobacco plant material. A further method of reducing the formation of matrix-bound NNK during the curing of tobacco plant material is described comprising reducing the amount of lignin therein prior to curing.

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METHODS FOR REDUCING MATRIX-BOUND NICOTINE-DERIVED NITROSAMINE KETONE IN TOBACCO PLANT MATERIAL

FIELD OF THE INVENTION

The present invention relates, in general, to methods for reducing the amount of nicotine-derived nitrosamine ketone or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) in tobacco plant material.

BACKGROUND OF THE INVENTION

During the manufacture and processing of tobacco products, by-products - such as tobacco stems, and leaf scraps - are produced. Tobacco stems and tobacco fines from manufacturing processes are unsuitable for use directly in the manufacturing of tobacco products. Since the stems and fines represent a substantial amount of raw material investment, processes have been developed to further convert these stems and fines into products - such as reconstituted tobacco materials (eg. reconstituted tobacco sheets) - which are then useable in relatively large amounts in a mixture with acceptable processed tobacco leaf. Reconstituted tobacco can be manufactured in a slurry or cast sheet process wherein pulp of mashed tobacco stems and other parts of the tobacco leaf are ground and mixed with a solution that might contain different additives. The resulting tobacco slurry is then sprayed to form a thin film, dried, rolled and diced into strips which are added to a filler.

Nitrosamines are organic compounds found in many consumer products - such as tobacco, food products and cosmetics. Nitrosamines have drawn intense scientific interest because some of the compounds in this class have been shown to be carcinogenic in laboratory animals. It has been reported that some cured tobaccos contain tobacco specific nitrosamines that can be found in smokeless tobacco, mainstream smoke and side stream smoke of cigarettes. In tobacco, at least four species of nitrosamines are produced at appreciable quantity. These are nicotine-derived nitrosamine ketone or 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), N-nitrosonornicotine (NNN), N-nitrosoanatabine (NAT), and N-nitrosoanabasine (NAB). Tobacco specific nitrosamines are not considered to be present in significant quantities in growing tobacco plants or fresh cut tobacco (green tobacco), but are formed during the tobacco curing process. In addition to the formation of tobacco specific nitrosamines during the curing process of green leaves, tobacco specific nitrosamines may also be formed during processes used to prepare aqueous tobacco slurries - such as processes used to prepare reconstituted tobacco.

In an attempt to reduce tobacco specific nitrosamines, various treatments of tobacco plants or harvested tobacco leaves have been suggested, including radiation treatments, chemical treatments and extractions. Other methods for reducing tobacco specific nitrosamines have been suggested by MacKown *et al.* (1988) *J. Agric. Food Chem.* 36, 1031-1035. These methods involve treatment using sterilization, microbial inhibitors, bases to increase pH, or ascorbic acid to

decrease the accumulation of tobacco specific nitrosamines during the production of reconstituted tobacco sheets. WO2012160133 describes a process for decreasing the levels of tobacco specific nitrosamines in tobacco homogenates by increasing the pH thereof, especially when elevated levels of nitrosamines are created by elevated nitrite levels.

One problem with trying to reduce the levels of tobacco specific nitrosamines in tobacco is that some of the nitrosamines in air-cured tobacco, including NNK, exist in a bound or matrix-bound form, which can be difficult to remove or extract. Matrix-bound NNK can be extracted with a 0.1N KOH solution from water-washed Burley filler. This alkaline treatment also decreases NNK levels in smoke (Keene, C.K., 1992, The Effect of Base Digestion on TSNA in Extractables-Depleted Fillers. Legacy Tobacco Documents). However, the treatment can introduce toxicologically relevant compounds into tobacco and can significantly deteriorate the quality of the tobacco, which is highly problematic for the tobacco industry.

WO2010/021809 describes a method for recuing nitrogen compounds and lignin in tobacco. Nitrogen compounds are removed by solvent extraction; lignin is removed in a separate step.

A need remains for an effective and cost efficient method for reducing matrix-bound NNK that is formed during the curing of tobacco. In particular, a cost-effective and simple method for reducing the levels of matrix-bound NNK in cured tobacco that does not introduce toxic or potentially toxic compounds and does not deteriorate the quality of the tobacco product is particularly desirable.

SUMMARY OF THE INVENTION

The present invention is based, at least in part, on the surprising finding that matrix-bound NNK co-localises with lignin in tobacco plants. In particular, the present inventors have observed that within cured parts of a tobacco plant – such as in the stems and midribs - matrix-bound NNK co-localises or co-localises predominantly or co-localises exclusively with lignin – such as lignified tissue, particularly in the vascular bundle (for example, the xylem) and not the surrounding tissues – such as the cortex. Therefore removal of lignin (for example, by separating lignified from non-lignified tissue) can reduce the amount of matrix-bound NNK and metabolites thereof in plant material. Matrix-bound NNK can be covalently or non-covalently linked to lignin. The plant material is expected to deliver smoke with reduced NNK levels and potentially improved sensory properties. The present disclosure can be applied to those plant materials which accumulate matrix-bound NNK or have the potential to accumulate matrix-bound NNK. In particular, the disclosure can be applied to low-value plant material comprising matrix-bound NNK that is used in certain tobacco processes. The methods described herein can be carried out without the use of any additives and thereby do not introduce additional toxicologically relevant compounds into the plant material. The removal of lignin can take place during or after curing of tobacco plant material. Lignin can be removed before curing to prevent, reduce or inhibit matrix-bound NNK co-localising with lignin.

One general object of this disclosure is to substantially decrease the amount of matrix-bound NNK and metabolites thereof in tobacco intended for smoking or consumption by other means. Another general object is to reduce the carcinogenic potential of tobacco products, including cigarettes, cigars, chewing tobacco, snuff and tobacco-containing gum and lozenges. Still another general object is to decrease or reduce the amount of matrix-bound NNK and metabolites thereof in tobacco plant material and in tobacco products. Another general object is to reduce the amount of matrix-bound NNK and metabolites thereof in cured – such as partially or fully cured - tobacco plant material. Another general object is to reduce the amount of NNK and metabolites thereof in aerosol, including smoke. Yet another object of this disclosure is to reduce the amount of NNK or metabolites thereof in humans who smoke, consume or otherwise ingest tobacco in some form, by providing a tobacco product suitable for human consumption which contains a reduced amount of NNK or metabolites thereof, thereby lowering the carcinogenic potential of such product.

In one aspect, there is provided a method of reducing the amount of matrix-bound NNK in a cured tobacco plant or in cured tobacco plant material comprising reducing the amount of lignin therein by separating lignified from non-lignified tissue, preferably, wherein the amount of lignin is reduced chemically and/or mechanically.

In one embodiment, the tobacco plant or the tobacco plant material is treated to expand non-lignified plant tissue. The amount of lignified tissue is reduced by separating the expanded and non-expanded plant tissue based on their different densities (for example, buoyant densities) and/or their different strengths and/or their different sizes and/or their different weight. The expanded plant tissue can be collected for further tobacco processing.

It has been observed that significant reduction in levels of NNK and associated TSNAs can be obtained by fractionating the lignified tissues. Separate chemical extraction of nitrogenous compounds and/or lignin is not necessary.

In one embodiment, the amount of lignin is reduced by removing the vascular bundle or xylem or lignified sclerenchymatic tissue or a combination of two or more thereof from the plant or plant material. The lignin can be located in the vascular bundle. The lignin can be located exclusively in the vascular bundle. The lignin can be located exclusively in the vascular bundle and not the surrounding tissue. The lignin can be located in the xylem. The lignin can be located exclusively in the xylem and not the surrounding tissue. The lignin can be located in sclerenchymatic tissue. The lignin can be located exclusively in sclerenchymatic tissue. The lignin can be located exclusively in the sclerenchymatic tissue and not the surrounding tissue. Lignified tissue is generally absent from the outer layer of plant midribs.

In one embodiment, the plant or plant material that is treated according to the present disclosure comprises or consists or consists essentially of plant midribs or plant stems or plant stalks or a combination of two or more thereof.

In one embodiment, the amount of lignin is reduced by harvesting the cortex – such as the outer cortex - from the plant or plant material.

In one embodiment, the method comprises the steps of: (a) providing a cured tobacco plant or cured tobacco plant material; (b) reducing the amount of lignin in the cured tobacco plant or the cured tobacco plant material by fractionating the tobacco plant material; and (c) obtaining a cured tobacco plant or cured tobacco plant material in which the amount of lignin is reduced and the amount of matrix-bound NNK is reduced as compared to the cured tobacco plant or the cured tobacco plant material provided in step (a).

In one embodiment, following step (a) there is a further step of measuring the amount of free NNK or matrix-bound NNK or a combination thereof, and optionally, wherein following step (b) there is a further step of measuring the amount of free NNK or matrix-bound NNK or a combination thereof.

In one embodiment, the method comprises the further step (d) of comparing the level of at least matrix-bound NNK measured following step (a) with the level of NNK measured following step (b), wherein a reduction in the amount of matrix-bound NNK in the tobacco material obtained in step (b) as compared to the tobacco material provided in step (a) is indicative that the amount of matrix-bound NNK in the tobacco material is reduced.

In a further aspect, there is provided a method of reducing the formation of matrix-bound NNK during the curing of a tobacco plant or tobacco plant material comprising reducing the amount of lignin therein prior to curing.

In one embodiment, the method comprises the steps of: (a) providing an uncured tobacco plant or uncured tobacco plant material; (b) reducing the amount of lignin in the uncured tobacco plant or the uncured tobacco plant material prior to curing; (c) curing the tobacco plant or the tobacco plant material provided in step (b); and (d) obtaining a cured tobacco plant or cured tobacco plant material in which the amount of matrix-bound NNK is reduced as compared to a control in which the amount of lignin has not been reduced.

In one embodiment, following step (a) there is a further step of measuring the amount of free NNK or matrix-bound NNK or a combination thereof, and optionally, wherein following step (b) there is a further step of measuring the amount of free NNK or matrix-bound NNK or a combination thereof and optionally, wherein following step (c) there is a further step of measuring the amount of free NNK or matrix-bound NNK or a combination thereof.

In one embodiment, following step (c) or step (d) said method comprises the further step of comparing the level of at least matrix-bound NNK measured following step (a) with the level of NNK measured following step (b) and/or step (c), wherein a reduction in the amount of matrix-bound NNK in the tobacco material obtained in step (b) or step (c) as compared to the tobacco

material provided in step (a) is indicative that the amount of matrix-bound NNK in the tobacco material is reduced.

In a further aspect, there is provided tobacco plant material obtained or obtainable by the method(s) described herein.

In a further aspect, there is provided the use of a tobacco plant or tobacco plant material in which the amount of lignin therein has been reduced as compared to control tobacco plant or control tobacco plant material for manufacturing tobacco with reduced levels of matrix-bound NNK, wherein said levels of matrix-bound NNK are reduced as compared to the control.

In a further aspect, there is provided a method for producing reconstituted tobacco comprising the steps of: (a) performing the method(s) described herein; (b) manufacturing the tobacco material obtained in step (a) into reconstituted tobacco; and (c) optionally incorporating the reconstituted tobacco into a tobacco product.

In a further aspect, there is provided reconstituted tobacco obtained or obtainable by the method described herein.

In a further aspect, there is provided a method for preparing tobacco for use as a tobacco cut filler comprising the steps of: (a) performing the method(s) described herein; and (b) rolling and cutting the tobacco material for use as a tobacco cut filler.

In a further aspect, there is provided cured tobacco plant material containing a reduced level of lignin as compared to control tobacco plant material in which the amount of lignin has not been reduced, and wherein the amount of matrix-bound NNK is about 3500 ng/g or less.

In one embodiment, the average particle size is greater than about 0.5 millimetres.

In one embodiment, the amount of free NNK is less than about 330 ng/g, optionally wherein the NNN content is less than about 1700 ng/g and optionally wherein the nicotine content is less than about 2610 µg/g.

In one embodiment, the cured tobacco plant material comprises, consists of consists essentially of plant cortex – such as outer plant cortex.

In one embodiment, vascular bundle or xylem or lignified sclerenchymatic tissue or a combination of two or more thereof is substantially absent from the cured tobacco plant material.

In one embodiment, the cured tobacco plant material comprises, consists of consists essentially of plant cortex – such as outer plant cortex – and vascular bundle or xylem or lignified sclerenchymatic tissue or a combination of two or more thereof is substantially absent therefrom.

In one embodiment, the cured tobacco plant material is obtained or obtainable from plant midribs or plant stems or plant stalks or a combination of two or more thereof.

In one embodiment, the average particle size is greater than about 0.5 millimetres.

In one embodiment, the amount of free NNK is less than about 330 ng/g.

In one embodiment, the NNN content is less than about 1700 ng/g.

In one embodiment, the nicotine content is less than about 2610 µg/g.

In a further aspect, there is provided a tobacco product or a reconstituted tobacco product comprising, consisting or consisting essentially of the plant material or the cured plant material described herein.

In a further aspect, there is provided a method of producing an aerosol in which the amount of NNK is reduced as compared to a control aerosol comprising the steps of: (a) providing a cured tobacco plant or cured tobacco plant material; (b) reducing the amount of lignin in the cured tobacco plant or the cured tobacco plant material; (c) obtaining a cured tobacco plant or cured tobacco plant material in which the amount of lignin is reduced and the amount of matrix-bound NNK is reduced as compared to the cured tobacco plant or the cured tobacco plant material provided in step (a); and (d) heating the cured tobacco plant or cured tobacco plant material from step (c) to produce an aerosol.

In a further aspect, there is provided a method of producing an aerosol in which the amount of NNK is reduced as compared to a control aerosol comprising the steps of: (a) providing an uncured tobacco plant or uncured tobacco plant material; (b) reducing the amount of lignin in the uncured tobacco plant or the uncured tobacco plant material prior to curing; (c) curing the tobacco plant or the tobacco plant material provided in step (b); (d) obtaining a cured tobacco plant or cured tobacco plant material in which the amount of matrix-bound NNK is reduced as compared to a control in which the amount of lignin has not been reduced; and (e) heating the cured tobacco plant or cured tobacco plant material from step (d) to produce an aerosol.

In a further aspect, there is provided a method of producing an aerosol in which the amount of NNK is reduced as compared to a control aerosol comprising the step of: (a) providing a tobacco product or a reconstituted tobacco product comprising, consisting or consisting essentially of the tobacco plant material or the cured plant material obtained or obtainable by the methods described herein; and (b) heating the tobacco product or the reconstituted tobacco product to produce an aerosol.

In a further aspect, there is provided an aerosol obtained or obtainable by the method(s) described herein.

In a further aspect, there is provided cured tobacco plant material consisting essentially of tobacco plant cortex and wherein the amount of matrix-bound NNK is reduced as described herein.

In a further aspect, there is provided a method for blending tobacco in which at least two different types of tobacco are blended so as to form a tobacco blend comprising the steps of: (a) providing a first cured tobacco plant material and reducing the amount of lignin therein; (b) measuring the total and/or matrix-bound NNK content of the first cured tobacco plant material and selecting cured tobacco plant material in which the total and/or matrix-bound NNK content is reduced as compared to the first cured tobacco plant material provided in step (a); (c) providing a second cured tobacco plant material which has a higher total and/or matrix-bound NNK content than the total and/or matrix-bound NNK of the first cured tobacco plant material obtained in step (b), and optionally

measuring the total and/or matrix-bound NNK content in the second cured tobacco plant material; (d) blending together the first and second cured tobacco plant materials from steps (b) and (c) and optionally measuring the total and/or matrix-bound NNK content in the blended tobacco plant material; and (e) obtaining a blended tobacco plant material in which the total and/or matrix-bound NNK content of the blended tobacco plant material is lower than the second cured tobacco plant material provided in step (c), optionally wherein steps (a) and (b) are performed after step (c).

In a further aspect, there is provided blended tobacco plant material obtained or obtainable by the method(s) described herein.

Each of the embodiments discussed above are disclosed as embodiments of each of the aspects of the invention. Combinations of one or of the embodiments are contemplated.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph illustrating the distribution of free NNK, matrix-bound NNK and lignin in lignified (L) and non-lignified (NL) tissues in cured Burley midribs or cured Burley stems.

Figure 2 is a cross-section of a hydrated cured Burley stem showing lignified (L) and non-lignified (NL) tissues. Lignified tissue is stained red with phloroglucinol.

Figure 3 is a graph illustrating the distribution of free NNK, matrix-bound NNK and lignin in lignified (L) and non-lignified (NL) tissues of green Burley midribs after nitrosation with sodium nitrite solution (1.5 mL (10 mg/mL in water) for 4 hours at room temperature with shaking).

Figure 4 is a graph illustrating free and matrix-bound NNK in sieving fractions of ground freeze-dried Burley tobacco plant stems.

Figure 5 is graph showing the correlation between matrix-bound NNK and lignin in sieving fractions of ground freeze-dried Burley tobacco plant stems.

Figure 6 is a graph showing the concentration of matrix-bound NNK (µg/g) in sclerenchymatic tissue (S) and in the outer layers of the midribs (NS) after nitrosation and washing in green midribs of TN90. Levels of pseudo-oxynictoine (PON) (µg/g) and nicotine (µg/g) are also shown. Matrix-bound NNK levels (ng/g) in lignified (CS) and non-lignified (CNS) parts of a commercial cured Burley stem sample are also shown. Levels of NNN (ng/g) and nicotine (µg/g) are also shown.

DEFINITIONS

The technical terms and expressions used herein are generally to be given the meaning commonly applied to them in the pertinent art of plant and molecular biology. All of the following term definitions apply to the complete content of this disclosure.

The word "comprising" does not exclude other elements or steps, and the indefinite article "a" or "an" does not exclude a plurality.

The terms "essentially", "about", "approximately" and the like in connection with an attribute or a value particularly also define exactly the attribute or exactly the value, respectively.

The term "plant" refers to any plant or any part thereof at any stage of its life cycle or development, and its progenies. In one embodiment, the plant is a tobacco plant, which refers to a plant belonging to the genus Nicotiana. Preferred species of tobacco plant are described herein.
A "plant cell" refers to a structural and physiological unit of a plant. The plant cell may be in the form of a protoplast without a cell wall, an isolated single cell or a cultured cell, or as a part of higher organized unit such as but not limited to, plant tissue, a plant organ, or a whole plant. In one embodiment, the plant cell is a tobacco plant cell.
The term "plant material" refers to any part of a plant or a mixture of different parts of plant or a mixture of different plants and includes without limitation plant tissues, leaf scraps, green leaf scraps, stems, dust created during plant processing, and leaf prime lamina strip and combinations thereof. In certain embodiments, the plant material will comprise, consist or consist essentially of a plant part or a mixture of plant parts containing lignin – such as plant midribs or plant stems or plant stalks or a combination or two or more thereof. Tobacco plant material can have the form of processed tobacco parts or pieces, uncured, cured or aged tobacco in essentially natural lamina or stem form, a tobacco extract or a mixture of the foregoing, for example, a mixture that combines extracted tobacco pulp with granulated cured and aged natural tobacco lamina. The plant material can be in solid form, in liquid form, in semi-solid form, in ground form, in crushed form, in sieved form, or in particulate form or the like or otherwise treated to reduce particle size. The plant material can be in the form of a homogenate that has been subjected to homogenization, including, but not limited to cutting or grinding or a combination thereof. The homogenate may be prepared from whole plants or from mixtures of plant components - such as a mixture of plant parts containing lignin, for example, midribs or stems or stalks or a combination of two or more thereof - that have been subjected to homogenisation. The plant material can be in the form of a slurry, including a suspension of plant material or a plant homogenate in an aqueous solution or solvent. The slurry can be a 5% (w/v), 10% (w/v), 15% (w/v), 20% (w/v) or 25% (w/v) or more mixture of plant material in an aqueous solution or solvent. In one embodiment, the plant material is that plant material which comprises, consists or consists essentially of lignin – such as lignified tissue. In one embodiment, the plant material is that plant material which comprises, consists or consists essentially of the vascular bundle. In one embodiment, the plant material is that plant material which comprises, consists or consists essentially of xylem. In one embodiment, the plant material is that plant material which comprises, consists or consists essentially of lignified sclerenchymatic tissue. In one embodiment, the plant material comprises, consists or consists essentially of plant midribs or plant stems or plant stalks or a combination of two or more thereof. In one embodiment, the plant material is tobacco plant material.
The term "tobacco product" includes smoking or smokable articles, and smokeless tobacco products.

The term “free NNK” refers to the NNK concentration calculated from the NNK content of extracts prepared by extracting said plant material with aqueous buffer(s) at room temperature. The free NNK content in such extracts can be determined using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).

The term “total NNK” refers to the NNK concentration calculated after subjecting the extraction mixtures to the methods described herein (for example, by heating to about 130°C for about 4 hours) and filtering aliquots of the extracts. The total NNK content in such extracts can be determined using UPLC-MS/MS.

The term “bound NNK” or “matrix-bound NNK” as used herein represents the difference between the “total NNK” and the “free NNK” concentration.

The terms "reduced lignin content" or "decreased lignin content" or “non-lignified” grammatical variations thereof refers to a measurable quantitative reduction in the amount of lignin in a plant when compared to the amount of lignin in a comparable control plant. A quantitative reduction of lignin can be readily ascertained by assays that are known in the art and include the Klason lignin assay (*Method in Enzymol.*, 161:87-101 (1988)), the acetyl bromide assay (*Wood Sci. Technol.*, 22:271-280 1988)) or the photometric method based on derivatisation with thioglycolic acid (*J. Chem. Ecol.*, *28*, 2483-2501 (2002)). In non-lignified tissue, the amount of lignin is decreased as compared to a comparable control plant and the amount of lignin can be completely, substantially or partially removed. In non-lignified tissue, a detectable amount of lignin can be present provided that there is a measurable quantitative reduction in the amount of lignin when compared to a comparable control plant in which the amount of lignin has not been reduced. In non-lignified tissue it may not be possible to detect any amount of lignin. Non-lignified tissue can contain less than 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2% or 1% of the total dry weight content of lignin in the plant or plant part from which it is removed.

A "control plant” or "control plant cell” refers to a plant or a plant cell - such as a native or naturally occurring plant or plant cell - having a lignin content and/or a NNK content that has not been manipulated or modified. Control plant material includes plant material obtained from, derived from or derivable from the control plant or the control plant cell or a combination thereof. The control plant or control plant cell can be the same type of plant or plant cell, for example, the same species of plant or plant cell as the plant or plant cell that it is being compared to. The control plant or control plant cell may correspond to a wild-type plant or wild-type plant cell.

The term “reduce” or “decrease” or grammatical variations thereof refers to a reduction of from about 10% to about 99%, or a reduction of at least 10%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or at least 100% or more of a quantity, amount or activity.

The term “inhibit” or grammatical variations thereof, refers to a reduction of from about 98% to about 100%, or a reduction of at least 98%, at least 99%, but particularly of 100%, of a quantity, amount or activity.

The term “increase” or grammatical variations thereof refers to an increase of from about 5% to about 99%, or an increase of at least 5%, at least 10%, at least 20%, at least 25%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 75%, at least 80%, at least 90%, at least 95%, at least 98%, at least 99%, or at least 100% or more of a quantity, amount or activity.

The term "about" in the context of a given numerate value or range refers to a value or range that is within 20 %, within 10 %, or within 5 % of the given value or range.

The term "at least a portion" or grammatical variations thereof includes at least about 5%, at least about 10 %, at least about 20%, at least about 30%, at least about 40%, at least about 50 %, at least about 60%, at least about 70%, at least about 75%, at least about 80%, at least about 90%, at least about 95%, at least about 98%, or at least about 99% of a quantity, amount or activity.

DETAILED DESCRIPTION

Generally speaking, the present disclosure can be applied to any form of tobacco plant material in which NNK or metabolites thereof or a combination thereof can form or have formed. Suitably, at least a portion of the NNK is in the bound form. At least a portion of the matrix-bound form co-localises with lignin – such as lignified tissue. Methods for measuring free nitrosamine(s) and matrix-bound nitrosamine(s) are well known in the art and described herein. Briefly, aliquots of tobacco samples can be extracted and the nitrosamine content therein can be analysed using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). Typically, one or more standards corresponding to the one or more nitrosamines that are being quantified will be incorporated into the aliquots of the tobacco samples. The sample concentration calculated from the extract corresponds to the “free nitrosamine(s)” concentration in the sample. After treating the extraction mixtures to the methods described herein (for example, by heating to about 130°C for about 4 hours) nitrosamine concentrations can again be measured by UPLC-MS/MS. From these values, the “total NNK” concentration in the samples can be calculated. The “matrix-bound NNK” concentration is the difference between the “total NNK” and the “free NNK” concentration.

Much research has been performed on tobacco, especially in relation to tobacco-specific nitrosamines. Freshly harvested tobacco leaves are referred to as "green tobacco" and are believed to contain no nitrosamines, but green tobacco is not suitable for human consumption. The process of curing green tobacco depends on the type of tobacco harvested. For example, Virginia flue (bright) tobacco is typically flue-cured, whereas Burley and certain dark strains are usually air-cured. The flue-curing of tobacco typically takes place over a period of five to seven days compared to one to two months for air-curing. Many major chemical and biochemical changes occur during the curing process and continue through the early phases of leaf drying.

The conversion of the tobacco from its yellow to brown colour generally results in formation and substantial accumulation of nitrosamines, including NNK, and an increased microbial content. The exact mechanism by which tobacco-specific nitrosamines, including NNK, are formed is not clear, but is believed to be enhanced by microbial activity, involving microbial nitrate reductases in the generation of nitrite during the curing process.

As described above, matrix-bound NNK has been found to co-localise with lignin in tobacco plants or in tobacco plant material. Lignin is the generic term for a large group of aromatic polymers resulting from the oxidative combinatorial coupling of 4-hydroxyphenylpropanoids. These polymers are deposited predominantly in the walls of secondarily thickened cells - such as fibers and tracheary elements - making them rigid and impervious. The mechanical rigidity of lignin strengthens these tissues so that the tracheary elements can endure the negative pressure generated from transpiration without collapse of the tissue. In addition to providing mechanical strength, lignin has protective functions. For example, the physical toughness and chemical durability of lignin may deter feeding by herbivores. Lignification is a frequent response to infection or wounding, which may provide a physical barrier to block the penetration of pathogens. The main building blocks of lignin are the hydroxycinnamyl alcohols (or monolignols) coniferyl alcohol and sinapyl alcohol, with typically minor amounts of *p*-coumaryl alcohol. The monolignols are synthesized from Phe through the general phenylpropanoid and monolignol-specific pathways. Phe is derived from the shikimate biosynthetic pathway in the plastid. Certain enzymes of the lignin biosynthetic pathway, namely the cytochrome P450 enzymes cinnamate 4-hydroxylase (C4H), *p*-coumarate 3-hydroxylase (C3H), and ferulate 5-hydroxylase (F5H), are membrane proteins thought to be active at the cytosolic side of the endoplasmic reticulum. Although metabolic channeling has been shown between phenylalanine ammonia-lyase (PAL) and C4H, it remains unknown whether the other pathway enzymes are also part of metabolic complexes at the endoplasmic reticulum. The units resulting from the monolignols, when incorporated into the lignin polymer, are called guaiacyl (G), syringyl (S), and *p*-hydroxyphenyl (H) units.

Lignin is commonly found in, for example, plant midribs, plant stems or plant stalks. Thus, the material for use in the present disclosure can include plant midribs or plant stems or plant stalks or a mixture thereof or a combination of two or more thereof and can be removed. Lignin is located in, for example, the vascular bundle of a tobacco plant which can be found in plant midribs, plant stems, plant stalks and the like. The vascular bundle is composed of a plurality of relatively hard, cellulose members closely secured together by fibrous vegetable connecting tissue. Surrounding this fibro-vascular bundle is the cortex which is formed of a relatively sponge-like vegetable tissue or covering constituting the larger portion of the stem and the portion which is closer in characteristics and properties to the lamina of the tobacco leaf. Lignin is generally located in the vascular bundle. The vascular bundle is a part of the transport system in vascular plants. The transport itself happens in vascular tissue, which exists in two forms, the xylem and phloem. Both

these tissues are present in a vascular bundle, which in addition will include supporting and protective tissues. Of these vascular tissues, lignin is to be found only in the xylem.

The amount of lignin can be substantially reduced in plant midribs, plant stems or plant stalks and the like or a mixture or a combination of two or more thereof. Lignin can be substantially removed from plant midribs, plant stems or plant stalks and the like or a mixture or a combination of two or more thereof. Suitably, the vascular bundle or the xylem or a combination thereof is substantially reduced in plant midribs, plant stems or plant stalks and the like or a mixture or a combination of two or more thereof. Suitably, the vascular bundle or the xylem or a combination thereof is substantially removed from plant midribs, plant stems or plant stalks and the like or a mixture or a combination of two or more thereof. Suitably, lignified sclerenchymatic tissue is substantially removed from plant midribs, plant stems or plant stalks and the like or a mixture or a combination of two or more thereof.

It is an advantage that matrix-bound NNK co-localises with lignin since lignin can be readily separated from other parts of the plant or other plant tissues. In one embodiment, the amount of lignin is reduced by separating lignified from non-lignified tissue. For example, the outer cortex can be readily separated from the vascular bundle containing lignin, thus obtaining plant material with reduced levels of lignin. The plant material containing reduced levels of lignin or substantially no lignin can be used in the manufacture of tobacco materials or tobacco products with reduced levels of matrix-bound NNK as described herein. Optionally, the separated plant material containing lignin co-localised with matrix-bound NNK can be discarded or used in other processes.

In one aspect, there is provided a method of reducing or decreasing the amount of matrix-bound NNK in a cured tobacco plant or in cured tobacco plant material comprising reducing the amount of lignin therein. According to this method, a cured tobacco plant or cured tobacco plant material is provided. The amount of lignin in the cured tobacco plant or the cured tobacco plant material is reduced. The lignin can be completely removed or partially removed. A cured tobacco plant or cured tobacco plant material is then obtained in which the amount of lignin is reduced and the amount of matrix-bound NNK is also reduced as compared to the cured tobacco plant or the cured tobacco plant material initially provided or as compared to a control.

The lignin to be completely or partially removed can be located in the vascular bundle. The lignin to be completely or partially removed can be located exclusively in the vascular bundle. The lignin to be completely or partially removed can be located exclusively in the vascular bundle and not the surrounding tissue. Thus, the amount of matrix-bound NNK in a cured tobacco plant or in cured tobacco plant material is reduced by reducing the amount of the vascular bundle in the cured tobacco plant or in the cured tobacco plant material.

The lignin to be completely or partially removed can be located in the xylem. The lignin to be completely or partially removed can be located exclusively in the xylem. The lignin to be completely or partially removed can be located exclusively in the xylem and not the surrounding

tissue. Thus, the amount of matrix-bound NNK in a cured tobacco plant or in cured tobacco plant material is reduced by reducing the amount of xylem in the cured tobacco plant or in the cured tobacco plant material.

The lignin to be completely or partially removed can be located in lignified sclerenchymatic tissue. The lignin to be completely or partially removed can be located exclusively in lignified sclerenchymatic tissue. The lignin to be completely or partially removed can be located exclusively in the lignified sclerenchymatic tissue and not the surrounding tissue – such as the outer layer of midribs. Thus, the amount of matrix-bound NNK in a cured tobacco plant or in cured tobacco plant material is reduced by reducing the amount of the lignified sclerenchymatic tissue in the cured tobacco plant or in the cured tobacco plant material.

In another aspect, there is provided a method of reducing, decreasing, preventing or inhibiting the formation of matrix-bound NNK during the curing of a tobacco plant or tobacco plant material comprising reducing the amount of lignin therein prior to curing. At least initially, the tobacco plant or tobacco plant material can be uncured or substantially uncured. The method can be used to reduce, decrease, prevent or inhibit the co-localisation of NNK with lignin that would otherwise occur during the subsequent curing process. According to this aspect, an uncured tobacco plant or uncured tobacco plant material or substantially uncured tobacco plant or substantially uncured tobacco plant material is provided and the amount of lignin therein is reduced prior to curing or during curing. The lignin can be completely removed or partially removed. The lignin can be located in the vascular bundle. The lignin can be located exclusively in the vascular bundle. The lignin can be located exclusively in the vascular bundle and not the surrounding tissue. Thus, the vascular bundle can be completely removed or partially removed. The lignin can be located in the xylem. The lignin can be located exclusively in the xylem. The lignin can be located exclusively in the xylem and not the surrounding tissue. Thus, the xylem can be completely removed or partially removed. The lignin can be located in the lignified sclerenchymatic tissue. The lignin can be located exclusively in the lignified sclerenchymatic tissue. The lignin can be located exclusively in the lignified sclerenchymatic tissue and not the surrounding tissue – such as the outer layer of midribs. Thus, the lignified sclerenchymatic tissue can be completely removed or partially removed. The lignin can be completely removed or partially removed. After subjecting the tobacco plant or the tobacco plant material to curing, using methods that are well known in the art, a cured tobacco plant or cured tobacco plant material in which the amount of matrix-bound NNK and the amount of lignin is reduced as compared to the starting material or as compared to a control can be obtained.

The amount of lignin in a tobacco plant or in tobacco plant material can be reduced using various methods that are well known in the art. In one method, a batch of stems or the like can be moistened or soaked in fluid, for example, water, which causes the cortex to soften and expand or swell whilst leaving the lignin in an unexpanded state. The cortex can be removed manually (*eg*.

by hand) and retained and the vascular bundle containing lignin can be discarded or used elsewhere. Accordingly, the cortex (for example, the outer cortex) can be separated from lignified tissue and retained and the unexpanded lignified tissue discarded. The cortex can then be used for further tobacco processing. There is also disclosed plant cortex (for example, plant outer cortex) or expanded plant cortex (for example, the expanded plant outer cortex) in which the amount of matrix-bound NNK is below detectable levels. Accordingly, non-lignified plant tissue can be expanded in order to separate lignified and non-lignified tissue. Suitably, the non-lignified tissue is selectively or preferentially expanded over the lignified tissue.

By way of further example, lignin can be separated using suitable decorticating machinery. A decorticator is a machine for stripping the bark, wood and plant stalks and the like.

In another method, mechanical separation can be used. For example, soaking of the batch of stems or the like is followed by freeze drying. In another example, soaking of the batch of stems or the like is followed by freeze drying, grinding and sieving. Suitably, tobacco plant material can be ground into a powder form using equipment and techniques for grinding, milling, or the like. Suitably, the tobacco plant material is relatively dry in form during grinding or milling, using various equipment - such as hammer mills, cutter heads, air control mills and the like.

The plant material can be reduced in size to form particles or particulate matter using various methods that are known in the art. The particles or particulate matter can be separated by size to obtain fractions with reduced levels of lignin and reduced levels of matrix-bound NNK. In one suitable method, plant material is treated by impact – such as by impact with one or more objects that are harder than the plant material to be treated. In one embodiment, impact with metal – such as metal balls - is used. The impact can be delivered using various methods, such as shaking. For example, plant material can be impacted with steel balls (2 steel balls, diameter 2 cm) with shaking at 300 rpm for 15 minutes. The particles or particulate matter can be separated by size using a sieve shaker into fractions of different particle size(s). Suitably, the average particle is greater than about 0.5 millimetres, greater than about 0.85 millimetres or greater than about 1 millimetre. These size fractions can have reduced levels of lignin and reduced levels of matrix-bound NNK.

The plant material can be ground or milled when the moisture content thereof is less than about 15 weight percent to less than about 5 weight percent. The tobacco plant material can be finely ground. Finely ground tobacco material typically has a particle size of from about 30 to 600 microns.

In one embodiment, the method comprises expansion, for example by contacting with a fluid (*eg.* by water soaking), followed by freeze drying, which will result in the expansion of plant material - which does not contain lignin or contains only low levels of lignin. Lignified plant material will retain a higher density, higher physical strength and a smaller particle size than the expanded plant tissue thereby permitting separation by size. In another embodiment, the method comprises

expansion, followed by freeze drying, followed by grinding (for example, by impact as discussed above), followed by sorting (for example, sorting by size) of the resulting fragments and selection of the fragments with reduced levels of lignin and reduced levels of matrix-bound NNK. Sieving may be used for this purpose. In another embodiment, the method comprises expansion, followed by grinding or crushing or a combination thereof, (for example, by impact, as described above), followed by sorting by size (using sieving or based upon density and/or mechanical strength, for example)the resulting particles and selection of the particles with reduced levels of lignin and reduced levels of matrix-bound NNK. The different sized fractions may also differ in their free-NNK content or their NNN content or their nicotine content or a combination of two of more thereof.

Following the complete or partial removal of lignin, the plant material can optionally be further processed for use in a tobacco product. By way of example, this material can be formed into an aqueous slurry. The resulting slurry can contain a substantial proportion of colloidal cortex particles dispersed therein. The conversion of the tobacco cortex into an aqueous slurry can be accomplished using a suitable type of mill - such as a ball mill or a colloid mill. The further processing of the cortex and aqueous slurry is described herein.

In certain embodiments, the plant material obtained or obtainable by the methods described herein comprises, consists or consists essentially of tissue surrounding the vascular bundle or the tissue surrounding the xylem or the tissue surrounding the lignified sclerenchymatic tissue or a combination of two or more thereof with the vascular bundle or the xylem or the lignified sclerenchymatic tissue or a combination of two or more thereof substantially absent. In certain embodiments, the plant material comprises, consists or consists essentially of the tissue surrounding the vascular bundle or the xylem or lignified sclerenchymatic tissue or a combination of two or more thereof and substantially no vascular bundle or no xylem or no lignified sclerenchymatic tissue or a combination of two or more thereof. In certain embodiments, the plant material comprises, consists or consists essentially of the tissue surrounding the vascular bundle or the xylem or the lignified sclerenchymatic tissue or a combination of two or more thereof and no vascular bundle or no xylem or no lignified sclerenchymatic tissue or a combination of two or more thereof. In certain embodiments, the plant material comprises, consists or consists essentially of the outer tobacco cortex. In certain embodiments, the plant material comprises, consists or consists essentially of the outer layers of plant midribs.

The methods described herein may comprise one or more further steps of measuring and optionally comparing the levels of free NNK or matrix-bound NNK or a combination thereof. Methods for measuring free NNK and matrix-bound NNK are described herein. In one embodiment, the amount of free NNK or matrix-bound NNK or a combination thereof is determined in a cured tobacco plant or in cured tobacco plant material. After reducing the amount of lignin in the cured tobacco plant or the cured tobacco plant material the amount of free NNK or matrix-bound NNK or a combination thereof can be measured again. The level of at least matrix-bound

NNK can be compared with the initial starting material to ascertain if the level of matrix-bound NNK has been reduced. In this step, the level of at least matrix-bound NNK previously measured can be compared with the level of NNK measured following the reduction of the amount of lignin. A reduction in the amount of matrix-bound NNK in the tobacco material obtained following the reduction of the amount of lignin as compared to the tobacco material initially provided is indicative that the amount of matrix-bound NNK has been reduced.

The methods described herein may comprise one or more further steps of measuring and optionally comparing the levels of free NNK or matrix-bound NNK or combinations thereof. In one embodiment, the amount of free NNK or matrix-bound NNK or a combination thereof is measured in the uncured tobacco plant or uncured tobacco plant material. Optionally, after reducing the amount of lignin in the uncured tobacco plant or the uncured tobacco plant material prior to curing the amount of free NNK or matrix-bound NNK or a combination thereof can be measured again. The method may include one or more comparison steps. By way of example, the method may comprise the further step of comparing the level of at least matrix-bound NNK initially measured, as discussed above, with the level of NNK later measured, wherein a reduction in the amount of matrix-bound NNK in the tobacco material as compared to the tobacco material initially provided is indicative that the amount of matrix-bound NNK in the tobacco material is reduced.

Free NNK or matrix-bound NNK or a combination thereof can be measured at the start of the method and/or at the end of the method and/or during the method. Free NNK or matrix-bound NNK or a combination thereof may be measured intermittently or at intervals. The intervals may be fixed intervals or random intervals. Free NNK or matrix-bound NNK or a combination thereof can measured at the end of the method to check that the free NNK or matrix-bound NNK or a combination thereof is present within a desired amount, concentration or range.

Lignin can be covalently or non-covalently bound to lignin. A complex comprising lignin covalently or non-covalently bound to NNK is described. A plant cell, plant tissue or plant or plant material comprising the complex is also disclosed. A method for reducing the amount of matrix-bound NNK in a cured tobacco plant or in cured tobacco plant material is also described comprising reducing the amount of the complex therein.

In a further aspect, there is provided cured plant tissue containing a reduced level of lignin, as compared to control plant tissue in which the amount of lignin has not been reduced, and wherein the amount of matrix-bound NNK is about 3500 ng/g or less. The amount of matrix-bound NNK can be about 3000 ng/g or less, about 2500 ng/g or less, about 2000 ng/g or less, about 2000 ng/g or less, about 1500 ng/g or less, about 1000 ng/g or less or about 500 ng/g or less. Suitably, the average particle size of this cured plant tissue can be greater than about 0.5 millimetres, greater than about 0.85 millimetres or greater than about 1 millimetre. Suitably, the amount of free NNK in this cured plant tissue can be about 330 ng/g or less, about 300 ng/g or less, about 250 ng/g or less, about 200 ng/g or less, about 150 ng/g or less, about 100 ng/g or less or about 50 ng/g or

less. Suitably, the amount of NNN in this cured plant tissue can be about 1700 ng/g or less, about 1500 ng/g or less, about 1300 ng/g or less, about 1100 ng/g or less, about 1000 ng/g or less, or about 500 ng/g or less. Suitably, the amount of nicotine in this cured plant tissue can be about 2600 µg or less, about 2300 µg or less or about 2100 µg or less. Suitably, the amount of lignin in this cured plant tissue can be about 6.5 % or less of the total dry weight content of the cured plant tissue, about 6 % of the total dry weight content of the cured plant tissue, about 5 % of the total dry weight content of the cured plant tissue, about 4 % of the total dry weight content of the cured plant tissue or about 3 % of the total dry weight content of the cured plant tissue.

In one embodiment, there is provided cured plant tissue containing a reduced level of lignin, as compared to control plant tissue in which the amount of lignin has not been reduced, and wherein the amount of matrix-bound NNK is about 3500 ng/g or less and the average particle size is about 0.5 mm or greater. Suitably, the amount of free NNK is about 300 ng/g or less. Suitably, the amount of NNN is about 1700 ng/g or less. Suitably, the amount of lignin in this cured plant tissue is about 6.4 % or less of the total dry weight content of the cured plant tissue. Suitably, the amount of nicotine is about 2600 µg or less.

In another embodiment, there is provided cured plant tissue containing a reduced level of lignin, as compared to control plant tissue in which the amount of lignin has not been reduced, and wherein the amount of matrix-bound NNK is about 1900 ng/g or less and the average particle size is between about 0.85 mm and about 1 mm. Suitably, the amount of free NNK is about 250 ng/g or less. Suitably, the amount of NNN is about 1270 ng/g or less. Suitably, the amount of lignin in this cured plant tissue is about 4.4 % or less of the total dry weight content of the cured plant tissue. Suitably, the amount of nicotine is about 2300 µg or less.

In another embodiment, there is provided cured plant tissue containing a reduced level of lignin, as compared to control plant tissue in which the amount of lignin has not been reduced, and wherein the amount of matrix-bound NNK is about 1600 ng/g or less and the average particle size is greater than about 1 mm. Suitably, the amount of free NNK is about 200 ng/g or less. Suitably, the amount of NNN is about 1100 ng/g or less. Suitably, the amount of lignin in this cured plant tissue is about 3 % or less of the total dry weight content of the cured plant tissue. Suitably, the amount of nicotine is about 2100 µg or less.

The tobacco plant or the tobacco plant material that is used at the start of the method(s) described herein can comprise or consist or consist essentially of uncured tobacco plant or uncured tobacco plant material or cured tobacco plant or cured tobacco plant material. Processes of curing tobacco – such as tobacco leaves, especially, green tobacco leaves are well known to those skilled in the art and include without limitation air-curing, fire-curing, flue-curing and sun-curing. The process of curing tobacco depends on the type of tobacco harvested. For example, Virginia flue (bright) tobacco is typically flue-cured, Burley and certain dark strains are usually air-cured, and pipe tobacco, chewing tobacco, and snuff are usually fire-cured. Although tobacco plants or tobacco

plant material from any type of tobacco may be used, certain types of tobacco are preferred. Particularly preferred tobacco materials are selected from the group consisting of: flue-Cured, Turkish, Burley, Virginia, Maryland, Oriental, or any combination of two or more thereof. The shape of the tobacco material is in general not limited. It can be in the form of homogenised tobacco material. Tobacco homogenates - such as but not limited to cured tobacco homogenates - may be prepared from tobacco material using various methods known in the art, for example, the tobacco may be in a shredded, granulated, ground or powder form. In certain embodiments, it is desirable not to begin with tobacco material in the ground or powder form since certain mechanical separation methods that can be used to separate lignin can require grinding and/or sieving steps.

The tobacco material used or obtained may comprise additives that include, but are not limited to, one or more of the following components as well as combinations thereof: flavourants, organic and inorganic fillers (for example, grains, processed grains, puffed grains, maltodextrin, dextrose, calcium carbonate, calcium phosphate, corn starch, lactose, manitol, xylitol, sorbitol, finely divided cellulose, and the like), binders (for example, povidone, sodium carboxymethylcellulose and other modified cellulosic types of binders, sodium alginate, xanthan gum, starch-based binders, gum arabic, lecithin, and the like), colorants (for example, dyes and pigments, including caramel colouring and titanium dioxide, and the like), humectants (for example, glycerin, propylene glycol, and the like), oral care additives, preservatives (for example, potassium sorbate, and the like), syrups (for example, honey, high fructose corn syrup, and the like used as flavourants), and disintegration aids (for example, microcrystalline cellulose, croscarmellose sodium, crospovidone, sodium starch glycolate, pregelatinized corn starch, and the like). Such additives are known to those having skill in the art and may be present in amounts and in forms known in the art.

The tobacco can be formed into reconstituted tobacco. Thus, in one embodiment, the methods described herein can be used in the preparation of reconstituted tobacco, such as reconstituted tobacco (leaf) sheets. These sheets are paper-like material that can be made from recycled tobacco fines, tobacco stems and "class tobacco", which consists of tobacco particles generally less than 30 mesh in size that are collected at any stage of tobacco processing. The reconstituted tobacco can be made by extracting the soluble chemicals in the tobacco by-products, processing the leftover tobacco fibers from the extraction into a paper, and then reapplying the extracted materials in concentrated form onto the paper. Reconstituted tobacco can generally be formed in a variety of ways. For instance, in one embodiment, band casting can be utilised to form the reconstituted tobacco. Band casting typically employs a slurry of finely divided tobacco parts and a binder that is coated onto a steel band and then dried. After drying, the sheet is blended with natural tobacco strips or shredded and used in various tobacco products, including as a cigarette filler. Some examples of processes for producing reconstituted tobacco are described in US 3,353,541, US 3,420,241, US 3,386,449, US 3,760,815 and 4,674,519. Reconstituted tobacco can also be formed by a papermaking process. Some examples of processes for forming reconstituted

tobacco according to this process are described in US 3,428,053, US 3,415,253, US 3,561,451, US 3,467,109, US 3,483,874, US 3,860,012, US 3,847,164, US 4,182,349, US 5,715,844, US 5,724,998; and US 5,765,570. For example, the formation of reconstituted tobacco using papermaking techniques can involve the steps of mixing tobacco with water, extracting the soluble ingredients therefrom, concentrating the soluble ingredients, refining the tobacco, forming a web, reapplying the concentrated soluble ingredients, drying, and threshing. Various ingredients - such as flavour or colour treatments - can be applied to the web.

The tobacco obtained or obtainable by the methods described herein may be formed into a tobacco sheet - such as a reconstituted tobacco sheet. According to this embodiment, the method may comprise the steps of: (a) obtaining tobacco material – such as a tobacco homogenate – according to the methods described herein; (b) preparing a slurry of tobacco homogenate; (c) casting the slurry of the tobacco homogenate; and (d) drying the slurry of the tobacco homogenate to form a reconstituted tobacco sheet. According to another embodiment, the method may comprise the steps of: (a) obtaining tobacco material – such as a tobacco homogenate – according to the methods described herein and preparing a tobacco slurry; (b) casting the slurry of the tobacco homogenate; and (c) drying the slurry of the tobacco homogenate to form a tobacco sheet. The step of casting the slurry of the tobacco homogenate may be performed using any of the casting or paper making processes that are known in the art. By way of example, casting processes are described in US 5,724,998 and US 5,584,306; paper-making processes are described in US 4,341,228; US 5,584,306 and US 6,216,706. Casting processes typically include casting the slurry onto a continuous stainless steel belt, drying the cast slurry to form a reconstituted tobacco sheet and removing said sheet. Paper-making processes typically include casting the aqueous slurry from a head box onto a wire screen for forming the desired sheet. The aqueous slurry may be separated into a soluble portion and a fibrous portion. Water is drained from the fibrous portion and a sheet is so-formed is subsequently treated and dried.

The tobacco slurries may further comprise one or more binders - such as gums and pectins. As described above, tobacco slurries that are used to prepare reconstituted tobacco sheets may further comprise common additives that include, but are not limited to, one or more of the following components as well as combinations of these: wood cellulose fibers, aerosol formers, sugars, and flavourants and binders. Additives of the list described above are known to those having skill in the art and may be present in these aqueous slurries in amounts and in forms known in the art.

Once prepared, the reconstituted tobacco sheets described herein may be cut in a similar fashion as whole leaf tobacco to produce tobacco filler suitable for cigarettes and other tobacco products. The reconstituted tobacco sheets described herein may be further trashed or flayed with mechanical fingers into sized pieces similar to natural tobacco lamina strips or cut into diamond shaped pieces, between about 50 to 100 mm on a side. The reconstituted tobacco sheet pieces described herein may be further blended with other tobaccos such as flue-cured tobacco, Burley

tobacco, Maryland tobacco, Oriental tobacco, rare tobacco, specialty tobacco, expanded tobacco and the like. The precise amount of each type of tobacco within a tobacco blend used for the manufacture of a particular cigarette brand varies from brand to brand. See, for example, Tobacco Encyclopaedia, Voges (Ed.) p. 44-45 (1984), Browne, The Design of Cigarettes, 3rd Ed., p.43 (1990) and Tobacco Production, Chemistry and Technology, Davis et al. (Eds.) p. 346 (1999). The entire blend may then be shredded into a cut filler and incorporated into a tobacco product.

According to a further aspect, there is provided a method for blending tobacco in which at least two different types of tobacco are blended so as to form a tobacco blend. The various tobacco blends have different recipes for blending different tobacco types. Tobacco types can be, by way of example, Burley, Flue Cured, Oriental, Bright and Reconstituted tobacco. Burley, Flue Cured and Oriental tobacco are specific types of tobacco, while Bright tobacco is a pre-blend of Flue Cured and Oriental tobacco. According to the method, a first (type of) cured tobacco plant material is provided and the amount of lignin therein is reduced. Any of the methods described herein can be used to reduce the amount of lignin. The total and/or matrix-bound NNK content of the first cured tobacco plant material can be measured and cured tobacco plant material in which the total and/or matrix-bound NNK content is reduced as compared to first cured tobacco plant material initially provided can be selected for further use. A second cured tobacco plant material which has a higher total and/or matrix-bound NNK content than the total and/or matrix-bound NNK of the first cured tobacco plant material is next provided. In some embodiments, the total and/or matrix-bound NNK content of this material may already be known so measurement of these values will not be required. In other embodiments, the total and/or matrix-bound NNK content of this material may not be known and so measurement will be required. Thus, measuring the total and/or matrix-bound NNK content in the second cured tobacco plant material is an optional step in this method. The first and second cured tobacco plant materials obtained from these steps can be blended together using processes that are well known in the art. Optionally, the total and/or matrix-bound NNK content in the final blended tobacco plant material can be measured. According to this method, a blended tobacco plant material can be obtained in which the total and/or matrix-bound NNK content of the final blended tobacco plant material is lower than the second cured tobacco plant material. Advantageously, this method can be used to provide a blend of tobacco material in which the overall NNK content of the blend is reduced. Essentially, the tobacco material in which the amount of lignin therein has been reduced is used to dilute or reduce the overall NNK content in the blended tobacco material.

The tobacco material obtained or obtainable according to this disclosure can also be used in tobacco cut filler and in a smoking article formed from a tobacco rod of the cut filler. Conventionally, cut filler tobacco products for smoking articles are formed predominantly from the lamina portion of the tobacco leaf, which is separated from the stem portion of the leaf during a threshing process. Much of the stem portion that remains after the lamina has been removed and

separated is not used. In order to increase the amount of the tobacco material that can be used commercially, some tobacco stems can be added back into the cut filler together with the lamina. In order to improve the taste and burning characteristics of the tobacco stem for use in the cut filler, the stems are often first subjected to one or more treatment procedures, which can include the procedures described herein. The rolling step can be carried out on tobacco stems that have been subjected to the method of the present disclosure. The stems can be rolled to a desired thickness – such as a mean thickness of about 0.6 mm to 0.8 mm. During subsequent processing and storage steps, the stems can expand to a final thickness of about 0.8 mm to about 1.0 mm. After rolling, the stems are dried and transferred to the tobacco production plant, where they are cut and added to the tobacco cut filler. In some cases, the rolling step may alternatively be incorporated as part of the on-line production process for cut filler. Typically the moisture content of the tobacco stems is about 28 % to about 34 % oven volatiles prior to rolling in order to prevent damage to the structure of the stems. If necessary, the tobacco stems can be conditioned prior to rolling in order to increase the moisture content to this level. Known processes for conditioning tobacco stems involve contacting the stems with water, steam or a mixture of water and steam. In methods where the rolling step is incorporated on-line and dried stems are used, the conditioning step will typically take longer and may require a soaking step in which the stems are soaked in water for a number of hours prior to rolling. The tobacco stems can be rolled using a one-step rolling process to reduce the thickness of the stems to the desired mean thickness. After rolling, the stems can be cut to a cut width of between 0.1 mm and 0.2 mm. The cut rolled stems are then optionally expanded using known stem expansion techniques, and then dried. Where the stems are pre-rolled and dried, it will typically be necessary to condition the stems prior to cutting in order to increase the moisture content of the tobacco stems back to between 28 % and 34 % oven volatiles. This increases the pliability of the tobacco stems in order to limit damage or breakage of the stems during cutting. Finally, the cut rolled stems are combined with tobacco cut lamina and any additional tobacco materials in order to form cut filler having at least 5 % by weight of the cut rolled tobacco stems.

Thus, in a further aspect, there is provided a method for preparing tobacco for use as a tobacco cut filler comprising the steps of: (a) performing the method(s) as described herein; and (b) rolling and cutting the tobacco material for use as a tobacco cut filler. There is also described a method of treating tobacco material – such as tobacco stems - for use in tobacco cut filler, the method comprising the steps of: (a) performing the method as described herein; (b) rolling the tobacco material; (c) cutting the rolled tobacco material; and (d) optionally drying the cut rolled stems. The rolled tobacco stems can be combined with tobacco lamina such that the steps are carried out on the combined tobacco stems and lamina. The cutting step can comprise cutting the rolled stems to a cut width of between about 0.3 mm and 1.3 mm. The method can comprise the steps of: removing stems from the tobacco leaf; cutting the stems to an average length of between about 15 mm and 80 mm; and rolling the stems to a thickness of between 0.1 mm and 0.5 mm. A method of

producing cut filler comprising rolled tobacco stems is also provided, the method comprising: treating tobacco stems using the method described herein; and blending the treated stems with at least one type of tobacco lamina, expanded tobacco or reconstituted tobacco to produce cut filler.
The tobacco cut filler obtained or obtainable by this method can comprise at least 60 %, and preferably at least 80 % by weight tobacco lamina having a mean cut width between 0.8 mm and 1.1 mm, suitably, about 0.9 mm, and a mean thickness of about 0.2 mm. The tobacco cut filler can comprise up to 95 % by weight tobacco lamina with a mean cut width between about 0.8 mm and 1.1 mm, more suitably about 0.9 mm, and a mean thickness of about 0.2 mm. The particles of tobacco lamina in the cut filler are therefore of similar dimensions to the particles of tobacco stem. As such, the tobacco stems are not visually distinct from the tobacco lamina, even at a high inclusion rate. In addition, the blend of tobacco stems and lamina can advantageously be transported and processed effectively without significant settling of the stems. Suitably, the mean cut width of the cut rolled tobacco stems is within about 0.1 mm, more suitably within about 0.05 mm of the mean thickness of the tobacco lamina in the cut filler. Cut fillers may be incorporated into a variety of smoking articles. For example, the cut filler may be used in the tobacco rod of a combustible smoking article, such as a filter cigarette, cigarillo or cigar. Alternatively, the cut filler may be used to provide the tobacco aerosol generating substrate in a distillation based smoking article, or an electrically heated smoking system. Alternatively, the cut filler may be used as a roll-your-own product, or loose tobacco product for example, for use in a pipe.

The tobacco material can be incorporated into various consumable products - such as tobacco products. Also encompassed are methods for making such tobacco products. Tobacco products include without limitation smoking articles or smokable articles and smokeless tobacco products, including non-combustible products, heated products, and aerosol-generating products. Non-limiting examples of smoking or smokable articles include cigarettes, cigarillos, cigars and pipe tobaccos. Non-limiting examples of smokeless tobacco products include chewing tobaccos, snuffs, and substrates for use in aerosol-generating products. Smokeless tobacco products may comprise tobacco in any form, including as dried particles, shreds, granules, powders, or a slurry, deposited on, mixed in, surrounded by, or otherwise combined with other ingredients in any format, such as flakes, films, tabs, foams, or beads. Liquid contents of smokeless tobacco products can be contained in a device or enclosed in a form, such as beads, to preclude interaction with a water-soluble wrapper. The wrapper may be shaped as a pouch to partially or completely enclose tobacco-incorporating compositions, or to function as an adhesive to hold together a plurality of tabs, beads, or flakes of tobacco. Exemplary materials for constructing a wrapper include film compositions comprising HPMC, CMC, pectin, alginates, pullulan, and other commercially viable, edible film-forming polymers. Other wrapping materials may include pre-formed capsules produced from gelatin, HPMC, starch/carrageenan, or other commercially available materials. Such wrapping materials may include tobacco as an ingredient. Wrappers that are not orally disintegrable may be

composed of woven or nonwoven fabrics, of coated or uncoated paper, or of perforated or otherwise porous plastic films. Wrappers may incorporate flavouring or colouring agents. Smokeless products can be assembled together with a wrapper utilizing any method known to persons skilled in the art of commercial packaging, including methods such as blister packing, in which a small package can be formed by a vertical form/fill/seal packaging machine.

The amount of matrix-bound NNK in these smokable articles, smokeless products and aerosols and the like may be at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, and 100% lower – such as about 200% or 300% lower - when compared to consumable products derived from control tobacco plant material. The amount of free-NNK may be substantially unchanged.

The amount of matrix-bound NNK in these smokable articles, smokeless products and aerosols and the like may be about 3500 ng/g or less, or about 3000 ng/g or less, or about 2500 ng/g or less, or about 2000 ng/g or less, or about 1500 ng/g or less, or about 1000 ng/g or less, or about 500 ng/g or less.

The amount of free NNK in these smokable articles and smokeless products and the like may be about 330 ng/g or less, about 300 ng/g or less, about 250 ng/g or less, about 200 ng/g or less, about 150 ng/g or less, about 100 ng/g or less or about 50 ng/g or less.

The amount of NNN in these smokable articles, smokeless products and aerosols and the like may be about 1700 ng/g or less, about 1500 ng/g or less, about 1300 ng/g or less, about 1100 ng/g or less, about 1000 ng/g or less, or about 500 ng/g or less.

The amount of nicotine in these smokable articles and smokeless products and the like may be about 2600 µg or less, about 2300 µg or less, or about 2100 µg or less, or about 2000 µg or less 1900 µg or less, or about 1800 µg or less.

The amount of lignin in these smokable articles and smokeless products and the like may about 6.5 % or less of the total dry weight content of the cured plant tissue, about 6 % of the total dry weight content of the cured plant tissue, about 5 % of the total dry weight content of the cured plant tissue, about 4 % of the total dry weight content of the cured plant tissue or about 3 % of the total dry weight content of the cured plant tissue.

In one embodiment, the amount of matrix-bound NNK in these smokable articles and smokeless products and the like is about 3500 ng/g or less, the amount of free NNK is about 300 ng/g or less, the amount of NNN is about 1700 ng/g or less, the amount of lignin is about 6.4 % or less of the total dry weight content of the cured plant tissue, and the amount of nicotine is about 2600 µg or less.

In one embodiment, the smokable articles or smokeless products and the like comprise about 3500 ng/g or less of matrix-bound NNK. Suitably, the amount of free NNK is about 300 ng/g or less. Suitably, the amount of NNN is about 1700 ng/g or less. Suitably, the amount of lignin in is about

6.4 % or less of the total dry weight content of the cured plant tissue. Suitably, the amount of nicotine is about 2600 µg or less. Suitably, the average particle size is about 0.5 mm or greater.

In another embodiment, the smokable articles or smokeless products and the like comprise about 1900 ng/g or less matrix-bound NNK. Suitably, the amount of free NNK is about 250 ng/g or less. Suitably, the amount of NNN is about 1270 ng/g or less. Suitably, the amount of lignin in this cured plant tissue is about 4.4 % or less of the total dry weight content of the cured plant tissue. Suitably, the amount of nicotine is about 2300 µg or less. Suitably, the average particle size is between about 0.85 mm and about 1 mm.

In another embodiment, the smokable articles or smokeless products and the like comprise about 1600 ng/g or less matrix-bound NNK. Suitably, the amount of free NNK is about 200 ng/g or less. Suitably, the amount of NNN is about 1100 ng/g or less. Suitably, the amount of lignin in this cured plant tissue is about 3 % or less of the total dry weight content of the cured plant tissue. Suitably, the amount of nicotine is about 2100 µg or less. Suitably, the average particle is greater than about 1 mm.

The tobacco material can be derived from tobacco plants, which include plants of the genus Nicotiana, various species of Nicotiana, including *N. rustica* and *N. tabacum*. The tobacco material can be derived from varieties of Nicotiana species, commonly known as flue or bright varieties, Burley varieties, dark varieties and oriental/Turkish varieties. In some embodiments, the tobacco material is derived from a Burley, Virginia, flue-cured, air-cured, fire-cured, Oriental, or a dark tobacco plant. In some embodiments, the tobacco material is derived, for example, from one or more of the following varieties: N. tabacum AA 37-1 , N. tabacum B 13P, N. tabacum Xanthi (Mitchell-Mor), N. tabacum KT D#3 Hybrid 107, N. tabacum Bel-W3, N. tabacum 79-615, N. tabacum Samsun Holmes NN, F4 from cross N. tabacum BU21 x N. tabacum Hoja Parado, line 97, N. tabacum KTRDC#2 Hybrid 49, N. tabacum KTRDC#4 Hybrid 1 10, N. tabacum Burley 21, N. tabacum PM016, N. tabacum KTRDC#5 KY 160 SI, N. tabacum KTRDC#7 FCA, N. tabacum KTRDC#6 TN 86 SI, N. tabacum PM021 , N. tabacum K 149, N. tabacum K 326, N. tabacum K 346, N. tabacum K 358, N. tabacum K 394, N. tabacum K 399, N. tabacum K 730, N. tabacum KY 10, N. tabacum KY 14, N. tabacum KY 160, N. tabacum KY 17, N. tabacum KY 8959, N. tabacum KY 9, N. tabacum KY 907, N. tabacum MD 609, N. tabacum McNair 373, N. tabacum NC 2000, N. tabacum PG 01 , N. tabacum PG 04, N. tabacum P01 , N. tabacum P02, N. tabacum P03, N. tabacum RG 11 , N. tabacum RG 17, N. tabacum RG 8, N. tabacum Speight G-28, N. tabacum TN 86, N. tabacum TN 90, N. tabacum VA 509, N. tabacum AS44, N. tabacum Banket A1 , N. tabacum Basma Drama B84/31 , N. tabacum Basma I Zichna ZP4/B, N. tabacum Basma Xanthi BX 2A, N. tabacum Batek, N. tabacum Besuki Jember, N. tabacum C104, N. tabacum Coker 319, N. tabacum Coker 347, N. tabacum Criollo Misionero, N. tabacum PM092, N. tabacum Delcrest, N. tabacum Djebel 81, N. tabacum DVH 405, N. tabacum Galpao Comum, N. tabacum HB04P, N. tabacum Hicks Broadleaf, N. tabacum Kabakulak Elassona, N. tabacum PM102, N. tabacum

Kutsage E1 , N. tabacum KY 14xL8, N. tabacum KY 171 , N. tabacum LA BU 21 , N. tabacum McNair 944, N. tabacum NC 2326, N. tabacum NC 71 , N. tabacum NC 297, N. tabacum NC 3, N. tabacum PVH 03, N. tabacum PVH 09, N. tabacum PVH 19, N. tabacum PVH 2110, N. tabacum Red Russian, N. tabacum Samsun, N. tabacum Saplak, N. tabacum Simmaba, N. tabacum Talgar 28, N. tabacum PM132, N. tabacum Wislica, N. tabacum Yayaldag, N. tabacum NC 4, N. tabacum TR Madole, N. tabacum Prilep HC-72, N. tabacum Prilep P23, N. tabacum Prilep PB 156/1, N. tabacum Prilep P12-2/1 , N. tabacum Yaka JK-48, N. tabacum Yaka JB 125/3, N. tabacum TI-1068, N. tabacum KDH-960, N. tabacum TI-1070, N. tabacum TW136, N. tabacum PM204, N. tabacum PM205, N. tabacum Basma, N. tabacum TKF 4028, N. tabacum L8, N. tabacum TKF 2002, N. tabacum TN90, N. tabacum GR141, N. tabacum Basma xanthi, N. tabacum GR149, N. tabacum GR153, and N. tabacum Petit Havana. The use of any species of the genus *Nicotiana* is disclosed, including *N. rustica* and *N. tabacum* (for example, LA B21, LN KY171, TI 1406, Basma, Galpao, Perique, Beinhart 1000-1, and Petico). Other species include *N. acaulis, N. acuminate, N. acuminate var. multiflora, N. alata, N. amplexicaulis, N. arentsii, N. benavidesii, N. benthamiana, N. bigelovii, N. bonariensis, N. cavicola, N. clevelandii, N. cordifolia, N. corymbosa, N. debneyi, N. excelsior, N. forgetiana, N. fragrans, N. glauca, N. glutinosa, N. goodspeedii, N. gossei, N. hybrid, N. ingulba, N. kawakamii, N. knightiana, N. langsdorffii, N. linearis, N. longiflora, N. megalosiphon, N. miersii, N. noctiflora, N. nudicaulis, N. obtusifolia, N. occidentalis, N. occidentalis subsp. hesperis, N. otophora, N. paniculata, N. pauciflora, N. petunioides, N. plumbaginifolia, N. quadrivalvis, N. raimondii, N. repanda, N. rosulata, N. rosulata subsp. ingulba, N. rotundifolia, N. setchellii, N. simulans, N. solanifolia, N. spegazzinii, N. stocktonii, N. suaveolens, N. sylvestris, N. thyrsiflora, N. tomentosa, N. tomentosiformis, N. trigonophylla, N. umbratica, N. velutina, N. wigandioides, and N. x sanderae.*

The use of tobacco cultivars and elite tobacco cultivars is also contemplated herein. Particularly useful *Nicotiana tabacum* varieties include Burley type, dark type, flue-cured type, and Oriental type tobaccos. Non-limiting examples of varieties or cultivars are: BD 64, CC 101, CC 200, CC 27, CC 301, CC 400, CC 500, CC 600, CC 700, CC 800, CC 900, Coker 176, Coker 319, Coker 371 Gold, Coker 48, CD 263, DF911, DT 538 LC Galpao tobacco, GL 26H, GL 350, GL 600, GL 737, GL 939, GL 973, HB 04P, HB 04P LC, HB3307PLC, Hybrid 403LC, Hybrid 404LC, Hybrid 501 LC, K 149, K 326, K 346, K 358, K394, K 399, K 730, KDH 959, KT 200, KT204LC, KY10, KY14, KY 160, KY 17, KY 171, KY 907, KY907LC, KTY14xL8 LC, Little Crittenden, McNair 373, McNair 944, msKY 14xL8, Narrow Leaf Madole, Narrow Leaf Madole LC, NBH 98, N-126, N-777LC, N-7371LC, NC 100, NC 102, NC 2000, NC 291, NC 297, NC 299, NC 3, NC 4, NC 5, NC 6, NC7, NC 606, NC 71, NC 72, NC 810, NC BH 129, NC 2002, Neal Smith Madole, OXFORD 207, PD 7302 LC, PD 7309 LC, PD 7312 LC‘ 'Periq’e' tobacco, PVH03, PVH09, PVH19, PVH50, PVH51, R 610, R 630, R 7-11, R 7-12, RG 17, RG 81, RG H51, RGH 4, RGH 51, RS 1410, Speight 168, Speight 172, Speight 179, Speight 210, Speight 220, Speight 225, Speight 227, Speight 234, Speight G-28,

Speight G-70, Speight H-6, Speight H20, Speight NF3, TI 1406, TI 1269, TN 86, TN86LC, TN 90, TN 97, TN97LC, TN D94, TN D950, TR (Tom Rosson) Madole, VA 309, VA359, AA 37-1, B 13P, Xanthi (Mitchell-Mor), Bel-W3, 79-615, Samsun Holmes NN, KTRDC number 2 Hybrid 49, Burley 21, KY 8959, KY 9, MD 609, PG 01, PG 04, PO1, PO2, PO3, RG 11, RG 8, VA 509, AS44, Banket A1, Basma Drama B84/31, Basma I Zichna ZP4/B, Basma Xanthi BX 2A, Batek, Besuki Jember, C104, Coker 347, Criollo Misionero, Delcrest, Djebel 81, DVH 405, Galpão Comum, HB04P, Hicks Broadleaf, Kabakulak Elassona, Kutsage E1, LA BU 21, NC 2326, NC 297, PVH 2110, Red Russian, Samsun, Saplak, Simmaba, Talgar 28, Wislica, Yayaldag, Prilep HC-72, Prilep P23, Prilep PB 156/1, Prilep P12-2/1, Yaka JK-48, Yaka JB 125/3, TI-1068, KDH-960, TI-1070, TW136, Basma, TKF 4028, L8, TKF 2002, GR141, Basma xanthi, GR149, GR153, Petit Havana. Low converter subvarieties of the above, even if not specifically identified herein, are also contemplated.

The following examples are provided as an illustration and not as a limitation. Unless otherwise indicated, the present invention employs conventional techniques and methods of molecular biology and plant biology.

EXAMPLES

Example 1

Method for analysis of free and matrix-bound NNK in tobacco

Aliquots of tobacco samples (for example, about 750 mg) are extracted with about 30 mL of Tris-HCl buffer (50 mM; pH 7.4) by shaking for about one hour at approximately room temperature. Internals standard (100 ng/mL NNK-d_4) are added. Samples (0.4 mL) of the extracts are filtered using a 0.2 µM filter and the NNK content is analysed using ultra performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS). The sample concentrations calculated from these extract concentrations correspond to the “free NNK” concentrations in the sample. After treating the extraction mixtures (for example, by heating to about 130°C for about 4 hours) and filtering aliquots of the extracts, NNK concentrations are again measured by UPLC-MS/MS. From these values, the “total NNK” concentration in the samples can be calculated. The “matrix-bound NNK” concentration is the difference between the “total NNK” and the “free NNK” concentrations.

An alternative method for “total-NNK” extraction comprises acidification of the extraction mixtures with concentrated HCl (for example, 3 mL of 37% HCl added to 30 mL) and incubation for 48 hours at 80°C. The acidic extracts are neutralised before filtration and UPLC analysis by adding NaOH solution (6N, 40 µL) and magnesium hydroxide suspension (10%; 40 µL) to 320 µL of extract.

Example 2

UPLC analysis

The column used is Waters Acquity BEH C18, 1.7 µm, 2.1 × 50 mm. The eluents used are: (A) ammonium bicarbonate (10mM; adjusted to pH 9.8 with ammonia) + 2% (v/v) acetonitrile; (B) acetonitrile. The gradient used is 0 min – 5 % B; 0.5 min – 5% B; 3.3 min – 18.3 % B. The flow that is used is 0.5 mL/min. The column temperature that is used is 50°C.

Example 3

MS/MS methodology

This analysis is carried out on a Waters TQ spectrometer using the following MRM transitions: NNK: 208.2 → 122.2; dwell time 100 ms; NNK-d4: 212.2 → 126.2; dwell time 100 ms; Capillary voltage: 0.6 kV; Cone voltage: 25 V; Collision energy: 11 eV; Source temperature: 120°C; Desolvation temperature: 400°C; Desolvation gas flow: 800 L/h.

Example 4

Distribution of matrix-bound NNK in lignified and non-lignified tissues of Burley stems

About 2 grams of midribs of cured Burley tobacco leaves are separated by hand into inner lignified tissue (36 % of total dry weight) and outer non-lignified tissue (64 % of total dry weight). In each of these samples the concentration of free NNK and total NNK is analysed by UPLC-MS, as described above. Matrix-bound NNK is calculated as the difference between free NNK and total NNK concentration. Lignin content is quantified using a photometric method based on derivatisation with thioglycolic acid (see Brinkmann *et al.* (2002) *J. Chem. Ecol.*, *28*, 2483-2501).

The results in Figure 1 show the distribution of free NNK, matrix-bound NNK and lignin in lignified (L) and non-lignified (NL) tissues of cured Burley stems. Figure 2 is a cross-section of a hydrated cured Burley stem showing lignified (L) and non-lignified (NL) tissues. Lignified tissue is stained red with phloroglucinol. The results in Figure 3 shows the matrix-bound NNK content of lignified and non-lignified tissues of green midribs after nitrosating with sodium nitrite solution.

These results show that matrix-bound NNK is distributed principally in lignified tissues of Burley tobacco stems and midribs.

Example 5

Enrichment of a fraction with low bound-NNK content from Burley Stem by freeze-drying, grinding and size separation

A sample of Burley Stems (52 g) is humidified with water (350 mL) and freeze-dried. A part of the resulting material (12 g) is ground by shaking with steel balls (2 balls, diameter 2 cm; 300 rpm; 15 min) and separated with a sieve shaker into fractions of different particle size ranging from greater than 1 mm to less than 0.25 mm.

Figure 4 and Table 1 show the free- and matrix-bound NNK, NNN, lignin and nicotine levels in sieving fractions of ground freeze-dried Burley stems. The analysis of free- and matrix-bound NNK in Figure 5 and Table 1 indicates that both the lignin content (as % dry weight of each fraction) and the matrix-bound NNK content is decreased in the fractions with particle size of greater than 0.5 mm, including fractions with particle size of 0.5 mm to 0.85 mm, 0.85 mm to 1 mm and greater than 1 mm. Figure 5 shows that lignin content correlates well with matrix-bound NNK thereby confirming the co-localisation of lignin and matrix-bound NNK.

Example 6

Localization of matrix-bound NNK precursor in green TN90 midribs and Burley stems

The relative distribution of matrix-bound NNK is measured in sclerenchymatic and non-sclerenchymatic tissue of TN90 midribs. The prediction of matrix-bound NNK being bound to lignin predicts a higher concentration of this precursor in the lignified sclerenchymatic tissue. In a second experiment the relative distribution of free and matrix-bound NNK in sclerenchymatic and non-sclerenchymatic tissue of (cured) Burley stems is investigated.

Materials & Methods

Green midribs

The midribs (only the proximal halves) of 15 mature TN90 leaves are manually separated in sclerenchymatic tissue (S) (the "center" of the midrib) and non-sclerenchymatic tissue (NS). Both are freeze-dried and finely ground. The water-insoluble fraction of the two materials is determined by extracting 1g, each, three times with 40 mL of methanol/water 1:3 (room temperature for 1 hour) and weighing the insoluble material (designated SW and NSW) after freeze-drying. Pseudo-oxynictoine (PON) and nicotine analysis in S and NS (n=5) is measured using the follow methods. Finely powdered plant material (~20 mg) is extracted by shaking at room temperature for 45 minutes with methanol/water (4:1) containing PON-methyl-d_3 as an internal standard (200 ng/mL). After filtration (0.2 µm) samples are subjected to LC-MS analysis using the following conditions: Column: Acquity UPLC BEH C18 column (1.7 µm, 50 × 2.1 mm; Waters); Column temperature:

50 °C; Eluents: Aqueous ammonium bicarbonate adjusted to pH 9.8 using NH_3 with acetonitrile (98:2, v/v; eluent A); acetonitrile (eluent B); Gradient: 0 min – 0 % B, 0.5 mL/min; 0.5 min – 0 % B, 0.5 mL/min; 6 min – 30 % B, 0.5 mL/min; MS detection: PON *m/z* 179.2 à *m/z* 106.1; PON-*methyl-d_3 m/z* 182.2 à *m/z* 106.1; UV detection: 260 nm. PON and nicotine are eluted after 2.6 and 4.1 minutes, respectively. For the quantification of nicotine the peak area at 260 nm and external calibration is used. In order to estimate the content of matrix-bound NNK precursor in S, NS, SW and NSW, aliquots (~20 mg; n=5) of these materials are nitrosated by incubation in NaNO2-solution (1.5 mL (10 mg/mL in water)) for 4 hours at room temperature with shaking, centrifuged and washed/centrifuged four times with 10 mL water. Then, the centrifugation sediment of each nitrosated sample is taken up in 4 mL Tris-HCl buffer (50mM pH 7.5; with NNK-d4 and NNN-d4 at 100 ng/mL), autoclaved (for 4 hours at 130°C) and analysed for NNK content using the methods described herein.

Cured Burley stems

A Burley stem sample (2g = four ~5 cm pieces) is manually separated into the outer (non-lignified) tissue (CNS) and the inner (lignified) part (CS). Both samples are finely ground in a mixer mill (Retsch "Tissuelyzer" for 2.5 minutes, 50 s-1). This results in 1183 mg and 651 mg of CNS and CS powder, respectively. Free NNK in the two samples is determined after extraction of ~50 mg aliquots (n=5) with 1.5 mL Tris-buffer (+IS) for 1 hour at room temperature. Total NNK is determined after autoclave extraction (130°C for 4 hours) of ~50 mg aliquots (n=5) in 5 mL Tris-buffer (+IS).

Results

The results of this experiment are shown in Table 2 and in Figure 6. After nitrosation and washing, a 7-fold higher concentration of matrix-bound NNK is found in the sclerenchymatic tissue (S) compared to the outer layers of the midribs (NS). PON and nicotine are both two-fold higher in NS than in S. The results of these artificial nitrosation experiments are corroborated by the bound-NNK levels in the lignified (CS) and non-lignified (CNS) parts of a commercially cured Burley stem sample. While free NNK is two-fold higher in CS, matrix-bound NNK is 7-fold higher in CS. Nicotine and NNN levels are higher in CS than CNS and less than NNK.

Conclusions

The presence of high-concentrations of matrix-bound NNK precursor in the lignified, sclerenchymatic tissue of green midribs indicates that matrix-bound NNK is covalently or non-covalently linked to lignin.

Any publication cited or described herein provides relevant information disclosed prior to the filing date of the present application. Statements herein are not to be construed as an admission that the inventors are not entitled to antedate such disclosures. All publications mentioned in the above specification are herein incorporated by reference. Various modifications and variations of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific preferred embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in cellular, molecular and plant biology or related fields are intended to be within the scope of the following claims.

TABLE 1

Sieving fraction	Fraction of total weight [%]	free NNK [ng/g]	NNN [ng/g]	NIC [ug/g]	bound NNK [ng/g]	Lignin [% d.w.]
A: > 1mm	16	200	1098	2091	1606	2.92
A: 0.85 - 1 mm	4	251	1269	2307	1905	4.41
A: 0.5 - 0.85 mm	16	328	1690	2608	3500	6.33
A: 0.25 - 0.5 mm	27	463	2305	2937	6218	11.31
A: < 0.25 mm	38	297	1732	3237	2936	4.81
total		329	1760	2839	3651	6.47

TABLE 2

	fresh weight [g]	dry weight [g]	dry matter [%]	before washing [mg]	after washing [mg]	water-insolubles [% dry weight]	water-insolubles [% fresh weight]
S	25	3.1	12.4	1010	668	66	8.2
NS	171	11	6.4	1010	414	41	2.6
CS		0.65					
CNS		1.18					

Sample	PON [ug/g]	NIC [ug/g]	PON/NIC *1000	NNK after autoclaving [ng/g]	NNN [ng/g]	Free NNK [ng/g]
S		265		13245	1660	
NS		116		184	111	
SW		331		17610	1595	
NSW		109		326	272	
PON-S	6.8	784	8.63			
PON-NS	14.0	1909	7.32			
CS-free		9496			1356	120
CNS-free		7985			895	64
CS-total		8459	2038			
CNS-total		7057	310			

CLAIMS

1. A method of reducing the amount of matrix-bound NNK in cured tobacco plant material comprising separating lignified tissue from non-lignified tissue, preferably, wherein the amount of lignin is reduced chemically and/or mechanically.

2. The method according to claim 1, comprising the steps of:

(a) providing cured tobacco plant material;

(b) separating lignified from non-lignified tissue in the cured tobacco plant material; and

(c) obtaining cured tobacco plant material in which the amount of lignin is reduced and the amount of matrix-bound NNK is reduced as compared to the cured tobacco plant material provided in step (a).

3. The method according to claim 2, wherein following step (a) there is a further step of measuring the amount of at least matrix-bound NNK, and optionally, wherein following step (b) there is a further step of measuring the amount of at least matrix-bound NNK.

4. The method according to claim 3, wherein said method comprises the further step (d) of comparing the level of at least matrix-bound NNK measured following step (a) with the level of matrix-bound NNK measured following step (b), wherein a reduction in the amount of matrix-bound NNK in the tobacco material obtained in step (b) as compared to the tobacco material provided in step (a) is indicative that the amount of matrix-bound NNK in the tobacco material is reduced.

5. A method of reducing the formation of matrix-bound NNK during curing of tobacco plant material comprising reducing the amount of lignin therein prior to curing, preferably, comprising the steps of:

(a) providing uncured tobacco plant material;

(b) separating lignified from non-lignified tissue in the uncured tobacco plant material prior to curing;

(c) curing the tobacco plant material provided in step (b); and

(d) obtaining cured tobacco plant material in which the amount of matrix-bound NNK is reduced as compared to a control in which the amount of lignin has not been reduced.

6. The method according to claim 5, wherein following step (a) there is a further step of measuring the amount of at least matrix-bound NNK, and optionally, wherein following step (b) there is a further step of measuring the amount of at least matrix-bound NNK and optionally, wherein following step (c) there is a further step of measuring the amount of at least matrix-bound NNK, preferably,

wherein following step (c) or step (d) said method comprises the further step of comparing the level of at least matrix-bound NNK measured following step (a) with the level of at least matrix-bound NNK measured following step (b) and/or step (c), wherein a reduction in the amount of matrix-bound NNK in the tobacco material obtained in step (b) or step (c) as compared to the tobacco material provided in step (a) is indicative that the amount of matrix-bound NNK in the tobacco material is reduced.

7. The method according to any of the preceding claims, wherein the tobacco plant material is treated to expand non-lignified plant tissue, preferably, wherein the amount of lignin is reduced by separating the expanded and non-expanded plant tissue based on their different densities and/or their different strengths and/or their different particle sizes and/or

wherein the amount of lignin is reduced by removing at least the vascular bundle or xylem or lignified sclerenchymatic tissue or a combination of two or more thereof from the plant material; and/or

wherein the plant material provided in step (a) comprises or consists or consists essentially of plant midribs or plant stems or plant stalks or a combination of two or more thereof.

8. Tobacco plant material obtained or obtainable by the method according to any of claims 1 to 7.

9. Use of tobacco plant material in which the amount of lignin therein has been reduced as compared to control tobacco plant material for manufacturing tobacco with reduced levels of matrix-bound NNK, wherein said levels of matrix-bound NNK are reduced as compared to the control.

10. A method for producing reconstituted tobacco comprising the steps of:

(a) performing the method according to any of claims 1 to 7;

(b) manufacturing the tobacco material obtained in step (a) into reconstituted tobacco; and

(c) optionally incorporating the reconstituted tobacco into a tobacco product.

11. Reconstituted tobacco obtained or obtainable by the method of claim 10.

12. A method for preparing tobacco for use as a tobacco cut filler comprising the steps of:

(a) performing the method according to any of claims 1 to 8; and

(b) rolling and cutting the tobacco material for use as a tobacco cut filler.

13. Cured tobacco plant material containing a reduced level of lignin as compared to control tobacco plant material in which the amount of lignin has not been reduced, and wherein the amount of matrix-bound NNK is about 3500 ng/g or less, preferably,

wherein the amount of free NNK is less than about 330 ng/g, optionally wherein the NNN content is less than about 1700 ng/g and optionally wherein the nicotine content is less than about 2610 µg/g; and/or

wherein the cured tobacco plant material comprises, consists of consists essentially of plant cortex – such as outer plant cortex; and/or

wherein vascular bundle or xylem or lignified sclerenchymatic tissue or a combination thereof is substantially absent from the cured plant tissue.

14. A tobacco product or a reconstituted tobacco product comprising, consisting or consisting essentially of the tobacco plant material according to claim 8 or the cured plant material according to claim 13.

15. A method for blending tobacco in which at least two different types of tobacco are blended so as to form a tobacco blend comprising the steps of:

(a) providing a first cured tobacco plant material and reducing the amount of lignin therein;

(b) measuring the total and/or matrix-bound NNK content of the first cured tobacco plant material and selecting cured tobacco plant material in which the total and/or matrix-bound NNK content is reduced as compared to the first cured tobacco plant material provided in step (a);

(c) providing a second cured tobacco plant material which has a higher total and/or matrix-bound NNK content than the total and/or matrix-bound NNK of the first cured tobacco plant material obtained in step (b), and optionally measuring the total and/or matrix-bound NNK content in the second cured tobacco plant material;

(d) blending together the first and second cured tobacco plant materials from steps (b) and (c) and optionally measuring the total and/or matrix-bound NNK content in the blended tobacco plant material; and

(e) obtaining a blended tobacco plant material in which the total and/or matrix-bound NNK content of the blended tobacco plant material is lower than the second cured tobacco plant material provided in step (c),

optionally wherein steps (a) and (b) are performed after step (c).

FIGURE 1

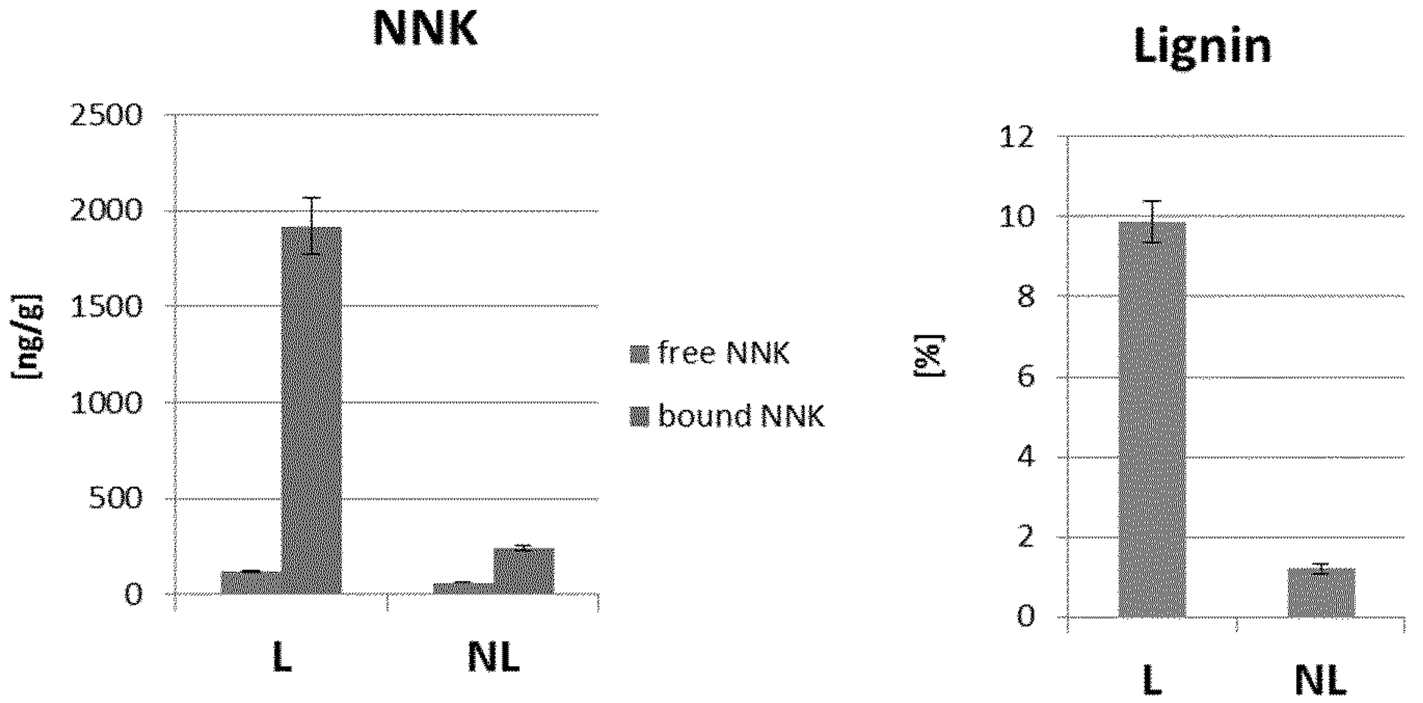

NNK
2500
2000
1500
1000
500
0
[ng/g]
free NNK
bound NNK
L
NL
Lignin
12
10
8
6
4
2
0
[%]
L
NL

FIGURE 2

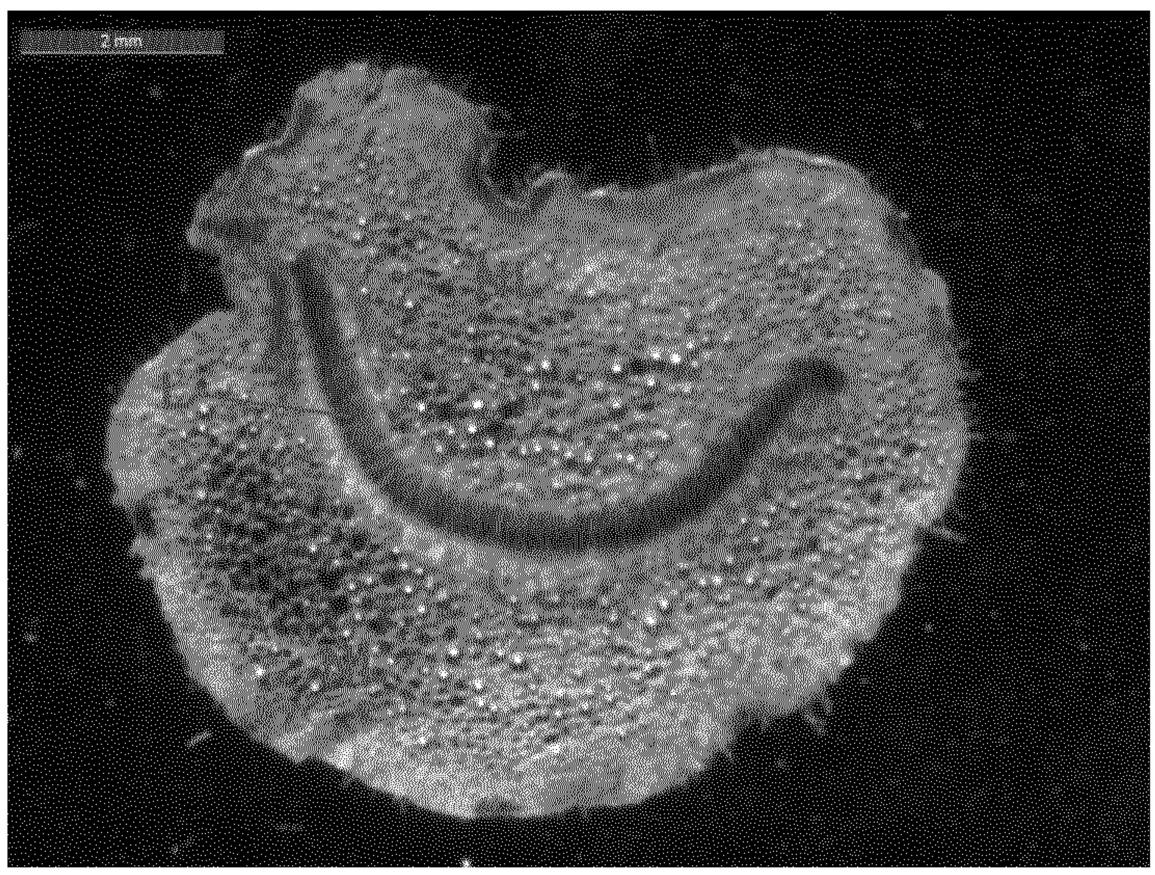


FIGURE 3

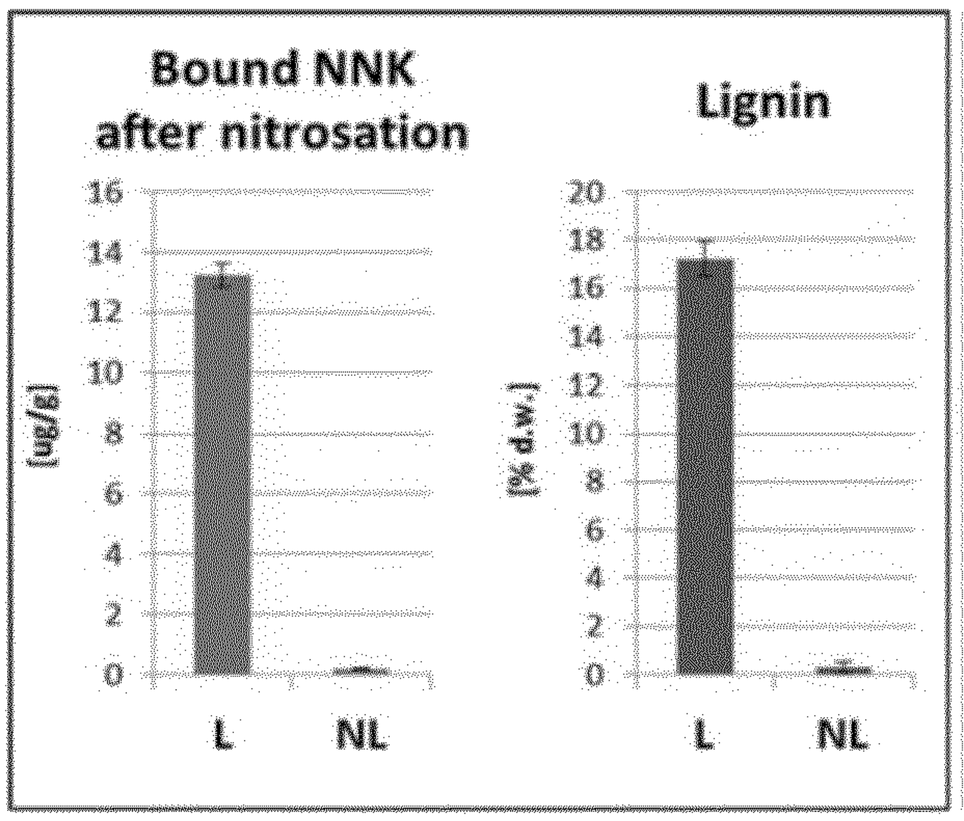
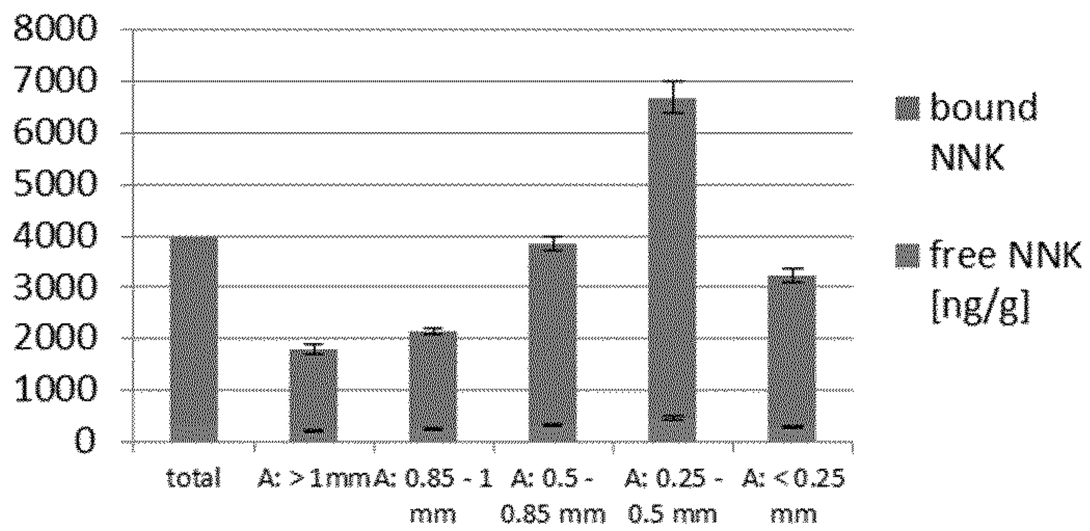

Bound NNK after nitrosation
[ng/g]
16
14
12
10
8
6
4
2
0
L
NL
Lignin
[% d.w.]
20
18
16
14
12
10
8
6
4
2
0
L
NL

FIGURE 4


8000
7000
6000
5000
4000
3000
2000
1000
0
total
A: > 1mm
A: 0.85 - 1 mm
A: 0.5 - 0.85 mm
A: 0.25 - 0.5 mm
A: < 0.25 mm
bound NNK
free NNK [ng/g]

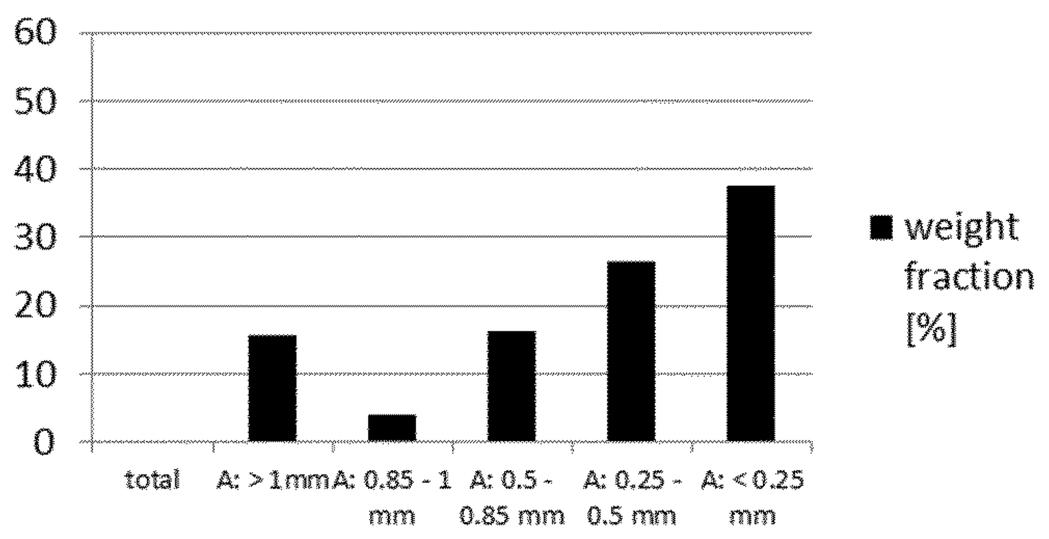

60
50
40
30
20
10
0
total
A: > 1mm
A: 0.85 - 1 mm
A: 0.5 - 0.85 mm
A: 0.25 - 0.5 mm
A: < 0.25 mm
weight fraction [%]

FIGURE 5

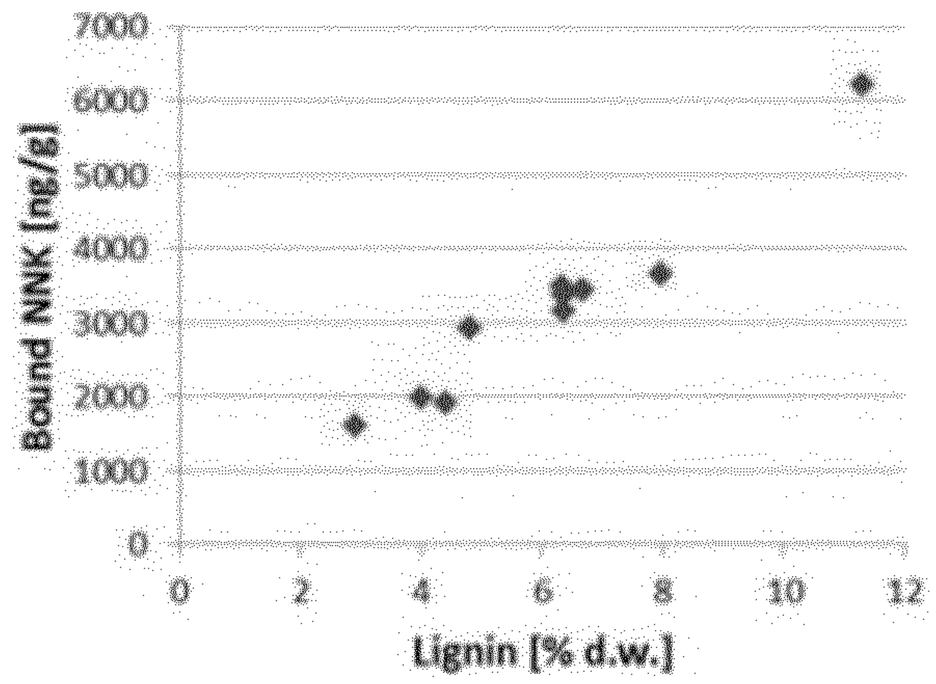

7000
6000
5000
4000
3000
2000
1000
0
Bound NNK [nB/g]
0
2
4
6
8
10
12
Lignin [% d.w.]

FIGURE 6

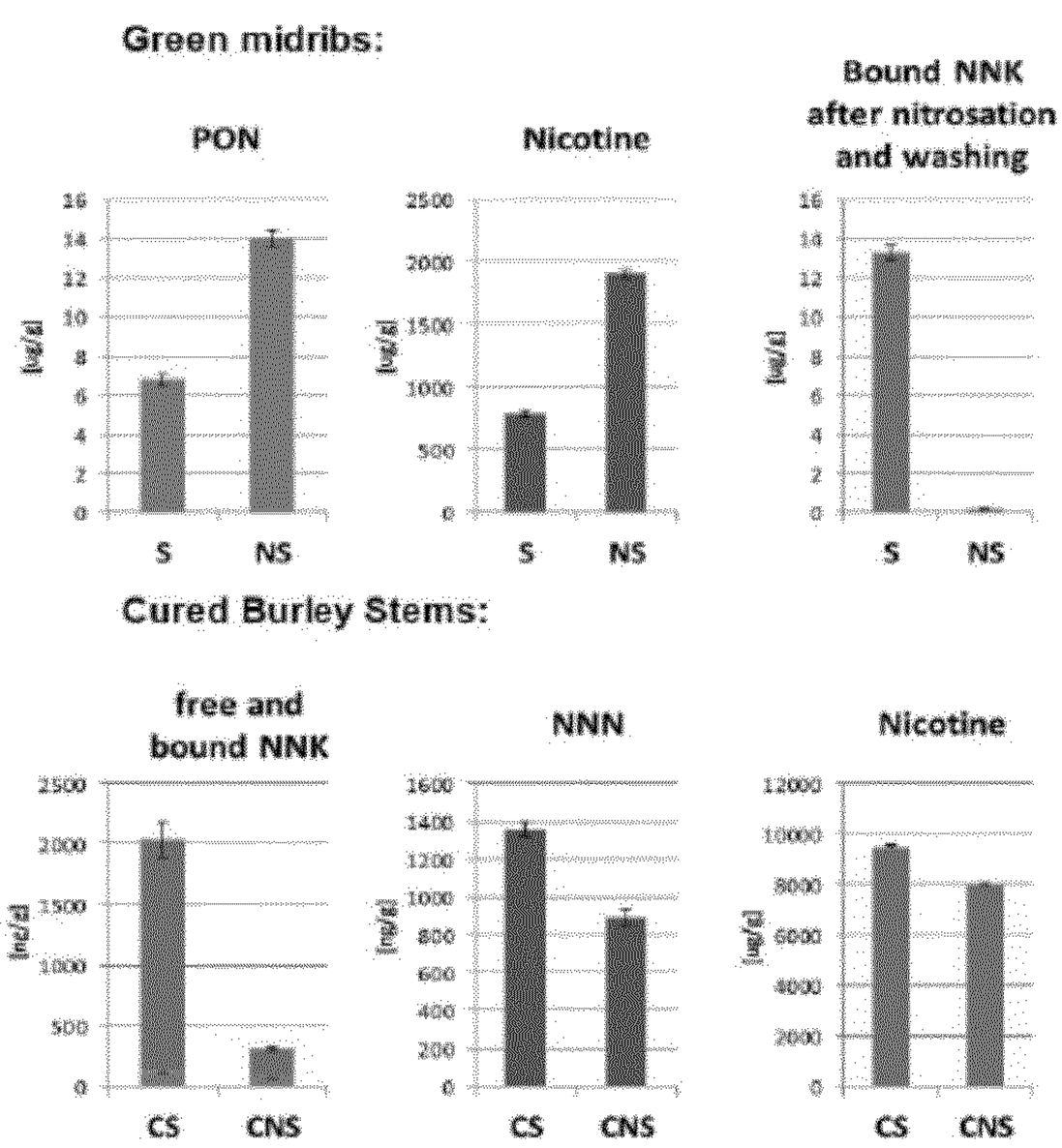
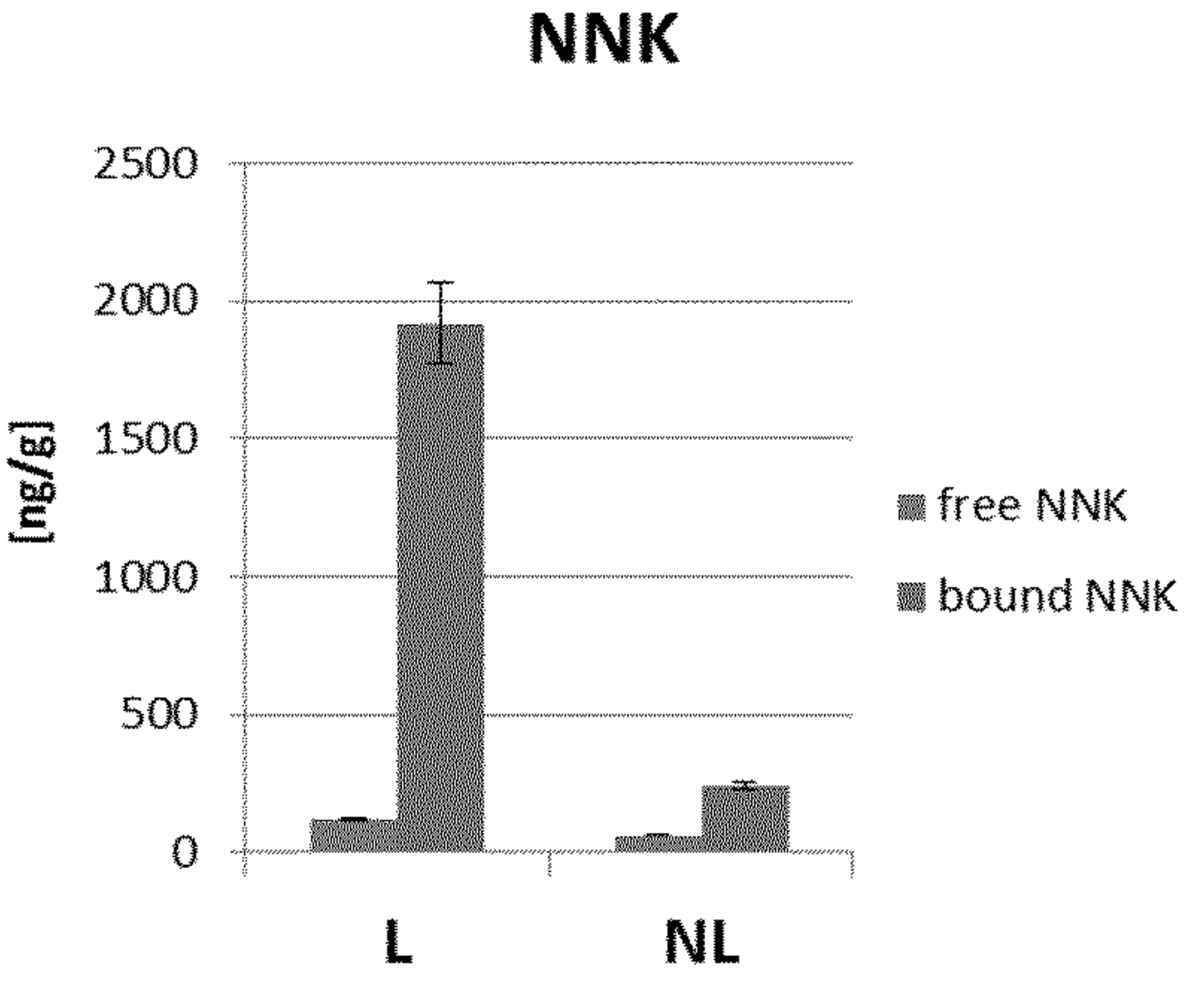
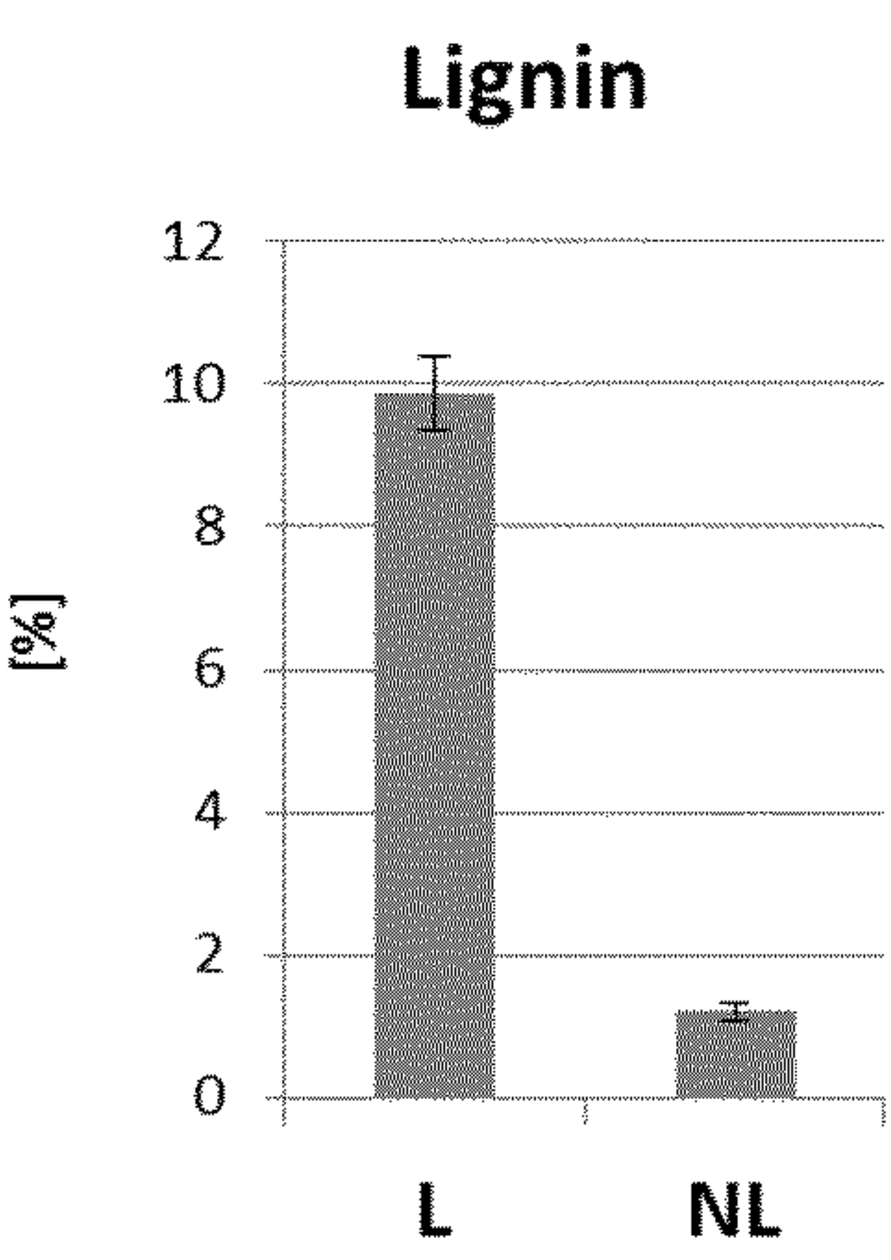

Green midribs:
PON
Nicotine
Bound NNK after nitrosation and washing
[µg/g]
S
NS
Cured Burley Stems:
free and bound NNK
NNN
Nicotine
CS
CNS

FIGURE 1


NNK
2500
2000
1500
1000
500
0
[ng/g]
free NNK
bound NNK
L
NL


Lignin
12
10
8
6
4
2
0
[%]
L
NL