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NEUTRAL VOLATILE COMPOUNDS IN ORIENTAL TOBACCO GROWN IN INDIA

C.V.N. SRIHARI*, K. SIVA RAJU AND C.V. NARASIMHA RAO

ICAR – Central Tobacco Research Institute, Rajahmundry 533105, India
*Ph.D.Scholar, Acharya Najarjuna University, Guntur

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Oriental tobacco also known as 'Turkish tobacco' or 'Aromatic tobacco' possesses typical aromatic smoking quality characterized by sweet and sour taste. The present study was taken up with the objective of assessing the status of neutral volatile compounds (NVCs) in Oriental tobacco grown in the Kurnool tract of Andhra Pradesh. Gas chromatograph-Mass spectrometry (GC-MS) analysis was carried out in the neutral fraction of steam volatiles and 89 NVCs were identified in the samples analysed. The NVCs were classified into eight groups *viz.*, norterpenoids (17%), norlabdanoids (2%), norcarotenoids (9%), cembranoids & thunberganoids (47%), neophytadiene (21%), Maillard reaction products (0.6%), phenylalanine related compounds (1.4%) and carbonyls, alcohols & esters (2%) thus accounting for ~99% of the NVCs identified. Norterpenoids, norlabdanoids and neophytadiene increased by 42, 100 and 31%, respectively in the samples from bottom to top positions on the stalk while cembranoids & thunberganoids decreased by 32%. There was no perceptible variation in the norcarotenoids among the leaf positions. The correlations observed were in tune with the increase of relative proportions in the case of norterpenoids, norlabdanoids and neophytadiene and decrease in the case of cembranoids & thunberganoids from bottom to top position of the plant. Neophytadiene, â-4,8,13-duvatriene-1,3-diol, thunbergol, solanone, epiglobulol, norsolanadione, isomers of megastigmatrienones, (1s,2E,4s,5R,7E,11E)-cembra-2,7,11-trien-4,5-diol, farnesyl acetone, â-damascone, hexahydrothunbergol, 8,13-epoxylabd-14-en-12-one, 8-epoxy-12-norambreinolide, (cis-A/B)-sclareoloxide and sclaral were identified as the important NVCs constituting the volatile profile of Oriental tobacco and their impact on the smoke flavour was documented. Identification of norlabdanoids viz., 8,13-epoxylabd-14-en-12-one, 8-epoxy-12-norambreinolide, (cis-A/B)-sclareoloxide, sclareolide and sclaral in the Oriental tobacco samples analysed in the present study is an important finding as these compounds are responsible for the typical cedar-amber notes of this tobacco.

Key Words: Oriental tobacco, Neutral volatile compounds, GC-MS

INTRODUCTION

A survey of the literature on aroma-bearing constituents reveals that the 300-odd volatile compounds, identified so far do not fall into a single class of compounds but represent a wide array of chemical entities like degradation products of thunberganoids, carotenoids and labdanoids, basic nitrogenous compounds, acidic substances, simple carbonyls and several types of neutral compounds. Wahlberg and Enzell (1987) reported that two major classes of diterpenoids *viz.*, monocyclic cembranoids and the bicyclic labdanoids are found in tobacco. Virginia and Burley tobaccos contain only the cembranoids, while Oriental and Cigar tobaccos contain both labdanoids and cembranoids. It is reported that about 41 compounds are found in substantial quantities to enable the smoker to perceive their impact on smoke flavour. The compounds are broadly classified as acids, alcohols, aldehydes, amides, anhydrides, esters, ethers, hydrocarbons, ketones and lactones. As the aroma-bearing constituents are basically volatile in nature, a micro-analytical technique like gas chromatograph-mass spectrometry (GC-MS) is generally employed as the individual component of the isolates is in minute quantity. Sakaki *et al.* (1986) observed that smoking quality of flue-cured tobacco can be evaluated by the relative abundance of the volatiles that are related to tobacco variety and stalk position.

Among the different tobacco types, Oriental tobacco also known as 'Turkish tobacco' or 'Aromatic tobacco' possesses typical aromatic smoking quality characterized by sweet and sour taste. The cheesy-sweety-buttery notes are attributed to the volatile acids *viz.*, â-methylvaleric, isovaleric and 2-methylbutyric acids, while the norlabdanoids *i.e.*, norambreinolide, dehydronorambreinolide, sclaral, ã-bicyclohomofarnesal and ambrox are reported/purported to be responsible for the cedar-amber notes. The best quality Oriental tobacco is produced in rocky, poor and infertile soils containing minimal amounts of nitrogen and organic matter (Gilchrist, 1999). Very small and bodied leaves are produced with low nicotine and high sugars.

In India, Oriental tobacco is naturally grown in limited area in the Kurnool district of Andhra Pradesh, without application of any of the inorganic fertilizers. Oriental tobacco is characterized by small size, aromatic, flavourful and readily combustible leaf with good filling capacity and used in blending with FCV tobacco in cigarette manufacture. Oriental tobacco leaves are harvested by priming 3-4 leaves at a time when the leaves mature and are air-cured. Air-curing is different from flue-curing where there is no control of temperature and moisture. Hence, formation of degradation derivatives in tobacco during air-curing is entirely different from flue-curing. As a part of comprehensive investigations on the chemical constituents responsible for smoke flavour in Indian tobacco, the present study was taken up with the objective of assessing the status of neutral volatile compounds (NVCs) in Oriental tobacco.

MATERIALS AND METHODS

The cured tobacco samples of Oriental tobacco (variety Izmir) were collected from the field experiments conducted in the Kurnool district of Andhra Pradesh during 2012 season. Leaf samples from different leaf positions on the stalk (bottom, middle and top) were collected separately from three locations as three replications in the experimental field grown with recommended package of practices. The mid-ribs were removed and dried in the hot air oven at 60 °C for 3 to 6 h, powdered and passed through 40 micron mesh. Analysis of nicotine and reducing sugars in the samples was undertaken by employing autoanalyezer (Harvey *et al.*, 1969).

Tobacco powder (10 g) and sodium sulphate (5 g) were taken in 500 ml distilling flask, 250 ml of phosphate buffer (pH 6.8) was added and distillate was collected into 500 ml volumetric flask containing 250 ml methylene chloride (dichlormethane) aqueous and organic layers were separated by using separating funnel organic layer was treated with 50 ml of in tartaric acid. Organic layer thus obtained was passed over anhydrous sodium sulphate. The final solution was made up to 1 ml for analysis (Wu *et al.*, 1992). The GC-MS analysis was carried out using a ZB-5 capillary column (length: 30 m, diameter: 0.25 mm, film thickness: 0.25 µm) fixed in a Shimadzu Model QP-2001 Plus GC-MS in Electron Ionisation (EI) mode.

The GC-MS analysis was carried out using a QP 2010 Plus GC-MS system equipped with AOC - 20i auto sampler (Single quadrupole, Shimadzu Corporation, Kyoto, Japan). A ZB-5 MS (5% Phenyl, 95% Dimethyl polysiloxane) (Zebron™ – Phenomenex, USA) capillary column of 30 m length, 0.25 mm internal diameter and 0.25 µm film thickness was used. The column oven temperature was programmed to rise from an initial temperature of 60°C (held for 1 min) to 140°C (held for 5 min) @ 6°C/min, from 140°C to 180°C (held for 5 min) @ 6°C/min and to a final temperature of 210°C @ 6°C/min, the final temperature was held for 14 min with a total run time of 50 minutes. Helium was used as the carrier gas with a flow rate of 1 ml/min. The inlet and interface temperatures were kept at 250°C. The Electron Ionisation (EI) source was operated at 200°C and the quadrupole temperature was 150 °C. All the samples were analysed in scan mode with a mass range of 50 to 500 units at a scan speed of 2500. One micro liter (µl) of the sample was injected in split-less mode by the auto-sampler. The obtained peaks were identified using US National Institute of Standards and Technology (NIST) standard mass spectral library database. As authentic standards of the compounds are not available for quantification, the area normalization method was adopted and the per cent of a particular compound in the total neutral volatile (NV) fraction was calculated.

RESULTS AND DISCUSSION

The NVCs were classified into eight groups viz., norterpenoids, norlabdanoids, norcarotenoids, cembranoids & thunberganoids, neophytadiene, Maillard Reaction Products, Phenylalanine related compounds and Carbonyls, Alcohols & Esters. The relative proportions of different groups and their composition was estimated. Based on the relative content of about 30 most important NVCs, volatile profile was formulated. Considering the mean values of all the samples, the results were discussed keeping in view 1) relative proportion of the seven groups of NVCs and the major compounds in each group, 2) volatile profile and 3) correlations among the groups.

In the present study, 89 NVCs were identified in the Oriental tobacco samples (original data not presented for brevity). The proportion of NVCs in the eight groups indicated above is as follows: norterpenoids (17%), norlabdanoids (2%), norcarotenoids (9%), cembranoids & thunberganoids (47%), neophytadiene (21%), Maillard Reaction Products (0.6%), Phenylalanine Related Compounds (1.4%) and Carbonyls, Alcohols & Esters (2%) thus accounting for 99% of the NVCs identified (Table1).

According to Fujimori *et al.* (1978), the medium-range boiling point fraction of Burley tobacco was composed of neophytadiene-related compounds: 46.2%, degradation products of carotenoids (megastigmatrienones, â- damascene etc.): 7.7%, degradation products of thunberganoids (solanone, duvatrienediol *etc.*): 3.6% and mono and sesquiterpenoids (solavetivone *etc.*): 4.7%. Huang *et al.* (2006) reported that 102 volatile compounds among 138 separated peaks were identified and quantified accounting for about 88.9% of the total content. Zhu *et al.* (2009) have identified and quantified 39 volatile components of the tobacco flavour samples accounting for 86.54% of the total content. In the FCV tobacco samples from NLS, KLS and SLS, the identified NVCs accounted for 83.4, 93.2 and 89.4% of the total fraction, respectively (Srihari *et al.*, 2013).

The relative proportion of norterpenoids increased with ascending leaf position on the stalk from bottom to top (14.44 to 20.80%). The increase (~42%) was more pronounced from middle to top positions. Nearly 100% increase was observed in the case of norlabdanoids from bottom (1.32%) to top (2.70%) positions. There was no perceptible variation in the norcarotenoids among the leaf positions (~8.50%). The proportion of cembranoids and thunberganoids was maximum (53.45%) in the leaf from bottom position and ~32% decrease was observed in the samples from top position (36.34%). Relative content of the principal NVC, neophytadiene increased by ~31% from the bottom position (18.16%) to the top position (23.86%). In all the above four groups of NVCs, only marginal differences were observed in the samples from bottom and middle positions. In respect of the other three groups of NVCs *viz.*, Maillard reaction products, phenylalanine related compounds and carbonyls, alcohols & esters, the differences among the plant positions were not significant (Table 1).

Highly significant and positive correlations were observed between norterpenoids and norlabdanoids (0.9767), neophytadiene (0.9768) while the correlation was negative with cembranoids & thunberganoids (-0.9996). Norlabdanoids had a negative correlation with cembranoids & thunberganoids (-0.9824) and a positive correlation with neophytadiene (1.000).

Table 1: Relative proportion (%) of important groups of NVCs, nicotine and reducing sugars in Oriental tobacco leaf from different plant positions

Group	Bottom*	Middle*	Top*	Overall mean
Norterpenoids	14.44	15.40	20.80	16.88
Norlabdanoids	1.32	1.81	2.70	1.95
Norcarotenoids	8.57	8.52	8.57	8.55
Cembranoids & Thunberganoids	53.45	50.37	36.34	46.72
Neophytadiene	18.16	20.18	23.86	20.73
Maillard Reaction Products	0.62	0.59	0.52	0.58
Phenylalanine Related Compounds	1.45	0.98	1.89	1.44
Carbonyls, Alcohols & Esters	1.99	2.14	1.26	1.80
Nicotine (%)	0.53	0.29	0.49	0.44
Reducing sugars (%)	11.69	18.63	11.10	13.81

*Mean of three replications

Neophytadiene had a negative correlation with cembranoids & thunberganoids (-0.9825). The correlations are in tune with the increase of relative proportions in the case of norterpenoids, norlabdanoids and neophytadiene and decrease in the case of cembranoids & thunberganoids from bottom to top position of the plant.

Davis (1976) reported that leaves from the upper stalk position contained greater quantities of neophytadiene and also its content was more in the flue-cured tobacco than air-cured tobacco. According to Grunwald *et al.* (1977) crude lipid, chlorophyll and carotenoids were higher in top leaves than in bottom leaves.

Based on the mean values of the relative proportion of the groups of NVCs, the major compounds in the respective groups are as follows: norterpenoids (epiglobulol, farnesyl acetone, hexahydrofarnesyl acetone and geranyl acetone); norlabadanoids (8,13-epoxylabd-14-en-12-one, 8-epoxy-12-norambreinolide, (cis-A/B)-sclareoloxide and sclaral); norcarotenoids (megastigmatrienone isomers, dihydroactinidiolide and â-damascone); cembranoids and thunberganoids (â-4,8,13-duvatriene-1,3-diol, thunbergol, solanone, norsolanadione and (1s,2E,4s,5R,7E,11E)-cembra-2,7,11-trien-4,5-diol); Maillard reaction products (methylethylmaleimide and indole); phenylalanine related compounds (phenethyl alcohol and phenylacetaldehyde) and carbonyls, alcohols & esters (palmitic aldehyde and 4-vinyl-2-methoxy-phenol). It is inferred from the results that both cembranoids and labdanoids are present in the samples analysed. Wahlberg and Enzell (1987) reported that two major classes of diterpenoids viz., monocyclic cembranoids and the bicyclic labdanoids are found in tobacco. Virginia and Burley tobaccos contain only the cembranoids, while Oriental and Cigar tobaccos contain both the labdanoids & cembranoids.

It is reported in the literature that the compounds responsible for the cedar-amber note of Oriental tobacco are derivatives of labdanoids. It is reported that *N. tomentosiformis*, one of the progenitors of *N. tabacum* produces diterpenoids of the labdane type (Reid, 1974). Schumacher patented norambreinolide (sclareolide) as an important constituent of Oriental tobacco and Kaneko (1971) isolated the compound from cigar tobacco leaf. Giles and Schumacher (1961) have reported the first C_{20} labdanoids in tobacco *i.e.* á- and â-levantenolides. These compounds were identified as the potential precursors of the five C_{16} norlabdanoids (norambreinolide, dehydronorambreinolide, ã-bicyclohomofarnesal, sclaral and ambrox) which contribute to the cedary odour of the Oriental tobacco (Schumacher and Vestal, 1974).

Taking into consideration the mean values of relative content of 28 NVCs in the samples, which accounted for ~93% of NVCs identified, the volatile profile of Oriental tobacco grown in the Kurnool tract of Andhra Pradesh was established (Table 2). It can be inferred that neophytadiene, â-4,8,13-duvatriene-1,3-diol, thunbergol, solanone, epiglobulol, norsolanadione, isomers of megastigmatrienones, (1s,2E,4s,5R,7E,11E)-cembra-2,7,11-trien-4,5-diol, farnesyl acetone, â-damascone, hexahydrothunbergol, 8,13-epoxylabd-14-en-12-one, 8-epoxy-12-norambreinolide, (cis-A/B)-sclareoloxide and sclaral are the important NVCs responsible for the smoke flavour of Oriental tobacco. The other minor compounds detected are listed in Table 3.

The important quality determinants of tobacco are leaf aroma and smoke flavour which refer to the sensory impressions perceived by the nasal passages. These distinct impressions are caused by the volatile constituents. Many biochemical and chemical degradation reactions occur in the leaf during post-harvest processing (curing, fermentation, ageing *etc.*) of tobacco ultimately generating the typical aroma. Many of the green leaf constituents undergo enzymatic, microbial, photochemical and oxidative reactions, the relative importance of which is dependent on the type of curing (flue-curing, air-curing or sun-curing) contributing to typical tobacco aroma.

Impact of some NVCs on the smoking quality has been reported in the literature (Green, 1977; Leffingwell *et al.*, 1972; Demole and Dietrich, 1977): neophytadiene (soothing, smoothing); isomers of megastigmatrienone (spicy, peppery, add body); solanone (smooth, ketonic); geranyl acetone (green, adds body); dihydroactinidiolide (slight cooling); phenylethyl alcohol (floral, rose), phenylacetaldehyde (intense floral); benzyl alcohol (weak, floral, smoothing), indole (smoothing, floral), duvatrienediol [oxidation products (solanone, oxysolanone, branched chain volatile acids) contribute to smoke flavour]; â-damascone (floral, adds body) and farnesyl acetone (sweet, green, flue-cured note).

Kimland *et al*. (1972) have studied the volatile fraction of the neutral oxygen-containing constituents of sun-cured Greek tobacco and identified 32 compounds (mainly ketones and aldehydes) using GC-MS. The important compounds identified were: â-cyclocitral, benzaldehyde, safranal, 6-methyl-3,5-heptadien-2-one, carvone, 2-acetylpyrrole, hexahydrofarnesyl acetone, phenylethylacetate, dibutylphthalate, benzyl alcohol, phenylethanol,damascenone, farnesyl acetone, 6-methyl-2-heptanone, solanone, geranyl acetone, 6-methyl-5-hepten-2-one and dihydroactinidiolide.

GC-MS analysis of volatile neutral fraction has focused attention on 20 compounds, which had the greatest chance of being transferred from tobacco to smoke and have a positive relation with smoke flavour and also correlate well with the sensory evaluation (Wu *et al*., 1992). The ionones, megastigmatrienones, damascones and damascenones are considered to be the most

Table 2: Relative content (%) of important NVCs in Oriental tobacco leaf from different plant positions contributing to the volatile profile

Rt (min)	Compound	Bottom*	Middle*	Top*	Mean
8.12	Benzyl alcohol	0.40	0.27	0.71	0.46
8.38	Phenylacetaldehyde	0.54	0.30	0.47	0.44
10.07	Phenethyl alcohol	0.48	0.41	0.70	0.53
12.92	Methylethylmaleimide	0.30	0.25	0.28	0.28
14.50	Indole	0.21	0.10	0.11	0.14
15.01	4-Vinyl-2-methoxy phenol	0.29	0.29	0.29	0.29
16.46	Solanone	4.59	4.10	3.79	4.16
19.58	Geranyl acetone	0.87	0.88	0.92	0.89
20.95	Norsolanadione	3.55	4.06	3.53	3.71
22.58	Dihydroactinidiolide	1.21	1.09	1.30	1.20
23.53	Megastigmatrienone isomers	3.53	2.73	5.09	3.78
25.67	(1s,2E,4s,5R,7E,11E)-Cembra-2,7,11-trien-4,5-diol	3.70	2.15	4.85	3.57
25.86	Tetrahydroionone	ND	1.37	0.71	1.04
26.73	Hexahydrothunbergol	0.67	1.07	0.93	0.89
27.72	Palmitic aldehyde	0.86	1.33	0.52	0.90
32.28	Neophytadiene	18.16	20.18	23.86	20.73
32.39	Hexahydrofarnesyl acetone	1.80	1.49	1.46	1.59
33.89	(cis-A/B)-Sclareoloxide	0.98	1.33	0.98	1.10
34.72	Farnesyl acetone	2.29	2.08	1.47	1.95
35.49	Isophytol	0.24	0.35	0.29	0.29
35.66	Sclaral (Sclareolide lactol)	0.70	1.03	0.76	0.83
37.56	8-Epoxy-12-norambreinolide	0.89	0.90	0.98	0.92
38.65	Thunbergol	19.89	18.75	3.26	13.97
42.17	Epiglobulol	3.12	2.23	4.58	3.31
44.42	Globulol	1.79	3.01	2.65	2.48
45.66	8,13-Epoxylabd-14-en-12-one	ND	1.02	1.28	1.15
46.13	Duvatriendiol	21.64	20.24	19.98	20.62
46.31	â-Damascone	1.76	1.74	1.21	1.57

*Mean of three replications; ND: Not detected important carotenoid derivatives found in cigarette smoke. The ionones and damascenones are primary aromatic compounds found in rose oil; therefore, these compounds add floral and woody-like notes to the aroma and taste of cigarette smoke (Roberts, 1988).

The predominant duvane found in tobacco is 4, 8, 13-duvatriene-1,3-diol (DVTol), which accounts for ~50% of the lipids in immature tobacco, produces degradation products by oxidation during senescence and curing. The degradation products like solanone, oxysolanone and branched chain volatile acids are reported to contribute to Burley tobacco flavour. Aasen *et al.* (1975) reported that the volatile degradation products of thunberganoids viz., á- and â-4,8,13-duvatriene-1-3-diols, solanone, norsolanadione etc. are organoleptically important. Neophytadiene has been suggested to be a tobacco flavour

Table 3: Minor NVCs identified in Oriental tobacco samples

Group/Compound	Group/Compound
Norterpenoids	**Norcarotenoids**
Dehydroaromadendrene	Dihydro-â-ionone
7,8-Dihydrolinalool	(E,Z-Pseudoionone)
Limonene dioxide 4	3-Oxo-á-ionol
Limonene dioxide 1	3-Oxo-7,8-dihydro-á-ionol
Longiborneol	á-Ionone
trans-Farnesol	Carotol
3,7,11,16-Tetramethyl-hexadeca-2,6,10,14-tetraen-1-ol	Dihydrooxophorone
Nootkatone	Â-Ionone
(Z,E)-Farnesal	Dihydro-á-Ionone
DL-6,7-Dihydro-2,cis-farnesol	Vitamin A Alcohol
Spathulenol	Â-iso Methyl ionone
Widdrol	4-(2,6,6-Trimethyl-2-cyclohexen-1-yl)- 2-butanone
17-Acetoxy-19-kauranal	Tetrahydroactinidiolide
d-Nerolidol	Â-iso Methyl ionone
Globulol	**Maillard Reaction Products**
Â-Cyclo homogeraniol	Furfural
(E)- Citral	Methylvinylmaleimide
8S,14-Cedran-diol	Benzaldehyde
(E)- Geranial	2-Acetylpyrrole
(E)-5-Isopropyl-8-hydroxy-8-methyl-non-6-en-2-one	**Carbonyls, Alcohols & Esters**
Isospathulenol	6-Methyl-5-hepten-2-one
Bicycloelemene	2-trans-6-cis-Nonadienal
3,7,11,16-Tetramethyl-hexadeca-2,6,10,14-tetraen-1-ol	trans-2-Nonenal
Nerolidol-epoxyacetate	4-Vinyl-2-methoxy-phenol
2,3-Epoxy-geranyl acetate	Diethyl phthalate
Citronellyl acetate	Myristaldehyde
Isocaucalol	Isobutyl phthalate
Caryophyllene oxide	Methyl hexadecanoate
Norlabdanoids	Myristaldehyde
Sclereodiol	Ethyl linoleolate
(11e,13z)-Labdadien-8-ol	Ethyl phthalate
13(16),14 Labdien-8-ol	Hendecanal

enhancer and is considered as a flavour carrier by entrapping volatiles in the tobacco smoke aerosol (Leffingwell and Leffingwell, 1988).

It is important to mention that norlabdanoids viz., 8,13-epoxylabd-14-en-12-one, 8-epoxy-12-norambreinolide, (cis-A/B)-sclareoloxide, sclareolide and sclaral were identified in the Oriental tobacco samples analysed in the present study. Even though Oriental tobacco contains many of the volatile compounds present in flue-cured and air-cured (Burley) tobaccos, majority of them are responsible only for the background flavour notes. The predominant flavour characteristics are attributed to the flavour compounds which are derivatives of labdanoids (e.g. norambreinolide, sclaral) possessing the cedar-amber notes.

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