

HPLC Analysis of *Nicotiana rustica* L. and Chewing Tobacco (Maraş Powder) Alkaloids

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Summary : A High Pressure Liquid Chromatographic Technique was used to quantitate the nicotine, nornicotine and anabasine levels in *Nicotiana rustica* L. leaves cultivated in Kahramanmaraş and Ankara, and in chewing tobacco (Maraş Powder) prepared from *Nicotiana rustica* L. The procedure involved the extraction of the milled tissue followed by "separation and quantitation" of the alkaloids on a reversed phase C₁₈ column with a mobile phase of 40 % methanol containing 0.2 % phosphoric acid buffered to pH 7.25 with triethylamine. It was found that the leaves of *Nicotiana rustica* L. cultivated in Southeast Anatolia and Ankara and chewing tobacco contained nicotine 7.7069, 2.1221, 1.1705 %, nornicotine 0.1112, 0.0705, 0.0401 %, and anabasine 0.1538, 0.0341, 0.0579 % respectively.

Key words: *Nicotiana rustica* L., Tobacco Alkaloids, RP-HPLC Analysis,

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Nicotiana rustica L. ve Hazırlanan Maraş Otu Alkaloidlerinin YBSK ile Analizi

Özet : Kahramanmaraş ve Ankara'da kültürü yapılan *Nicotiana rustica* L. yapraklarındaki ve *Nicotiana rustica* L.'dan hazırlanan çiğneme tütünündeki (Maraş Otu) nikotin, nornikotin ve anabazin alkaloidlerinin kantitatif analizi YBSK kullanılarak yapılmıştır. Toz edilmiş yaprakların ekstraksiyonunu takiben alkaloidlerin ayrımı ters-faz C₁₈ kolonunda Trietilaminle pH=7.25'e tamponlanmış % 0.2 fosforik asit içeren % 40'lık Metanol kullanılarak analiz gerçekleştirilmiştir. Güneydoğu Anadolu'da, Ankara'da kültürü yapılan *Nicotiana rustica* L. yapraklarının ve Maraş otunun sırasıyla % 7.7069, 2.1221, 1.1705 nikotin, 0.1112, 0.0705, 0.0401 nornikotin, % 0.1538, 0.0341, 0.0579 anabazin içerdiği bulunmuştur.

Anahtar kelimeler: *Nicotiana rustica* L., Tütün Alkaloidleri, TF-YBSK Analizi

INTRODUCTION

Three species of tobacco plant are found in Anatolia; among these *Nicotiana tabacum* L. is widely cultivated, *Nicotiana glauca* Graham is naturalized and *Nicotiana rustica* L. has a very limited cultivation in Southeast Anatolia around Gaziantep and Maraş where it is called "Delitütün" or "Hasankeyf"¹. The leaves of *Nicotiana rustica* L. are commonly used to prepare chewing tobacco (Maraş Powder). Sun dried *Nicotiana rustica* L. leaf powder and oak ash are mixed in 1:2 or 1:3 proportions. To this mix-

ture, water is sprinkled for humidification. This mixture is kneaded until it is a greenish powder. This chewing tobacco is used by placing a pinch of the powder between teeth and lower lip for relaxing and giving up smoking cigarettes. For this reason, people who are used to chewing tobacco powder have dropped and bruised lips. Habituation occurs approximately after one month's usage. During our questionnaire studies, addicts informed that itching on the gingiva, irritability, uneasiness, and aggressive behaviour are observed when they stop using it². In another study; analgesic, antiaggressive,

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exploratory and locomotor effects of total alkaloid extract of *Nicotiana rustica* L. were compared with those of nicotine. No difference was observed between the effects of Maraş powder and buccal nicotine use³.

Epidemiological and screening investigations have also been made on Maraş powder users. We have evaluated cytological smears obtained from lower lip mucosa lesions in eighty Maraş powder users and punch biopsies were performed. Oral cancer among Maraş powder users appeared to be related to the length of use as it was only observed in subjects with 15 or more years of exposure⁴.

It is known that *Nicotiana rustica* L. leaves contain higher amounts of nicotine than *Nicotina tabacum* L.^{2,5} However, there have been no reports on the quantitative determination of tobacco alkaloids in Turkish *Nicotiana rustica* L. and Maraş Powder.

Many GC methods have been published for determining alkaloids in tobacco; which although sensitive, do not allow rapid analysis of alkaloids; temperature programming being required or replicate analysis of samples at a higher temperature. High pressure reversed phase liquid chromatography is an effective and dependable method for the separation of tobacco alkaloids⁶.

In our research, we aimed to determine the amount of alkaloids in cultivated *Nicotina rustica* L. leaves and in prepared chewing tobacco (Maraş Powder). The amount of nicotine, nornicotine and anabasine were determined in *Nicotina rustica* L. samples. In recent years, there appears to be more interest in Maraş Powder because of its intensive use in South-east Anatolia for giving up smoking.

EXPERIMENTAL

Apparatus

The HPLC system consisted of a Waters (Milford, MA, USA) model U6 K injector, a M-45 solvent pump and a model 440 detector equipped with a 254 nm filter.

The tobacco alkaloids were quantitatively separated on a Waters Novapak C₁₈ reversed - phase column

10 µ (15 cm x 3.9 mm) eluted with an isocratic mobile phase of 40 % (v/v) methanol containing 0.2 % (v/v) phosphoric acid buffered to pH 7.25 with triethylamine⁷. Nicotine was determined at a flow rate of 0.3 ml/min in all samples. Nornicotine and anabasine were determined at a flow rate of 0.2 ml/min in *Nicotiana rustica* L. samples and Maraş Powder.

All solvents and samples were filtered through a 0.45 µm. Millipore filter prior to use. Nicotine, nornicotine and anabasine were quantitated by a Waters 745 B Data module which automatically integrated peak areas and compared them with those of authentic standards.

Chemicals

(s)-(-) Nicotine was obtained from Aldrich Chemical Company (Milwaukee WIS 53233 USA), while (±) Nornicotine and (±) Anabasine were obtained from Sigma Chemical Company (ST. Louis, MO 63178 USA).

Chromatographic solvents were HPLC grade from Merck Chemical Company (D-6100 Darmstadt, F.R. Germany).

Material

Samples subjected to alkaloid analysis were as follows: *Nicotina rustica* L. leaves cultivated in Kahramanmaraş and cultivated in our experimental garden in Ankara. Three chewing tobacco (Maraş Powder) samples were supplied in commercial packets of Kahramanmaraş bazaars.

Nicotine analysis

The milled samples were weighed into 0.5000 g lots and extracted with 10 ml of 25 mM sodium phosphate buffer (pH 7.8) at 30°C for 24 h with constant agitation. The aqueous extract was filtered under reduced pressure through a Whatman No. 2 filter paper and diluted ten fold with water. Each extract was filtered through a 0.45 µm Millipore filter and 1 µl samples were injected⁷.

Nornicotine and anabasine analysis

The milled samples were weighed into 5.0000 g lots

and extracted twice with 150 ml of 1N HCl for 24 h maceration and 2 h constant agitation; acidic aqueous extracts were filtered and combined. The combined acidic extract was basified with 6 N NaOH to pH 11 and extracted three times with 150 ml dichloromethane. The dichloromethane layer was removed and gently evaporated to dryness under nitrogen⁸. The crude alkaloid fraction was dissolved in 10 ml methanol and 1 µl samples were injected onto the column.

Quantification

An external standard method based on peak area was used for quantitative determinations. The calibration curves were prepared by analysing five dilutions (n=5) of authentic samples of nicotine (0.4 - 0.05 mg/ml), nornicotine (0.5 - 0.025 mg/ml) and anabasine (0.5 - 0.025 mg/ml). The linearity of nicotine, nornicotine and anabasine were confirmed by regression analysis. The correlation coefficients were found to be $r^2=0.9968$ for nicotine, $r^2=0.9990$ for nornicotine and $r^2=0.9999$ for anabasine. Results are expressed as the mean of three determinations.

RESULTS AND DISCUSSION

This report describes a RP-HPLC analysis of alkaloids applied to *Nicotiana rustica* L. of Turkish origin (Table 1).

Table 1. Alkaloid Content of *Nicotina rustica* L. samples from Turkey

| Sample | % Nicotine ± S.D. | % Nornicotine ± S.D. | % Anabasine ± S.D. |
|--|-------------------|----------------------|--------------------|
| Leaves of <i>Nicotina rustica</i> L. cultivated in Kahramanmaraş | 7.7069 ± 0.1090 | 0.1112 ± 0.0063 | 0.1538 ± 0.0074 |
| Leaves of <i>Nicotina rustica</i> L. cultivated in Ankara | 2.1221 ± 0.1039 | 0.0705 ± 0.0043 | 0.0341 ± 0.0070 |
| Chewing Tobacco (Maraş Powder) | 1.1705 ± 0.0337 | 0.0401 ± 0.0042 | 0.0579 ± 0.0134 |

Each value is the average of three runs ± Standard Deviation (S.D.)

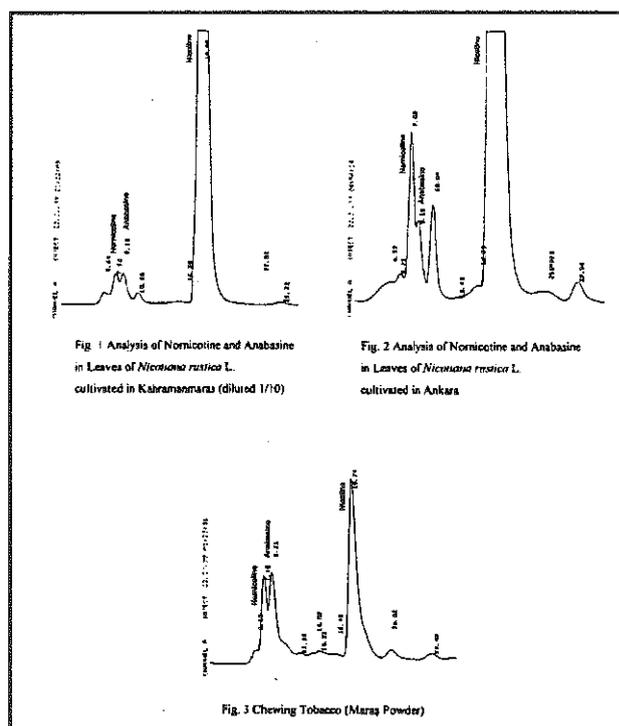
The extraction method of Saunders and Blume was used to determine nicotine, but this method was not sufficient for minor alkaloids. Different extraction methods were used for nornicotine and anabasine

analysis. The extraction method is given in the experimental section.

It is seen from our findings that leaves of *Nicotiana rustica* L. cultivated in Southeast Anatolia contain nicotine 7.7069 %, nornicotine 0.1112 % and anabasine 0.1538 % (fig.1).

However nicotine, nornicotine and anabasine contents may vary due to the effect of climate and soil conditions. Thus, leaf samples from cultivated plants in our experimental garden in Ankara are found to contain low amounts of nicotine 2.1221 %, nornicotine 0.0705 % and anabasine 0.0341 % (fig.2) during the flowering stage. This decrease may be due to various environmental factors such as sunlight, soil, climate, fertilization and others⁹⁻¹¹. Similar results were observed previously for *Nicotiana rustica* L. where the nicotine content was found to decrease from 6.6 - 8.8 % in greenhouse plants, to 1.54 - 2.64 % in field plants¹².

On the other hand chewing tobacco (Maraş Powder) prepared from *Nicotiana rustica* L. leaves was found to contain low amounts of nicotine 1.1705 %, nornicotine % 0.0401 and anabasine 0.0579 % (fig.3) when compared with the leaves. This decrease depends partially on the mixture of dried leaf powder with oak ash in various unknown proportions (1:3



or 1:2) and partially on the preparation technique, where nicotine may be lost during humidification after mixing with the ash; nicotine presumably evaporates partly in a mild alkaline medium.

These findings are in accordance with the reports on nicotine content in *Nicotiana rustica* L. leaves 0.747 %⁸, 1.54 - 2.64 %¹², 3.60 - 5.83 %¹³, 4.52 - 8.61 %¹⁴ and 7 - 8 %⁵. The nor nicotine level was reported to be 0.0069 %⁸, 0.18 - 0.48 %¹⁴ and 0.51 - 0.89 %¹³, and anabasine level was reported to be 0.0085 %⁸ in a single study.

Analgesic, antiaggressive, exploratory and locomotor effects of total alkaloid extract of *Nicotiana rustica* L. and carcinoma of buccal mucosa in Maraş Powder users have been discussed in detail in our previous reports^{3,4}.

The carcinogenic effects of the various types of smokeless tobacco have been attributed to the presence of N-nitrosornicotine (NNN). NNN is produced in tobacco by bacterial or enzymatic activity and it has been reported to cause oral and pharyngeal cancer experimentally^{15,16}. Our research will continue on NNN content of *N. rustica* L. leaves and Maraş Powder.

It is clear that using Maraş Powder is not a safe substitute for smoking cigarettes. It can cause cancer and a number of non-cancerous oral conditions and also can lead to nicotine addiction and dependence.

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