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Identification and quantification of methyl nicotinate in rice (*Oryza sativa* L.) by gas chromatography–mass spectrometry ☆

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Abstract

During the course of analysis of popular rice varieties cultivated in India towards identification of their aroma compounds, methyl nicotinate (MN), a medicinal and flavour additive compound, was identified for the first time in rice samples. A simple direct solvent extraction method using 300 mg of the sample is developed to extract MN in rice samples and detected by capillary gas chromatography–mass spectrometry analysis. Quantitative analysis of MN is performed for polished rice, brown rice and rice bran samples from five rice varieties that are widely produced in India by using GC–MS operating under SIM mode (*m/z* 106). The quantity of MN is in the range of 0.63–1.30 μg/g, 1.37–3.99 μg/g and 1.87–12.04 μg/g for polished rice, brown rice and rice bran samples, respectively. Breeding programmes for rice with high concentrations of MN can be greatly facilitated by establishing the concentrations of the MN in new cultivars. This method is more economic with less time consumption and enables fast screening of a large number of samples.

Keywords: Methyl nicotinate; Rice; *Oryza sativa* L.; Solvent extraction; Gas chromatography–mass spectrometry; Flavoring agent; Medicinal compound

1. Introduction

Rice (*Oryza sativa* L.) is the most important food crop of the world and has a great influence on human nutrition and food security. Rice contains a number of bioactive compounds and ingredients that can be used in medicinal formulations. The medicinal values of rice have been described in ancient Indian books and rice is considered to be ocrid, oleaginous, tonic, aphrodisiac, fattening, diuretic and useful in biliousness (Caius, 1986). Rice bran is an important source of minerals, amino acids, proteins, carbohydrates, lipids, tocopherols, anthocyanins and tocotrienols (Escribano-Bailon, Santos-Buelga, & Rivas-Gonzalo, 2004; Jariwalla, 2001; Orthoefer, 1996; Qureshi, Mo, Packer, & Peterson, 2000; Qureshi, Qureshi, & Wright, 1991; Saunders, 1985). More than 100 compounds have been identified in the aroma of rice, among them 2-acetyl-1-pyrroline was identified as the principal aroma compound (Buttery, Ling, Juliano, & Turnbaugh, 1983; Tanchotikul & Hsieh, 1991; Tsugita, 1986; Widjaja, Craske, & Wootton, 1996). 2-Acetylpyridine was also established, for the first time, as the characteristic aroma compound of Xiangjing-8618 rice (a scented rice, *Oryza sativa* L.) (Jianming, 2002). Among the compounds that have high medicinal values, Oryzanol has been extensively studied (Fang, Yu, & Badger, 2003; Juliano, Cossu, Alamanni, & Piu, 2005; Miller, Frenzel, Schmarr, & Engel, 2003; Xu & Godber, 1999).

During the analysis of rice samples for aroma and bioactive compounds in rice, we observed the presence of

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methyl nicotinate (MN) for the first time. It is an active ingredient in a number of medicinal products as a rubifacient and vasodilator for the treatment of a variety of diseases such as respiratory, vascular and rheumatoid disorders (Koivukangas, Oikarinen, Salmela, & Lahti, 2000; Wilkin et al., 1985). MN is a non-immunological compound and it helps in removing wrinkles, rejuvenates the skin (Caselli et al., 2003), induces local skin vasodilation (http://www.eudra.org//emea.html), and dilates the blood vessels for quicker, better absorption and it gives a warming sensation. It has been widely used in cosmetics as an ingredient in skin products like soaps, shampoo and creams due to its rubefacient nature (Riviere, Qiao, Baynes, Brooks, & Mumtaz, 2001). *Aenictus* species utilizes MN in its chemical communication system (Oldham, Morgan, Gobin, & Billen, 1994). MN is also an active flavour compound in fruits like Cupuacu (*Theobroma grandiflora*) (Franco & Janzantti, 2005; Pelissier et al., 1994), Soursop (*Annona muricata*) (Wong & Khoo, 1993), Mammee apple (*Mammea americana*) (Morales & Duque, 2002), Strawberries (*Fragraria Vesca*) (Pyysalo, Honkanen, & Hirvi, 1979), Papaya, Guava, headspace of the living orchids Calanthe izu-insularis Ohwi et Satomi (Orcidaceae), *Calanthe sieboldii* Decne (Orcidaceae) and Tuberose and flowers like calanthe (Orchidaceae) (Awano, Ichikawa, Tokuda, & Kuraoka, 1997). In the 63rd meeting of the Joint FAO/WHO experts committee on food additives (JECFA), MN has been accepted as a flavouring agent and there is no safety concern for intake (FAO/WHO Expert Committee on Food & Additives, 2004).

In the present work, we report the presence of MN, a medicinal and flavour additive compound, for the first time in the rice samples. MN was extracted by using direct solvent extraction technique from the polished rice, brown rice and rice bran using less amount of the sample, and its quantity has been estimated by GC–MS in selected ion monitoring (SIM) mode.

2. Materials and methods

2.1. Rice samples

Five rice varieties viz., Taroari basmati, Vasumathi, Yamini, Krishna hamsa, IR-64, which are popular rice varieties in India, were grown during kharif 2004 at Directorate of Rice Research, Hyderabad, India. The rice grains were stored at 4 °C in sealed polypropylene bags prior to analysis.

2.2. Chemicals

Methanol, acetone, acetonitrile, hexane, isopropyl alcohol, toluene, ethanol, tetrahydrofuran and dichloromethane used in this work are of HPLC grade and were procured from Merck (Mumbai, India). Methyl nicotinate (99%) was purchased from Sigma–Aldrich (Steinheim, Germany).

2.3. Equipments

Ultrasonic bath Sonarex super 10P (Bandelin, Germany) was used for the extraction of MN from rice and rice bran samples. The generator of the ultrasonic bath has an output of 150 W and a frequency of 35 kHz. Standards and samples were weighed on BP1215 Sartorius analytical MIC balance (Sartorius, Germany).

2.4. Preparation of standard MN solutions

A 1000 mg/L standard solution of MN was prepared in methanol by dissolving 10 mg of MN in 10 mL of methanol. This solution was diluted with methanol to obtain the necessary concentrations (10 μg/mL to 100 ng/mL) to draw a calibration curve for quantification of MN in the samples.

2.5. Sample preparation

The rice samples collected from five varieties were hulled by a Class 35A rice machine (Satake, Japan) to get brown rice samples. The brown rice samples were milled for 90 s using a Class 05 grain-testing mill (Satake, Japan) to obtain polished rice samples and rice bran samples. Both brown rice and polished rice samples were grounded using disc grinder (Swantech International, France) with a 0.5 mm mesh screen prior to solvent extraction.

2.6. Solvent extraction

Solvent extraction was carried out initially by taking 10 g of brown rice sample and extracted with 10 mL of solvent (methanol). The mixture was sonicated for 60 min at room temperature and filtered through 0.45 μm membrane filter. The filtrate was concentrated by using a gentle stream of nitrogen at room temperature. In order to reduce total weight of sample, the method was later optimized by decreasing the amount of rice sample. Finally the method was optimized with 300 mg of rice sample and 500 μL of methanol for extraction. Powdered (polished and brown rice)/bran samples (300 mg) were transferred into 2 mL crimp cap vials (12 × 32 mm) and 500 μL methanol was added to each vial. The vials were crimp sealed tightly by using PTFE septa and aluminum seals. The vials were placed in the ultrasonic water bath and sonicated at various temperatures (30, 40, 50, 55, 60, 70 and 80 °C) for different time periods (30–150 min) to extract maximum MN. The optimized extraction temperature and time is 50 °C for 120 min. The vials were removed from the sonicator and kept at 4 °C for 10 min. The cooled vials were centrifuged at 5000*g* rpm for 20 min and 1 μL of the supernatant was injected into GC–MS system using an autosampler.

2.7. GC–MS analysis

The GC–MS analyses were carried out on Agilent 6890 N GC equipped with 5973 inert mass selective detector (Agilent Technologies, USA), Agilent 7683 Series auto sampler and a SPB-624 (Supelco, USA) capillary column of length 30 m, 250 μm internal diameter and 1.4 μm film thickness. The column oven was programmed initially from 40 °C with 2 min hold up time to the final temperature of 240 °C with 10 °C/min ramp. The final temperature hold-up time was 8 min. Helium was used as carrier gas in constant flow mode at a flow rate of 1.0 mL/min. The inlet and GC–MS interface temperatures were kept at 280 °C. The temperatures of the EI source and quadrupole analyzer were kept at 230 °C and 150 °C, respectively. The MSD scan range was 30–600 u. The quantification of MN was done in the SIM mode for the ion m/z 106 and the dwell time was set to be 25 ms. The samples (1.0 μL) were injected in to GC–MS by using auto sampler in splitless injection mode with 0.2 min splitless time and each extract was analyzed three times to obtain an average peak area.

2.8. Quantitative analysis

The amount of MN in different rice samples was estimated by using external calibration method. To plot calibration curve, standard solutions of MN with concentrations 0.1, 0.5, 1, 2, 4, 6, 8 and 10 μg/mL were prepared, and 1 μL of each solution was injected by an auto sampler into GC–MS system operated in a SIM mode. Each sample was injected five times and the average of peak area was calculated for each concentration. The calibration curve generated by a least-squares linear regression analysis of the MN response versus the concentration of MN.

2.9. Method validation

The linearity of response of GC–MS in SIM mode was checked with calibration standards in the range of concentrations from 0.1 to 10 μg/mL which corresponds to 0.17 to 16.70 μg/g of MN in rice sample. The recovery of MN in solvent extraction was evaluated by spiking 300 mg of rough rice sample with 500 μL of MN (1 μg/mL, in methanol). Methanol was evaporated by a gentle stream of nitrogen and MN was extracted from rice by solvent extraction as described in Section 2.6. The analysis was compared to a control sample without adding MN. The recovery was calculated on the basis of the peak area obtained by MS detection after analysis of the spiked and un-spiked samples.

3. Results and discussion

The aroma and bioactive compounds are generally present in very low quantities in foods and hence the extraction step is crucial for the highest recovery of the compounds. The previously reported methods needed huge amount of the samples (up to 1.25 kg or 100 mL juice) and solvents for extraction of MN from the fruits and flowers and involve many steps (Wong & Khoo, 1993; Awano et al., 1997). Such kinds of methods are highly undesirable for breeding experiments on rice where very less quantity of samples is available for the analysis. The solvent extraction used in the current method reduces the amount of sample and solvent and efficient for the extraction of MN from the small quantity of rice samples.

3.1. Method development

3.1.1. Solvent extraction

In order to select the suitable solvent for the extraction of MN from rice, the solvent extraction was individually carried out by using different solvents such as methanol, acetone, acetonitrile, hexane, isopropyl alcohol, toluene, ethanol, tetrahydrofuran and dichloromethane. Maximum recovery of MN was obtained when methanol was used as an extractant; hence methanol is used as extractant for all the samples. The total ion chromatogram (TIC) obtained from the GC–MS analysis of the methanol extract in full scan mode indicated the presence of MN at 15.52 min. MN was confirmed by comparing the data with that of the authentic compound under similar analytical conditions. The TICs obtained from the analysis of rice extract and the authentic MN are shown in Fig. 1. The EI mass spectrum of MN obtained from the analysis of rice extract is fairly matching with that of authentic MN (Fig. 2).

3.1.2. Effect of extraction temperature and time

When solvent extraction was performed at different extraction temperatures (30–80 °C), the extraction efficiency of MN initially increased with increasing temperature and was observed to be maximum at 50 °C and was stable from 50 to 55 °C. The extraction efficiency was decreased there after (>55 °C), that may be due to leakage of vapors as the vapor pressure of solution increases with increase of temperature. Hence, the extraction temperature is set at 50 °C for the solvent extraction method. The experiments at different extraction time (30–150 min at 50 °C) showed maximum extraction of MN at 120 min and it was found constant later on. Thus, the extraction method was finally optimized with methanol by sonication for 120 min at 50 °C.

3.1.3. Quantitative analysis

The calibration curve was generated by a least-squares linear regression analysis by plotting the average of peak area against the concentration of MN. The plot was linear with regression coefficient $r^2 = 0.9987$ and the linear equation $y = 541132x + 12664$. The GC–MS-SIM chromatogram of methanol extract obtained from rice samples show a single peak that proves no interference in the region of interest was detected in rice samples. The linearity of

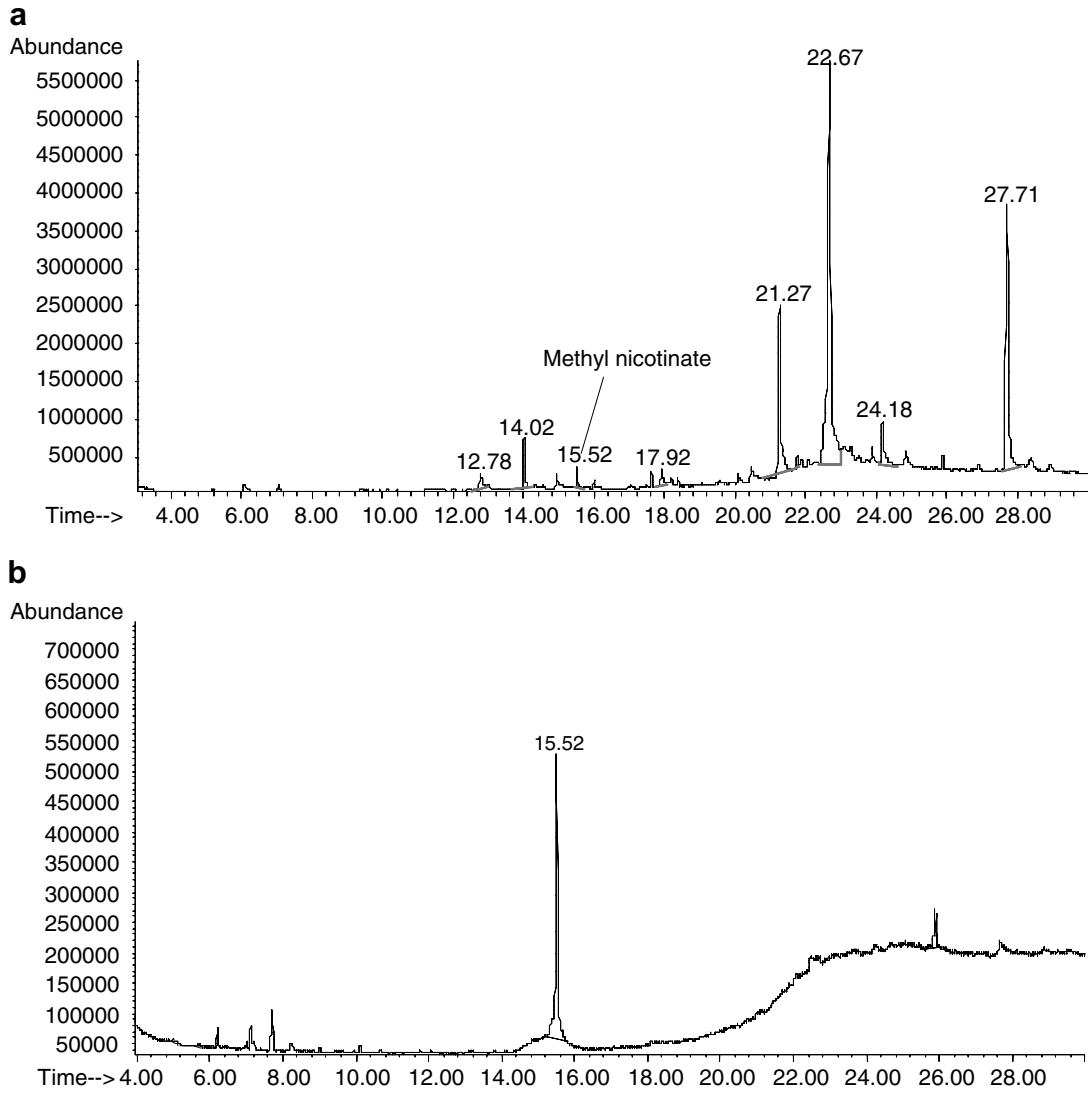

Fig. 1. TIC obtained from the GC–MS-EI analysis of (a) the methanol extract of rice and (b) authentic MN (8 μg/mL).

response of GC–MS in SIM mode was checked with calibration standards in the range of concentrations from 0.1 to 10 μg/mL.

3.1.4. Method validation

The recovery of MN in solvent extraction was evaluated by spiking MN in rough rice (See Section 2.9) and it was found to be $98 \pm 5\%$ ($n = 5$). Repeatability of peak area for MN expressed as relative standard deviation (RSD) was in the range of 2% ($n = 5$). Limit of detection (LOD) is the smallest measured concentration of an analyte from which it is possible to deduce the presence of the analytes in the test sample with acceptable certainty. This determination should consider matrix related interferences with an instrumental signal to noise (S/N) ratio greater than 5:1 or the concentration determined by a factor of 3 standard deviations of the signal response for blank matrix, whichever is less (Food Standards Agency Information Bulletin, 2004). LOD of MN was found to be 0.1 ppm in SIM mode at a signal-to-noise ratio of 7:1.

3.2. Methyl nicotinate in various rice varieties

The developed analytical method was applied to determination of MN in polished rice, brown rice and rice bran samples. We analysed five rice varieties which are popular in India. The concentrations of the MN in the five rice varieties determined by GC–MS-SIM analyses are shown in Table 1. The concentrations of MN found in brown rice, polished rice and rice bran of five varieties are in the range of 1.37–3.99 μg/g, 0.63–1.30 μg/g and 1.87–12.04 μg/g, respectively. Maximum amount of MN was found in the rice bran followed by brown rice and polished rice. The difference in MN levels between the polished rice, brown rice and rice bran samples suggests that this compound is not equally distributed in the bran and endosperm. The results clearly show that most of the

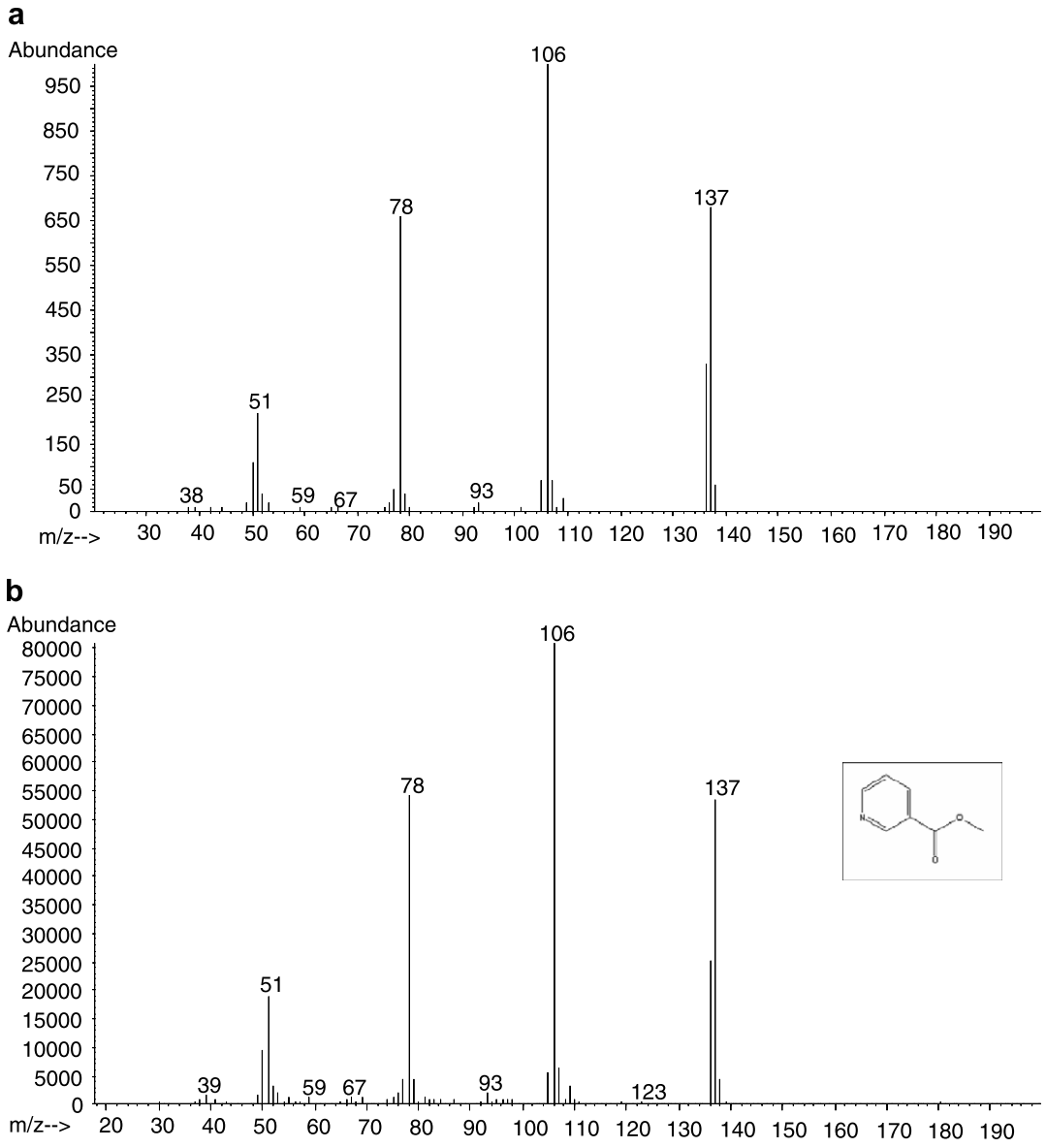

Fig. 2. EI mass spectrum obtained from the GC–MS analysis of (a) the methanol extract of rice and (b) authentic MN.

Table 1
Concentration of MN in various types of rice ($n = 3$)

Variety	Brown rice (μg/g)	Polished rice (μg/g)	Rice bran (μg/g)
Taroari basmati	3.01 ± 0.12	1.19 ± 0.09	9.22 ± 0.16
Vasumathi	1.44 ± 0.09	0.77 ± 0.06	2.69 ± 0.09
Yamini	3.99 ± 0.14	1.30 ± 0.11	12.04 ± 0.23
Krishna hamsa	2.94 ± 0.13	1.14 ± 0.08	9.07 ± 0.19
IR-64	1.37 ± 0.07	0.63 ± 0.06	2.87 ± 0.07

MN is accumulated in the aleurone layer. The content of MN varied significantly between different rice varieties. Among the five varieties, Yamini showed the highest, followed by Taroari basmati, Krishna hamsa, and IR-64, Vasumati had the lowest MN content. Potential reasons for these variations include differences in genotypes and are subject of current investigations employing the newly developed GC–MS approach.

4. Conclusions

The medicinal compound MN is reported for the first time in rice and rice bran. A simple, rapid, and economic (requires less sample) method is developed for the extraction and determination of MN in rice samples. The concentration of MN is found to be more in rice bran followed by brown rice and polished rice. The concentration of MN is varied among the different varieties of rice studied and is attributed to the genotypes of the rice varieties. Breeding programmes for rice with high concentrations of MN can be greatly facilitated by establishing the concentrations of the MN in new cultivars.

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