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Short communication

Development and validation of reverse phase high performance liquid chromatography for citral analysis from essential oils

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ABSTRACT

Citral is a widely used monoterpene aldehyde in aromatherapy, food and pesticide industries. A new validated reverse phase high performance liquid chromatography (RP – HPLC) procedure for the detection and quantification of *cis-trans* isomers of citral was developed. The RP-HPLC analysis was carried out using Enable C – 18G column (250 × 4.6 mm, 5 μ), with acetonitrile and water (70: 30) mobile phase in isocratic mode at 1 mL/min flow. A photodiode array (PDA) detector was set at 233 nm for the detection of citral. The method showed linearity, selectivity and accuracy for citral in the range of 3–100 μg/mL. In order to compare the new RP-HPLC method with the available methods, one of the commercially available essential oil from *Cymbopogon flexuosus* was analyzed using new RP-HPLC method and the same was analyzed using GC–MS for the comparison of the method for the detection of citral. The GC–MS analysis was done using mass selective detector (MSD) showed citral content to be of 72.76%; wherein the new method showed to contain that same at 74.98%. To prove the application of the new method, essential oils were extracted from lemongrass, lemon leaves and mosambi peels by steam distillation. The citral content present in the essential and also in the condensate was analyzed. The method was found to be suitable for the analysis of citral in essential oils and water based citral formulations with a very good resolution of its components geranial and neral.

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1. Introduction

Lemongrass oil is widely used in aromatherapy for relieving pains, in skincare and cosmetic products. It is also used in food industry and pesticide industry due to its excellent biocompatibility and inherent antibacterial, anti-fungal, insecticidal, antiseptic, and anti-inflammatory properties [1]. The tea made from its leaves is popularly used in Brazil as an antispasmodic, analgesic, anti-inflammatory, antipyretic, diuretic and sedative [2]. The quality of lemongrass oil and many lemon scented herbs is due to its citral content [3]. Moreover, the aldehyde with molecular formula $C_{10}H_{16}O$ i.e. citral is responsible for the lemon odour. Citral is a mixture of two stereoisomeric monoterpene aldehydes. In lemongrass oil, the *trans* isomer geranial predominates over the *cis* isomer neral as observed with the GC–MS analysis [4]. Citral also acts as a precursor for vitamin A synthesis [5]. Similarly, it was found that essential oil from lemon leaves consisted of limonene/β-pinene, geranial, neral and linalool, linalyl acetate, α-terpineoletc. The essential oil extracted from the leaves of the Egyptian navel orange (*Citrus sinensis* (L.) Osbeck var. Malesy) was found to contain 33 compounds as analyzed by GC–MS and was found to elicit antimicrobial action against *Streptococcus pyogenes*, *Staphylococcus aureus*, *Salmonella typhimurium* and *Aspergillus fumigatus* [6]. In lime peel oils four types of components such as limonene, limonene/γ-terpinene, limonene/β-pinene/γ-terpinene and limonene/γ-terpinene/β-pinene/oxygenated products are present [7].

The aldehydes and alcohols in the essential oils are highly reactive against microbes because of ready solubility of these small molecules in the peptidoglycan membranes of bacteria and chitosan membranes of fungi [8]. Citral showed antimitotic activity by targeting the microtubules in *A. thaliana* [9]. In addition to their antimicrobial property, the anticancer properties of citral have also been studied hitherto [10–13].

The analysis of essential oils [14,15] is usually done by GC–MS because of the ionization properties of the volatile components

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present in the essential oils. In many products intended for flavouring agents and pesticides, citral is present in the aqueous medium. However, the quality analysis of water based compounds by GC–MS analysis are known to face problem of longer methods of sample preparation. Further, the water needs to be removed in vacuum systems as it reduces the GC-column life [16], which in many cases is not possible. Thus in this regards HPLC method is preferred for measurement of citral in essential oils and also in water-based formulations. Validated RP-HPLC method is becoming one of the most accepted analytical techniques for identification and quantification of essential oils and its constituents due to their ease of sample preparation for water based samples [17–21].

Further, capillary liquid chromatography and micellar electrokinetic chromatography is also used for the separation of monoterpenes from orange essential oils [22]. More recently, HP-TLC method has been reported for the separation of geranial and neral [23]. However, the HPLC method reported in the present study is developed using the C-18G column and the isocratic mobile phase conditions which are applicable for the detection of citral with the separation of geranial and neral. Geranial was reported to be significantly more potent than neral or citral in eliciting anticancer effect [24]. This demands a need for the analysis of these isomers specifically in the synthetic and natural mixtures. There are only few HPTLC [23] and HPLC [25,26] methods to separate and analyze these isomers. The current method deals with the measurement of the citral along with the accurate measurement of the *cis* and *trans* isomers of citral.

ISO definition for validation of an analytical method is “method validation is the confirmation via the provision of objective evidence, that the requirements for specifically intended use or application have been met” [27]. The systematic guidelines and definitions are given by USFDA, ICH, USP and European Medicines Agency (EMEA) etc. ‘WHO’ has given the series of technical guidelines for herbal medicines implemented with parts of its traditional medicine strategy. The guidelines focus on quality control methods for medicinal plant materials, good agricultural and collection practices, storage, trade and distribution and finally their assessment by HPLC, GC and other analytical techniques [28–33]. In the present study, we demonstrated the analytical method development and validation for citral estimation from the essential oil and the water soluble fraction of citral during the oil extraction process. Further, we also show that even the isomers present in citral viz. geranial and neral can also be quantified using this method.

2. Experimental

2.1. Chemicals and reagents

The citral (assigned purity of 95%) and geraniol (assigned purity of 98%) were obtained from Sigma Laboratory, India. The lemongrass oil (*Cymbopogon flexuosus*) was obtained from Perfect Herbals and Oils Company, Madhya Pradesh, India. For the oil extraction, the plants viz., lemongrass, lemon leaves and mosambi peels were collected from the local farms, Bangalore, India. The lemongrass obtained was identified as *C. citratus* from National Ayurveda Dietics Research Institute, Bangalore, India. The solvents such as acetonitrile and HPLC grade water were obtained from Spectrochem laboratory, Bangalore, India.

2.2. Apparatus, chromatographic conditions

The HPLC system included a Shimadzu LC-20AP with Prominence SPD-M20A photodiode array detector (PDA) with D2 and tungsten lamp, with Prominence system controller (CBM 20A) and Rheodyne injector with position sensing switch and 20 μL loop. The HPLC separation was achieved by using Enable C-18G column (250 mm × 4.6 mm, 5 μm). The optimized mobile phase was contained acetonitrile and water (70:30) in isocratic mode at a flow rate of 1 mL/min. The chromatographic runs were carried out at 22 °C, while the detector cell temperature was set to 40 °C and detector slit width 1.2 nm for the high resolution of the spectral lines.

2.3. Gas chromatography analysis

Gas chromatography (GC) analysis was done by Agilent technologies 5975 Inert Mass Selective Detector GC–MS. The separation was achieved through 5% phenylmethyl polysiloxane column (HP5, 30 m length × 0.250 mm diameter × 0.25 μm film thickness). The temperature was programmed in the range from 75 °C to 250 °C at 4 °C/min increment. Both inlet and the detector temperatures were 250 °C with a split ratio of 1:100. The helium was used as a carrier gas at a flow rate of 1 mL/min. The data obtained from the experiments were analyzed using Chemstation. In all instances, experiments were carried out in triplicates of the standard solutions or samples. Then each solution or sample was injected into the GC system also in triplicates. The peak areas of the three injections were averaged and the relative standard deviations were calculated.

2.4. HPLC method development

After several trials of gradient and isocratic combinations of solvents, the isocratic flow rate sat 1 mL/min with acetonitrile and water (70:30) was found to be the best for separation of neral and geranial peaks present in citral. The optimum wavelength for the detection of citral was found to be 233 nm and PDA chromatogram was taken at 233 nm for the quantification of citral. However, for the recovery studies, the contaminant geraniol peak could not be detected at 233 nm. Therefore 200 nm was used to check the presence of the geraniol peak.

3. Methods

3.1. Preparation of standard samples

A 10 mg of citral standard was taken in a volumetric flask containing 1.0 mL of acetonitrile giving 10 mg/mL stock. The stock solution was diluted to get the citral concentration range between 3 and 100 μg/mL using acetonitrile. The diluted samples were used as standards for linearity study.

3.2. Extraction of essential oils

Freshly collected lemongrass, lemon leaves and mosambi peel were washed with distilled water to remove the dust and dried at 45 °C for 24 h. The dried materials were crushed into small pieces using agitate grinder. In order to remove the moisture content, the ground plant products were kept in hot air oven at 45 °C for 24 h. Then, the small pieces of dried products were further ground into fine powder. Then 20 g of powder was transferred to Clevenger apparatus containing 200 mL distilled water and the essential oils were extracted by steam distillation method at atmospheric pressure and temperature at 80 °C. The extracted oils were further diluted to the theoretical concentrations of 100 μg/mL using acetonitrile. From this, 65 μg/mL were injected for analysis. However, for water soluble citral content measurement, 30 μL of condensate obtained during oil extraction was diluted with 70 μL of acetonitrile to make up the final solvent content.

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