

COMPARATIVE STUDY OF MODIFIED TECHNIQUES FOR LIMONENE EXTRACTION FROM AGRO-WASTE

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Abstract: Essential oil such as limonene is found in abundance in citrus plants but there are limitations in its industrial production with respect to its yield, purity, safety and grade. Such problems could be circumvented by some alternative methods of extraction. Non-edible portion such as peels of citrus fruits, orange, lemon, sweet lime, pomelo and leaves of eucalyptus, lemon grass was used in this study. Various techniques such as Soxhlet, rotary shaker, sonication and Clevenger were used to extract the limonene content and analysis was done by Gas Chromatography (GC) technique. Limonene was found to be present in significant amount in all the samples with highest yield in orange, nutmeg and eucalyptus i.e. 347.21mg/g, 330.58mg/g and 250.99mg/g respectively. Sonication was found to be advantageous over the other methods due to its higher yield, less time of extraction and with reduced usage of organic solvent.

Index Terms - Limonene, Extraction techniques, Gas chromatography, Nutmeg.

I. INTRODUCTION

Limonene is a naturally occurring monoterpene present in most of the citrus fruits including oranges, lemon, sweet lime, grapefruits, etc. It is a colorless liquid aliphatic hydrocarbon classified as a cyclic monoterpene. It is widely used as a flavor and fragrance and is listed to be generally recognized as safe in food by the FDA. There are also other plants which naturally synthesize limonene as a secondary metabolite. Plant parts such as eucalyptus leaves, lemon grass, nutmeg peels also show presence of aromatic compounds indicating presence of monoterpenes. Limonene is currently produced as a by-product of citrus juice processing. It is an essential oil which can be extracted from various sources using different analytical techniques. Limonene value may increase when new potential applications in the polymer and fuel industries lead to a higher demand that cannot solely be met by the citrus industry. Moreover, essential oils produced from citrus materials may contain significant amounts of pesticides, which limit the application in food and household products. Here, the need arises of some alternative methods to obtain high yield as well as cost effective method (Nichkova M, et al, 2009). Till date only citrus fruit peels were tapped for extraction of limonene. But, there are other sources such as lemon grass, eucalyptus leaves and nutmeg peels which too contribute to limonene content which is not being explored much.

Wastes and by-products from citrus fruits contain large amounts of value added compounds such as pectin, antioxidant and essential oils and show a variety of opportunities in the technological and health promoting domains (Predrag Putnik 1). New strategies for processing citrus wastes are required to address high disposal costs, a lack of disposal sites, and concerns related to negative environmental impacts from the high organic content of citrus waste and its fermentability (Tripodo M M, et al, 2004).

One of the main reasons for the low levels of the citrus agro-industry residues utilization is the lack of efficient and cost-effective extraction methods for compounds with the required quality. Till recently agro-waste was only processed for production of biogas. (Abbas, M. N. 2014). Traditionally essential oils have been extracted using techniques such as steam distillation, organic solvent extraction or Soxhlet method. All these methods require large amount of solvent and time. Ultrasound extraction involves the use of ultrasound with frequencies ranging from 20 kHz to 2000 kHz; this increases the permeability of cell walls and produces cavitation. Although the process is useful in some cases, its large-scale application is limited due to the higher costs. One disadvantage of the procedure is the occasional but known deleterious effect of ultrasound energy (more than 20 kHz) on the active constituents of medicinal plants through formation of free radicals and consequently undesirable changes.

The present study focuses on identifying rich sources of limonene containing non-edible plant sources. The comparative study between the sources, extraction techniques and time of extraction was done to optimize the product yield.

II. MATERIAL AND METHODS

2.1 Chemicals:

Standard D-limonene was procured from TCI chemicals. HPLC grade n-hexane, Standard BSA (Bovine serum albumin) was procured from Merck India.

2.2 Collection of samples:

The non-edible portion such as citrus fruit peels of orange, sweet lime, lemon, pomelo, were procured from various fruit vendors and juice center. Eucalyptus, lemon grass leaves were obtained from local garden in Mumbai. Nutmeg shells were obtained from ayurvedic vendors.

2.3 Sample preparation:

One kg of sample was shade dried and then ground to coarse powder, passed through mesh to obtain coarse powder and further subjected to extraction and analysis techniques.

2.4 Proximate analysis:

2.4.1 Moisture: (AOAC. Official Methods of Analysis, 2005)

2.4.2 Ash: (AOAC Official Method 942.05)

2.4.3 Carbohydrate: (Roe J.H, 1955)

2.4.4 Protein: (AOAC Official Method 942.05)

2.4.5 Phytochemical:

2.4.5.1 Test for alkaloids: Test was done using Meyer's reagent method (Harborne JB. Phytochemical methods) Yellow precipitate indicates presence of alkaloids.

2.4.5.2 Test for tannins: Green or blue- black coloration indicates presence of tannins (Harborne JB. Phytochemical methods).

2.4.5.3 Test for saponins: The formation of stable persistent froth shows the presence of the saponins in extract (Harborne JB. Phytochemical methods)

2.4.5.4 Test for terpenoid (Salkowski test): A reddish-brown coloration of the interface was formed to show positive results for the presence of terpenoids (Harborne JB. Phytochemical methods)

2.4.5.5 Test for flavonoids: A yellow coloration indicates the presence of flavonoids (Arora, M., & Kaur, P. 2013).

2.5 Extraction of limonene:

Four different extraction methods Soxhlet, rotary shaker, clevengers, sonication was used for comparative study and are described below.

2.5.1 Soxhlet method: 5g of sample was taken in a whatmann filter paper thimble. One and a half cycle of solvent i.e. 200ml of n-hexane was added for extraction. Extraction was carried out for 6hrs at 80°C. Excess of solvent was evaporated using rotary evaporator for concentrating. The extract was reconstituted in 0.5ml of HPLC grade n-hexane and stored in GC vials, analysed using GC for estimating limonene concentration.

2.5.2 Rotary shaker: One gm of sample was mixed with 10ml of n-hexane. Kept for 48h on rotary shaker for extraction. Excess of solvent was evaporated using rotary evaporator concentrating the extract and the extract was reconstituted in 0.5ml of HPLC grade n-hexane and stored in GC vials.

2.5.3 Sonication: Three gm of sample was mixed with 20ml of n-hexane and placed in sonication bath at 20KHz for varying time interval from 30, 45, 60, 75, 90 minutes. The obtained slurry was then filtered using whatmann filter paper no. 41. The excess solvent was then evaporated at room temperature. The extract was then reconstituted in 0.5ml of HPLC grade n-hexane and stored in GC vials.

2.5.4 Clevenger: 25g of fresh samples were used for extraction of limonene. The sample was soaked in 200ml of distilled water in the round bottom flask. Extraction was carried out at 60°C for 3h. The extract was collected in the narrow receiver tube. Further the extract was collected in air tight vials by opening the oil delivery cock.

2.6 Gas Chromatography analysis:

The GC analyses was performed on a SHIMADZU 2014 Gas Chromatograph equipped with a Flame Ionization Detector (FID). Fused silica capillary Rtx-5 (Crossbond- 5% diphenyl – 95% dimethyl polysiloxane) column (30m x 0.25mm i.d., film thickness 0.25 µm) was used with nitrogen as carrier gas at the flow rate of 1.0 ml/min. Injector and detector temperature were set at 220°C and 250°C, respectively. Oven temperature was kept at 90°C then raised to 220°C at the rate of 20°C/ min and finally held isothermally for 5 min. One microliter of the diluted samples was injected manually (split mode, split ratio – 1:10).

III. RESULTS & DISCUSSION:

Proximate analysis of samples was carried out for moisture, ash, carbohydrate and proteins.

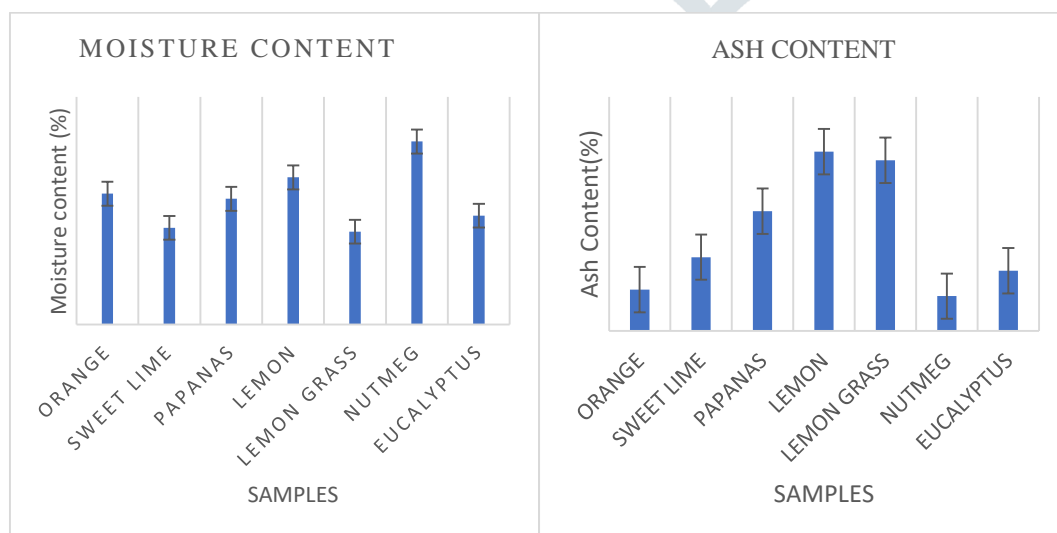


Figure 1: Moisture content Figure 2: Ash content

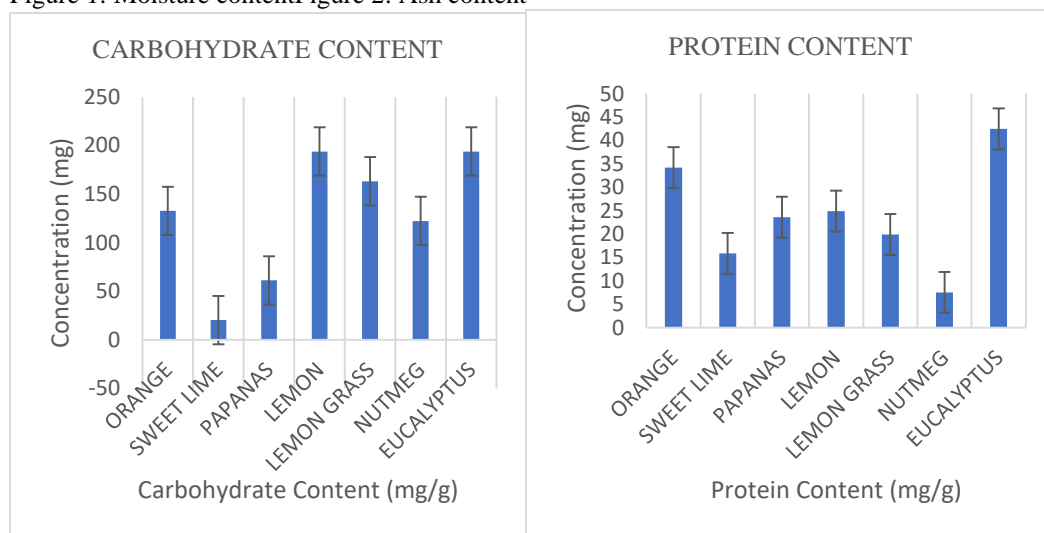


Figure 3: Carbohydrate content

Figure 4: Protein content

Moisture content of the sample ranged from 6% to 13% (fig.1). The samples were subjected to drying for five days to reduce the moisture content. The obtained values of moisture lie in the reported range from 3% to 12%. Lowest moisture content i.e. 6.52% was found to be in lemon grass and highest in nutmeg i.e. 12.86%. Vacuoles are present in peels of fruits and leaves. Vacuoles act as a storage house. More moisture present in the peels occupy the space required for the storage of volatile compounds, leading to faster evaporation of the volatiles (M’hiri, N. et al, 2015).

The mineral content determined as ash value was between 1% to 10% (fig.2). Nutmeg showed the lowest mineral content indicating the possibility of good source of limonene. High inorganic matter content affects the activity of oil leads to decrease in the conversion to volatile hydrocarbons. Complete removal or decrease in the inorganic content, leads to increase in the yield of total volatile hydrocarbons. The presence of mineral also reduces the diffusion of volatiles (Ballice, L.2005).

The carbohydrate content was found to be in the range of 1% to 7% (fig.3). Nutmeg showed the lowest carbohydrate content. Carbohydrates can form structures that sterically entrap the compounds and can form an immobile matrix that restricts the movement of volatile compounds and limits migration to the gaseous phase. The protein content was found to be ranging from 0.5% to 5% (fig.4). Proteins generally have a lesser impact as compared to carbohydrates. It can form linkages to hydrogen and hydrophobic compounds (Fisk, I. D, et al, 2012).

Table 1: Phytochemical analysis of samples

Sample	Orange	Sweet lime	Lemon	Papanas	Eucalyptus	Lemon grass	Nutmeg
Analysis							
Terpenoids	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+
Tannins	-	-	-	-	+	+	-
Alkaloid	-	-	-	-	+	+	+
Saponin	+	+	+	+	+	+	+

Legend :

+ - present

- - absent

The phytochemical tests of all the samples showed that it is very rich in secondary metabolites (Table 2) which it indicates that the peels are rich in monoterpenes, as it is one of the products of secondary metabolite.

Table 2: Yield of limonene using various techniques.

Sample Extraction method	Orange	Sweet lime	Lemon	Papanas	Eucalyptus	Lemon grass	Nutmeg
	Concentration of limonene (mg/g)						
Rotary (48h)	0.024	1.454	0.0046	0.017	0.021	0.020	-----
Soxhlet	14.710	4.432	7.684	2.558	40.047	1.85	238.87
Sonication	347.21	57.62	3.88	5.70	250.99	10.020	330.58
Clevenger	88.48	67.76	13.715	-----	15.486	9.35	0.270

Various conventional and modified techniques were used to extract limonene from the peels and leaves. Other than traditional Soxhlet and rotary method sonication was also used for extraction of limonene from the above-mentioned samples. A significant difference was seen in yield of limonene content when same sample was subjected to different techniques such as sweet lime gave yield of 1.454mg/g when extracted from rotary shaker, 4.432mg/g when extracted using soxhlet method, 57.62mg/g using sonication and 67.76mg/g using clevenger extraction technique (Table 2).

By using soxhlet method highest limonene content was found to be in nutmeg i.e. 238.87mg/g which is quite high than the reported values i.e. 50mg/g (Saputro, M, et al, 2016). Nutmeg was also found to have lowest amount of mineral content, protein and carbohydrate content as well. This explains the influence of proximate contents on limonene. When same sample was subjected to rotary shaker no limonene was extracted from nutmeg. From the obtained result, it can be said that the appropriate extraction method is needed to be chosen for high yield of limonene.

Among all the extraction methods, sonication proved to yield the highest limonene concentration. When samples such as orange, eucalyptus, nutmeg were subjected to sonication it gave a significant increase in yield of limonene as compared to other methods (Table 2). Orange gave yield of 14.710mg/g using soxhlet method whereas it gave a yield of 347.21mg/g using sonication method. Similarly, eucalyptus gave a yield of 40.047mg/g using soxhlet and 250.99mg/g using sonication and nutmeg gave a yield of 238.87mg/g using soxhlet and 330.58mg/g using sonication.

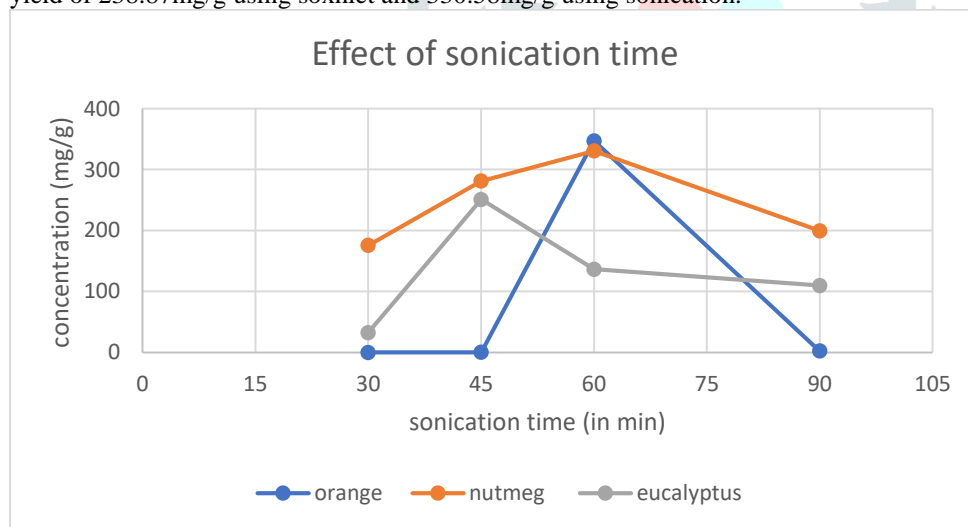


Figure 5: Effect of sonication time on limonene concentration.

Time of sonication also affected the yield of limonene significantly (Fig 5). Sweet lime showed highest limonene content at 90min, eucalyptus at 45min, nutmeg at 60min, orange at 60min, papanas at 90min, lemon at 30min and lemon grass at 90min. It also indicates that there is a possibility in few samples with increasing time volatiles or monoterpenes may undergo degradation. A decrease in limonene content was observed with increased time of sonication in some cases. Sonication can be used as an effective alternative method to soxhlet. The time and amount of solvent required for sonication is 10 times less than soxhlet method. Also, sonication yields a 10-fold higher limonene content. Soxhlet and rotary extraction requires 6h and 48h respectively. Clevenger extraction is also found to be a useful and cost-effective alternative for limonene extraction. Since, it is free from use of organic solvents, it may be useful for food grade limonene production. Extraction time required in this method is 2hrs. The time required is also less than soxhlet method and rotary extraction.

CONCLUSION:

1. The present study describes non-edible parts of eucalyptus and nutmeg as novel sources of high limonene content.
2. Sonication can be used as a cost effective, efficient extraction method for limonene extraction. Time of sonication is a critical parameter in yield of limonene and prolonged sonication may lead to degradation or loss of limonene.

3. Use of clevegers extraction can be a very good option for eco-friendly green chemistry technique where use of organic solvents can be avoided.

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