



# New method development and validation for Simultaneous determination of 3 terpenoids (Terpineol, Limonene, $\alpha$ -Terpinene) in Black cardamom by HPLC

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## Abstract

A HPLC method was developed and validated for simultaneous determination of Terpineol, Limonene,  $\alpha$ -Terpinene in Black cardamom using a Hypersil bds C18 column (250 mm $\times$ 4.6 mm, 5  $\mu$ m) with the mobile phase consisting of Methanol and 0.1% triethylamine, 0.05M  $\text{KH}_2\text{PO}_4$  used as mobile phase in the ratio of 45%, 10%, 45%. The linear ranges of three compounds were determined and method validation was evaluated completely. The established method is rapid and accurate with high repeatability.

**Key words:** Black cardamom, HPLC, Determination, Quality control, Terpineol, Limonene,  $\alpha$ -Terpinene

## 1. Introduction

Terpineol is a naturally occurring monoterpene alcohol that has been isolated from a variety of sources such as cajuput oil, pine oil, and petitgrain oil. There are four isomers, *alpha*-, *beta*-, *gamma*-terpineol, and terpinen-4-ol. *beta*- and *gamma*-terpineol differ only by the location of the double bond. Terpineol is usually a mixture of these isomers with *alpha*-terpineol as the major constituent. Terpineol has a pleasant odour similar to lilac and is a common ingredient in perfumes, cosmetics, and flavours.  $\alpha$ -terpineol is one of the two most abundant aroma constituents of lapsang souchong tea; the  $\alpha$ -terpineol originates in the pine smoke used to dry the tea.

Limonene is common in cosmetic products. As the main odor constituent of citrus (plant family Rutaceae), *D*-limonene is used in food manufacturing and some medicines, e.g. as a flavouring to mask the bitter taste of alkaloids, and as a fragrant in perfumery; it is also used as botanical insecticide, particularly the (*R*)-(+)-enantiomer is most active as an insecticide. It is added to cleaning products such as hand cleansers to give a lemon-orange fragrance (see orange oil) and because of its ability to dissolve oils. In contrast, *L*-limonene has a piney, turpentine-like odour. In natural and alternative medicine, *D*-limonene is marketed to relieve gastroesophageal reflux disease and heartburn.

Limonene is increasingly being used as a solvent for cleaning purposes, such as the removal of oil from machine parts, as it is produced from a renewable source (citrus oil, as a by product of orange juice manufacture). It is used as a paint stripper and is also useful as a fragrant alternative to turpentine. Limonene is also used as a solvent in some model airplane glues and as a constituent in some paints. All-natural commercial air fresheners, with air propellants, containing limonene are used by philatelists to remove self-adhesive postage stamps from envelope paper.

Limonene is also finding increased use as a solvent for filament-fused 3D printing. Printers can print the plastic of choice for the model, but erect supports, and binders from HIPS, a polystyrene plastic, that are easily solvable in Limonene. As it is combustible, limonene has also been considered as a biofuel.

In preparing tissues for histology or histopathology, *D*-limonene is often used as a less toxic substitute for xylene when clearing dehydrated specimens. Clearing agents are liquids miscible with alcohols (such as ethanol or isopropanol) and with melted paraffin wax, in which specimens are embedded to facilitate cutting of thin sections for microscopy.

Limonene is adenosine agonist which may explain its anti-stress and sedative properties.  $\alpha$ -Terpinene has been isolated from cardamom and marjoram oils, and from other natural sources.

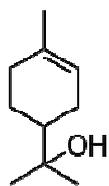


Fig.1 Terpineol

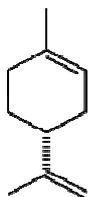
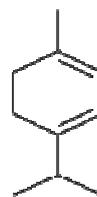


Fig.2 Limonene

Fig.3  $\alpha$ -Terpinene

## 2. Experimental

### 2.1. Chemicals and reagents

Three reference compounds are Terpineol, Limonene,  $\alpha$ -Terpinene purchased from sigma Aldrich. Acetonitrile, water, Methanol (HPLC grade), the other solvents were all of Analytical Reagent (AR) grade and purchased from Merck.

### 2.2. Samples preparation

All tested samples were collected from cochin, Goa, Medikeri Madurai, punjab India 5 packets of tested samples were dried at 50 °C until constant weight was attained. Approximately 1.0 g dried powder of each sample was accurately weighed, moistened with 0.2 mL ammonia water for 15 min and then extracted with 25 mL of 4:1 (v/v)  $\text{CHCl}_3$ :MeOH for 45 min under Ultrasonic vibration. The supernatants were filtered through 0.45  $\mu\text{m}$  micro porous membranes into HPLC vials for future test. 20  $\mu\text{L}$  was injected for analysis.

### 2.3. Chromatographic conditions

Analyses were performed using a PEAK-7000 HPLC system. The HPLC detector was an PEAK UV detector with 229 nm. A hypersil bds c18 column (250 mm $\times$ 4.6 mm, 5  $\mu\text{m}$ ) column was combined with a guard column of the same stationary phase (10 mm $\times$ 4.6 mm $\times$ 5  $\mu\text{m}$ ). Column temperature was maintained at room temperature. The mobile phase consisting of Methanol and 0.1% triethylamine, 0.05M  $\text{KH}_2\text{PO}_4$  used as mobile phase in the ration of 45%,10%,45% at a flow rate of 0.5 mL/min. The injection volume was 20  $\mu\text{L}$ . Data was collected and processed using peak software on a Samsung computer.

### 2.4. Calibration curves

Terpineol, Limonene,  $\alpha$ -Terpinene are dissolved in methanol and prepared 100  $\mu\text{g/ml}$  concentrated 3 stock solutions. The stock solutions are diluted 1, 2, 4, 8, 16 and 32 times to six different concentrations. Each concentration was injected three times. Calibration curves are constructed by plotting the logarithm of peak areas versus the logarithm of concentration of each analyte, and the results are shown in Table 1.

**Table 1. Calibration data of Terpenoid alkaloid standards.**

Standards	Linear range ( $\mu\text{g/ml}$ )	Regression equation	Correlation factor ( $\gamma$ )	LOQ ( $\mu\text{g/ml}$ )	LOD ( $\mu\text{g/ml}$ )
Terpineol	0.8–1.6	$Y=1.2489X+0.254$	0.9991	0.3	0.01
Limonene	0.2–1.2	$Y=1.5362X+0.315$	0.9994	0.05	0.02
$\alpha$ Terpinene	0.5–3.0	$Y=1.8956X+0.651$	0.9998	0.1	0.05

## 2.5. Quantification

Each sample solution was injected in triplet and the peak areas of the three analytes were recorded. Quantification was based on the two point's external standard method using calibration curves fitted by linear regression analysis.

## 2.6. Method validation

Method validation including precision, repeatability, stability and sample recovery was evaluated. Precision and repeatability were assessed by the successive analysis of five injections of the same sample solution and five replicates of the same patch of sample, respectively. The analysis of six time period within a day (0, 2, 4, 8, 16, 24 h) was used to evaluate the stability of sample solution in 24 h. Recovery was determined by adding known quantities of standards to the tested samples, and calculated by comparing the added and obtained quantities using external standard linear regression.

## 3. Results and discussion

### 3.1. Optimization of sample pre-treatment

As for the low polarity and weak alkalinity of the analytes, multiple related extraction conditions were designed and evaluated, which involved the following factors and corresponding levels: extraction method (ultra sonication or reflux), extraction repetitions (1 or 2 times), extraction solvents (CHCl<sub>3</sub>:MeOH:DCM 3:1:1, 1:1:3 2:1:2, 2:2:1, and 1:3:1 v/v), solvent volume (25, 50, or 100 mL), extraction time (15, 30, 45 or 60 min) and volume of 0.01% KH<sub>2</sub>PO<sub>4</sub> (0.1, 0.2, 0.3, 0.4 or 0.5 mL). By comparison of the contents of five analytes determined by the above experiments, the final optimized pre-treatment method for *D- trichoforum* was selected as follows: The accurately weighed sample powder was moistened with 0.2 ml 0.01% KH<sub>2</sub>PO<sub>4</sub> for 15 min and then extracted with 25 mL of 2:1:2 (v/v) CHCl<sub>3</sub>:MeOH"DCM for 45 min under Ultrasonic vibration.

### 3.2. Optimization of HPLC conditions

Several HPLC parameters affecting the results of separation and content precision of the content determination were optimized, including mobile phase i.e Methanol and 0.1% tri ethylamine, 0.05M KH<sub>2</sub>PO<sub>4</sub> with different ratios of at a flow rate of 0.5 mL/min. Column temperature was maintained at room temperature (25–35 °C).

### 3.3. Validation of methodology

The values of relative standard deviation (RSD) of peak area (PA) of analytes for replicated injections of the same sample were in the range of 0.2–3.0%. Meanwhile, the RSD values for method repeatability were from 1.52% to 1.90%. Moreover, the RSD values for sample stability test were less than 2.0%. The results of sample recovery indicated that the average recovery of the analytes was from 99.80% to 99.916% and the RSD values were less than 2.0% (see Table 2). All these results indicated that the established method of determination was valid and feasible.

Table 2.Recovery of the three analytes.

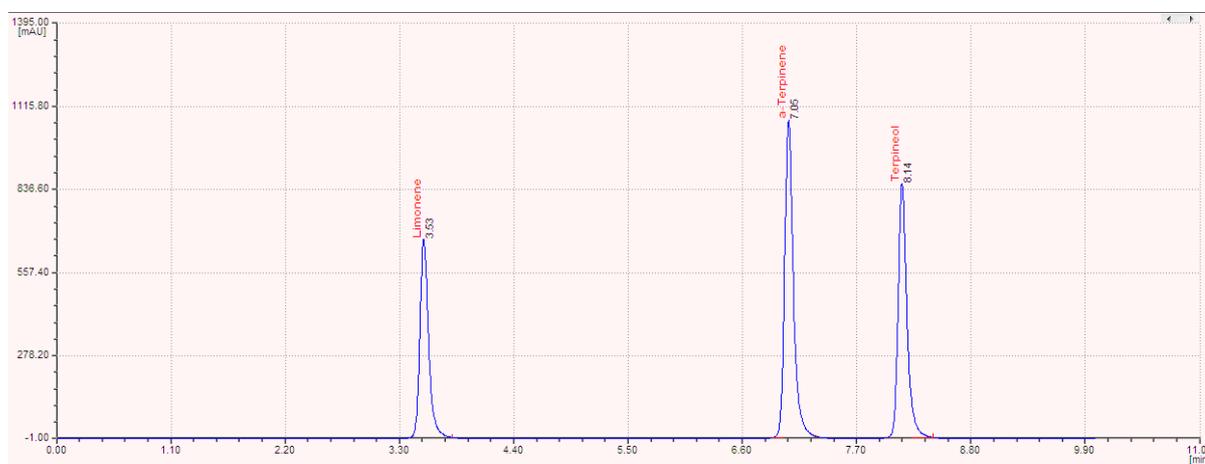
Analyte	Standard concentration ( $\mu\text{g/ml}$ )	Recovery ( $\mu\text{g/ml}$ )	%of recovery	% of RSD
Terpineol	1.2	1.199	99.916	1.59
Limonene	0.6	0.598	99.666	1.64
$\alpha$ -Terpinene	2.0	1.996	99.80	1.89

#### 3.4. Analysis of Terpineol, Limonene, $\alpha$ -Terpinene in Black cardamom

The three analytes were identified in 5 samples by comparing their retention time ( $t_R$ ) with standards and quantified by the external standard method using calibration curves fitted by linear regression analysis. The contents of all tested samples are listed in Table 3 and the chromatograms of representative samples are shown in Fig. 4 and Fig.5. T-test was used to analyze the variance of contents of the three analytes and total terpenoids of samples from different collection locations and times.

Table 3.Contents of the five components in 5 tested samples.

S.NO	Sample	Terpineol ( $\mu\text{g/ml}$ )	Limonene ( $\mu\text{g/ml}$ )	$\alpha$ -Terpinene ( $\mu\text{g/ml}$ )
1	Cochin	0.52	0.82	1.46
2	Medikeri	0.48	1.32	1.32
3	Goa	0.69	0.92	1.25
4	Ludhiana	0.53	0.76	1.39
5	Madurai	0.32	0.89	1.31

Fig. 4. The chromatogram of Terpineol, Limonene,  $\alpha$ -Terpinene reference compounds

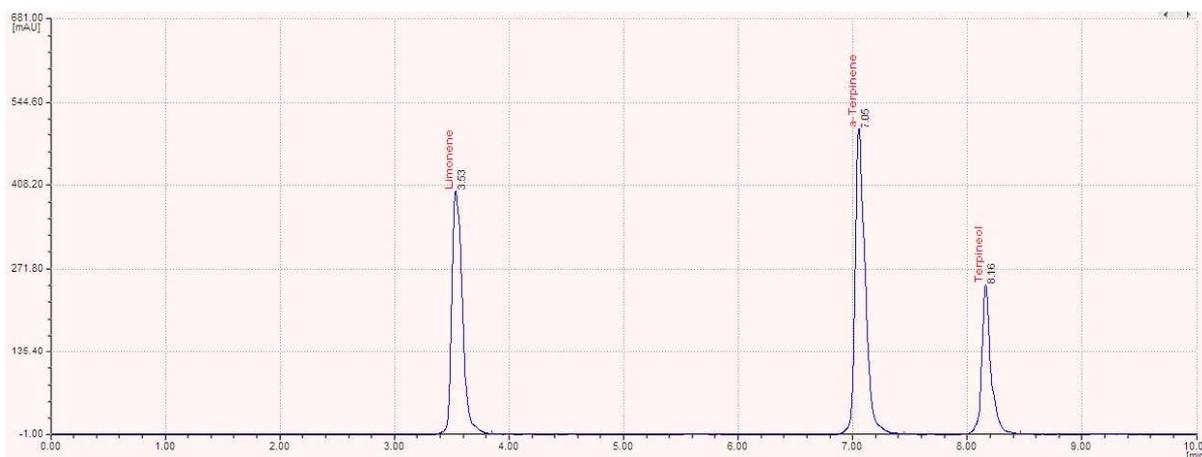


Fig.5 The chromatogram of representative Terpeneol, Limonene, and  $\alpha$ -Terpinene sample of Black cardamom

**Conclusion:** The method was very useful for qualitative and quantitative analysis of Terpeneol, Limonene and  $\alpha$ -Terpinene sample. The method was very accurate and precise.

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