

## Development of HPLC method for the quantification of verbenone in rosemary extracts

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### Abstract

Rosemary extract is commercially used as an effective natural antioxidant solution for Food & Beverage and Cosmetic products. To achieve the maximum flexibility of usage in all over the world, it is necessary to reduce the aroma of the rosemary extracts. The reference key volatiles in the rosemary extracts are usually quantified by GCMS method. In this study, a reliable high performance liquid chromatography (HPLC) method was developed for the quantification of Verbenone (a major reference key volatile in Rosemary extract). The HPLC system consists of C18 column and isocratic mode with Acetonitrile: 0.1% Phosphoric acid in Water (60:40) with a flow rate of 1.0 ml/min. This method was validated with linearity (0.9997), accuracy (101.4, 101.14, and 99.64) and repeatability (RSD = 0.2749%). Data obtained from this HPLC method was also compared with GCMS analysis.

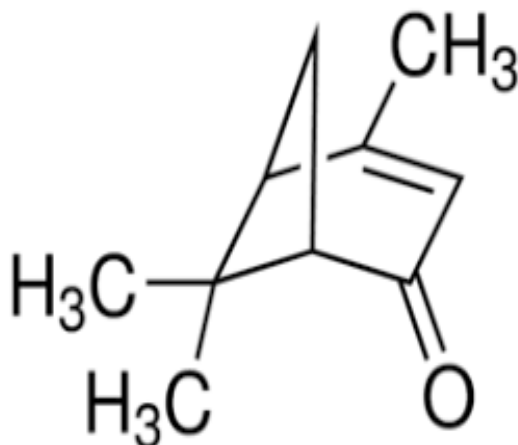
**Keywords:** - HPLC, GCMS method, UV Vis.

### 1. Introduction

Mediterranean region was fond of using Rosemary as a culinary spice and since 1955 the extracts of Rosemary have been considered as an antioxidant food additive. Different studies were published regarding the antioxidant activity of rosemary extracts. Phenolic diterpene carnosic acid and its homologues like carnosol, rosmanol, rosmariquinone and rosmaridiphenol are the key components behind the antioxidant activity of the Rosemary extract [1]. Also, the studies observed 90% of the antioxidant efficacy from Carnosic acid and carnosol [2].

However, the volatile oil components co-extracted from the raw material is undesirable while using the extract in so many food applications. Process developments were started since 1970s itself to reduce the aroma/taste of the extracts. US patent 3950266 by Stephen S Chang described the steam distillation process to reduce the aroma of the extract. There are several studies described different methods for the reduction of volatile oil molecules like hydro distillation, instant pressure drop, microwave pre-treatment, super critical extraction etc [3,4,5]. Recently EU legislation (Commission Regulation 231/2012) [6] characterized the rosemary extracts by mentioning the reference key volatile molecules (Borneol, Bornyl Acetate, Camphor, 1, 8-Cineol, Verbenone) and ensure the reduction of these volatile components by keeping Antioxidant/Volatile ratio greater than 15 [7]. The major challenge is to maintain the low aroma content along with the antioxidant activity while doing the reduction of the volatile oil components in industrial process [8].

Among the reference key volatile mentioned in the EU regulation, Bornyl acetate has the highest boiling point 231<sup>o</sup> C, but this molecule is very low in the Rosemary extracts while comparing with other volatile molecules. Boiling point of Borneol, Camphor, 1, 8-Cineol is 213, 209 and 177<sup>o</sup> C respectively. Verbenone (Figure 1) has a boiling point of 227 – 228<sup>o</sup> C and it is a natural bicyclic ketone terpene. This data of boiling points clearly indicates that the measurement of Verbenone in the product gave a very good assumption regarding the reduction of total volatile oil molecules in the rosemary extract as Verbenone is the major volatile molecule having least volatility.



**Figure-1 Structure of Verbenone**

As per EU directives 231/2012 for Rosemary extracts, Gas chromatography with Mass spectrometry detector is the method for quantifying the reference key volatile components. The equipment and method are more complex in nature and requires more time. High Performance Liquid Chromatography (HPLC) with Photo Diode Array (PDA) detector is one of the most advanced and precise instruments for the quantitative determination of volatile and non-volatile compounds in the natural extractives. In this study our aim was to develop and validate an HPLC method to quantify the verbenone content in the rosemary extracts.

## **2. Materials and Methods**

### **2.1 Rosemary Extract**

Dried leaves of Rosemary were collected from Morocco and the crude extract was prepared by extraction with Acetone followed by removal of the solvent.

### **2.2 Chemicals**

Verbenone standard was purchased from Sigma Aldrich (India). All the solvents were purchased from Merck (India)

### **2.3 Instrumentation**

1. Waters UPLC system (Model–AcquityUPLC – H Class plus system with PDA detector) used for the quantification was equipped with a vacuum degasser, quaternary solvent mixing, auto-sampler and a Photodiode array detector. Instrument controlling, collection of data and processing of data were done by the software Empower 3. The column used was ODS column (make: Kinetex from Phenomenex (4.6 × 250 mm, 5 μm) and mobile phase was Acetonitrile: 0.1% Phosphoric acid in Water (60:40) in isocratic mode with a flow rate of 1.0 ml/min. Injection volume for all samples and standard solutions was 10 μl. UV spectra were collected across the range of 200–900 nm, while extracted the chromatograph at 254 nm.

2. Agilent GCMS system (model – G1888) was used for the comparison of data obtained by newly developed HPLC method. Column used in the GC was DB 5-MS or equivalent (30m x 0.25mm x 0.25 μm). The carrier gas used was Helium at a flow rate of 1 ml/min and a split ratio 50:1. The column temperature was programmed at 40°C for 10 min and then heated to 280°C at a rate of 10°C/min and injector temperature was 200°C. Mass detector temperature was 230°C at source and 150°C at Quad. Head space conditions were fixed with 100°C as oven temperature, 105°C as loop temperature and 110°C as transfer line temperature. Vial equilibration time was 30 min.

### **2.4 Quantification of Verbenone in Rosemary Extract by HPLC**

#### **2.4.1 Preparation of Standard/Working Standard**

0.01 g of standard Verbenone was weighed into a 50ml standard flask and 30 ml HPLC grade Methanol was added into the flask. The flask was sonicated for 20 seconds for complete dissolution by using an ultrasound sonicator and then made up to the mark with HPLC grade methanol. The solution was filtered through 20-micron filter and kept in the auto sampler for injection.

#### **2.4.2 Preparation of Sample**

0.06 g of sample was weighed into a 50ml standard flask and 30 ml HPLC grade methanol was added into the flask. The flask was sonicated for 60 seconds for complete dissolution by using an ultrasound sonicator and then made up to the mark with HPLC grade methanol. The solution was filtered through 20-micron filter and kept in the auto sampler for injection.

#### **2.4.3 Validation**

The reliability of the method for quantification of Verbenone by HPLC was established by evaluating the linearity, accuracy and repeatability.

### 2.4.3.1 Establishment of Linearity

Preparation of stock solution: -2000 ppm Verbenone solution was prepared as a standard stock solution and diluted into different levels of concentration, namely 80 ppm, 120 ppm, 160 ppm, 200 ppm, 240 ppm, 300 ppm and 400 ppm. Six replicate runs were carried out and mean response was taken for calculating the Correlation coefficient.

### 2.4.3.2 Establishment of Accuracy

Accuracy of assay was established over 100 ppm, 200 ppm and 300 ppm Verbenone solutions by doping with 10 ppm Verbenone reference solution and then the recovery percentage was measured.

### 2.4.3.3 Repeatability

Repeatability was established by six replicate injections of 200 ppm Verbenone reference solution.

## 2.5 Quantification of Verbenone in Rosemary Extract by GCMS

### 2.5.1 Blank Preparation

5.0 ml of N, N- Dimethyl formamide was pipetted out into a HS vial and crimped tightly.

### 2.5.2 Standard Preparation

#### 2.5.2.1 Stock Solution

100±10 mg of (1S)-(-)- Verbenone was weighed accurately in a 10 ml volumetric flask containing 1 ml of Ethanol and made up to the mark with Ethanol.

#### 2.5.2.2 Working Standard

1.0 ml of the stock solution was pipetted out in to a 100 ml volumetric flask containing 10 ml N, N-Dimethyl formamide and made up to the mark. 5.0 ml of this working standard solution was pipetted out into a HS vial and crimped tightly.

## 3. Results and Discussion

Gas chromatography with mass spectroscopy is the method suggested by the regulatory directives of European Union for the measurement of reference key volatile oil components of the Rosemary extracts. But this method is not easy to conduct and the instruments require high care. The current study was aimed to develop and validate an easy method for the quantification of verbenone by HPLC. The results of HPLC method were also compared with those of GCMS method.

### 3.1 Method Development and Validation

Selection of mobile phase was done by comparing the selectivity of different ratios of Acetonitrile, Water and Phosphoric acid. Acetonitrile:0.1% Phosphoric acid in Water (60:40) was found to be optimal for better separation and the flow rate was optimized to 1 ml/min. The best separation of molecules was observed while using C 18 column by Phenomenex. UV spectrum of Verbenone showed maximum absorption at 254 nm; therefore, the compounds were monitored at this wavelength using photodiode array detector.

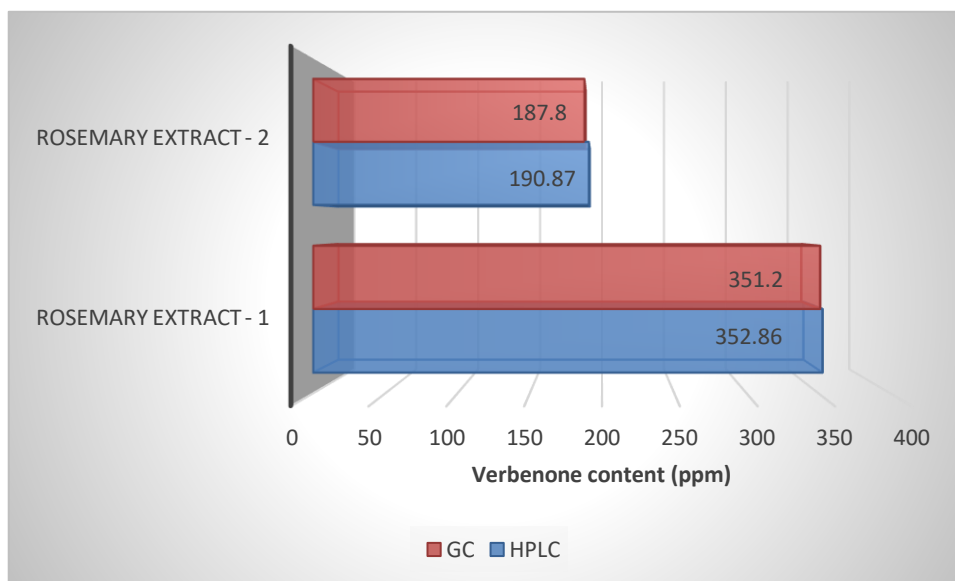
The results obtained in the validation study for Verbenone by HPLC method according to linearity, accuracy and repeatability showed that the proposed method was reliable [Table 1]. Good linearity for Verbenone for the concentrations 80 ppm to 400 ppm with  $r^2 = 0.9997$  was obtained. By doping 10 ppm Verbenone to different concentration of 100 ppm, 200 ppm and 300 ppm of Verbenone reference solution this method showed an accuracy value of 101.4%, 101.14% and 99.46% respectively. RSD of 0.2749% in the repeatability also proved high consistency of this method.

Table 1	
HPLC method validation factors for quantification of Verbenone	
Parameters	Results
Linearity ( $r^2$ )	0.9997
Accuracy of 10 ppm solution + 100 ppm solution	101.40%
Accuracy of 10 ppm solution + 200 ppm solution	101.14%
Accuracy of 10 ppm solution + 300 ppm solution	99.64%
Repeatability of 100% solution (n=6, RSD%)	0.2749%

### 3.2 Comparison of GCMS Data

GCMS data of Verbenone content was almost similar with the data by HPLC method as mentioned in the Graph- 1.

Graph – 1: Verbenone content by HPLC and GCMS methods



### 4. Conclusion

This newly established HPLC method for the quantitative measurement of Verbenone was validated statistically by measuring the linearity, accuracy and repeatability. Comparison of the results obtained by HPLC method with GCMS method established the reliability of the developed HPLC method.

### 5. Acknowledgement

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### 6. Conflicts of Interest

The authors declare that there is no conflict of interest

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