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ORIGINAL ARTICLE

Simultaneous Determination of Terpenoids in *Mentha longifolia* leaves by HPLC

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ABSTRACT
A rapid and simple high-performance liquid chromatographic method has been developed for simultaneous determination of two terpenoids; piperitone and pulegone in Mentha longifolia leaves. Reversed-phase liquid chromatography was performed on C_{18} column with methanol: water, 60:40 v/v, as mobile phase at a flow rate of 1.0 mL min^{-1}. Total run time of chromatography was less than 20 min. The Detection wavelength was kept as 279 nm; well separated and sharp peaks were obtained for piperitone and pulegone at retention times of 5.9 ± 0.3 and 9.9± 0.3 min. respectively. Linearity of piperitone was found to be in the range of 10–500 µg/mL; while pulegone was found to be 20–500 µg/mL. The method has shown good correlation coefficients of 0.9950 and 0.9990 for piperitone and pulegone respectively. The detection (LOD) and quantification (LOQ) limits were found as 3.5 and 11 µg mL^{-1} for piperitone; 8.0 and 20 µg mL^{-1} for pulegone respectively. The HPLC method showed good linearity, recovery, high precision and robustness for both markers. The rapid HPTLC method provides a new and powerful approach for the simultaneous quantification of pulegone and piperitone as phytomarkers in the extract as well as its commercial formulations for routine quality control.
Key words: *Piperitone, Pulegone, HPLC, Mentha longifolia*

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INTRODUCTION

The family Labiatae, or the mint consist of about 200 genus and between 2000 or 5000 species of fragrant plant and short shrubs having a large diversity and around 22 species are considered to be medicinal plants. The ground fresh biomass and dried leaves of the plant are used as spice and herbal tea, and cultivated commercially in the entire world [1, 2, 3]. *Mentha* spp. have been used as a folk remedy for treatment bronchitis, flatulence, anorexia, ulcerative colitis and liver complaints due to their antiinflammatory, carminative, antiemetic, diaphoretic, antispasmodic, analgesic, stimulant and anticatharral activities [4].

The major components in the essential oil of *M. longifolia* found vary considerably as per different localities and chemotypes. The chief component of the essential oil of *M. longifolia* from South Africa appears to be the monoterpene ketone and menthone. Interestingly carvone is not reported, which is common in other chemotypes from to those in other parts of the world [5]. Ciscarveol (53 to 78%) was reported as the major constitute of Iranian samples [6], whereas, the main compounds in the collected samples from Sudan and India were Carvone (50 to 70 %) and piperitenone oxide (40-60%) [7, 8]. Hafedh *et al.,* 2010 reported that the most important components of essential oil from *M. longifolia* were: menthol (32.51%), menthone (20.71%) and pulegone (17.76%) [9]. Maffei, 1998 reported that essential oil of a new chemotype of *M. longifolia*, growing wild in the Piedmont valley (Italy), was rich in piperitenone oxide (77.43%) [10].

Since, this many variations in the chemical constituents present in the various chemotypes present all over the world, it is important to qualitatively and quantitatively analyze the components present in the *Mentha* species which is widely used for food and pharmaceutical purposes. Therefore, a well validated

and effective analytical method needs to develop for employing these compounds. To our knowledge, this is the first time that HPLC was used for simultaneous quantification of these piperitone and pulegone (Figure 1) in *Mentha longifolia.* Among the chromatographic fingerprinting applied to the authentication, qualitative and quantitative evaluation of botanical products over the past HPLC emerges to be the most widely used method attributed to its convenience and efficiency.

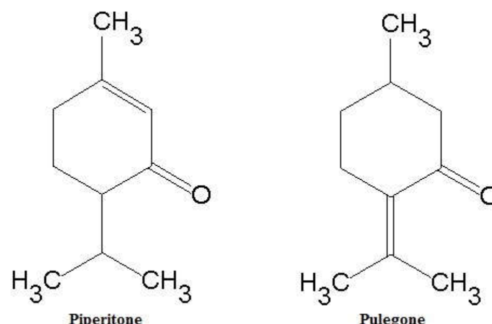

Figure 1 Chemical structure of piperitone and pulegone

MATERIALS AND METHODS

The fresh leaves of *Mentha longifolia* was procured from the Local Market of Al-Kharj and Riyadh, Saudi Arabia. The voucher specimens were deposited at Department of Pharmacognosy, College of Pharmacy, Salman Bin Abudlaziz University.

HPLC-grade acetonitrile, methanol and acetic acid were purchased from Sigma Chemical Co. (USA) and Merck (Germany). HPLC grade water used throughout the experiment prepared by Milli-Q purification system.

HPLC Instrumentation

The HPLC analysis has been carried out on a Waters separating module (Waters Co., MA, USA) with UV detector. The instrument was controlled by use of "BREEZE" software installed with equipment for data collection and acquisition. The separation was achieved by using column with 250 x 4.6 mm, particle size 5 µm C18 reverse phase column (Merck, Germany) maintained at room temperature. The mobile phase used was methanol and water (60:40, *v/v*). All analyses were carried out in isocratic eluation mode with a flow rate of 1.0 mL min^{-1}. Solutions and mobile phase were freshly prepared at the time of use. The eluate was monitored by the UV detector set at 279 nm

Preparation of standard solution for linearity

Working solutions of pulegone and piperitone were prepared daily by dissolving drug standards in methanol to yield a final drug concentration of 500 µg/mL. Further, different concentration of solutions was obtained by serial dilutions of stock solutions with methanol. Aliquots (10 µl) of each diluted sample were subjected to HPLC (in triplicate), and the areas under the peaks of both standard samples were reported.

Preparation of calibration graph for piperitone and pulegone

A set of six standard solutions was prepared in the concentration range of 10 to 500 and 20 to 500 µg/mL for piperitone and pulegone respectively, and each was analyzed in duplicate. Calibration curves were constructed by plotting peak areas versus concentrations of pulegone and piperitone, and the calibration equation was calculated using linear regression analysis. Aliquots (20 µL) of each solution were injected under the operating chromatographic conditions described above (Table 1).

Sample preparation

Accurately weighed 0.5 g quantity each of powder of *Mentha longifolia* leaves were separately extracted with methanol under reflux (30 min each time) in a water bath. A known amount of extract (20 mg) was taken and dissolved in methanol (10 mL) and filtered through 0.45 lm filter for HPLC analysis.

Validation of the method

The proposed method was validated as per ICH [11] guidelines for different parameters like linearity, accuracy, precision, LOD and LOQ and robustness [12, 13, 14].

Accuracy

Accuracy test was performed by adding standard solutions of pulegone and piperitone at three concentration levels (50, 100, and 150%) to crude extract of *Mentha longifolia* (100 µg/mL) with a known content of this compound. Three determinations were carried out for each solution. The amounts of drug recovered were determined by applying these values to the regression equation of the calibration curve.

Precision

In order to evaluate precision, repeatability and intermediate precision were carried out. Repeatability of the method was checked by injecting replicate injections of the combined solution and intermediate precision was determined from repeating analysis on the same day (intra-day precision) and on three consecutive days (inter- day precision). Assay for each analysis was calculated and % RSD was determined.

Robustness

Robustness was studied during method development by determining the effects of small variations done in mobile phase composition, flow rate and column temperature. The robustness of the method was assessed in terms of % RSD.

LOD& LOQ

LOD (signal to noise ratio = 3.3) and LOQ (signal to noise ratio = 10) for the method has been determined based on signal-to-noise ratio for both compounds as per ICH guidelines.

RESULTS AND DISCUSSION

Method Development

Many trials had been carried out for the development of suitable mobile phase which can give sharp peaks with maximum resolution. Different buffers solution (phosphate and acetate) of acidic pH (2.5-4.0) were tried in combination with methanol and acetonitrile. But it was found that there was problem with peak shape. So the buffer was avoided from the mobile phase even it was giving good resolution. The chromatographic separation of was carried out in the isocratic mode using a mixture of methanol: water (60:40, *v/v*) as mobile phase. The column temperature was ambient. The mobile phase of methanol and water 60:40 (% *v/v*) results in sharp peaks with satisfactory resolution at the Rt of 5.9±0.3 for piperitone and 9.9 ± 0.3 for pulegone for both standard and sample (Figure 2 & 3). The column was equilibrated with the mobile phase flowing at 1.0 mL min^{-1} for about 30 min prior to injection. Subsequently, the chromatographic detections of piperitone and pulegone were monitored with a UV detector at 279 nm.

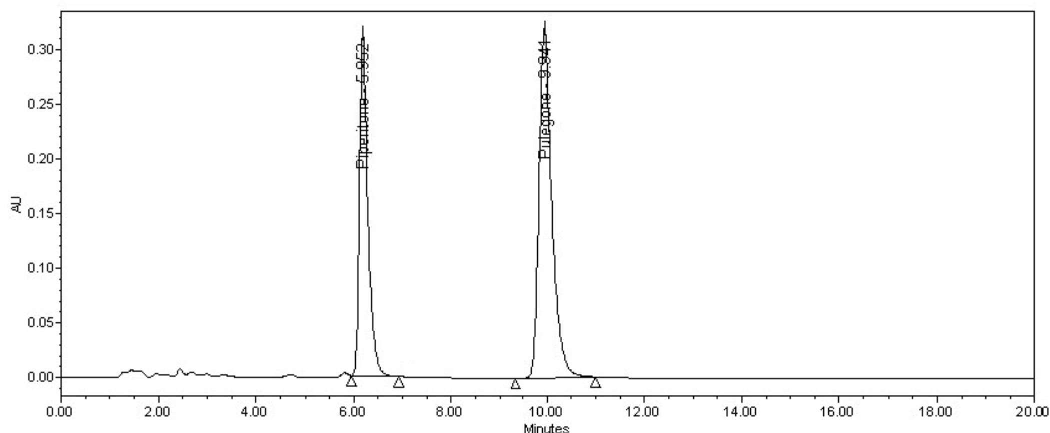

Figure 2 HPLC chromatogram of standard piperitone (R_t 5.952) and pulegone (R_t 9.941) at 279 nm

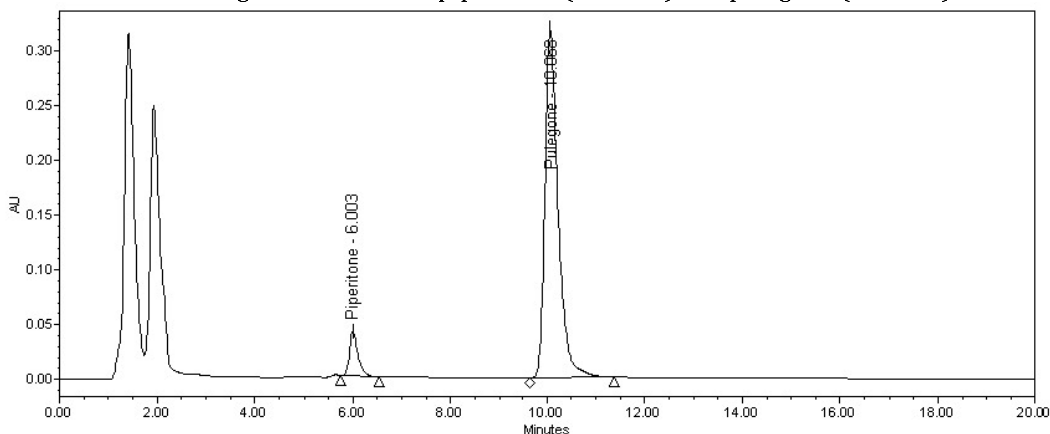

Figure 3 HPLC chromatogram of Mentha longifolia at 279 nm showing piperitone (R_t 6.003) and puligone (R_t 10.068).

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Method validation

The developed methodology was validated for its linearity, precision, accuracy, sensitivity, and robustness in accordance with the guidelines of ICH Q2 (Revision 1).

Accuracy

The recoveries of the method were found in the range of 97.69-101.39% and 96.99-102.71% for piperitone and pulegone, respectively. High recovery indicated that the proposed method was reliable and reproducible (Table 2).

Precision

The % RSD of repeatability for peak area of piperitone and pulegone was found to be 0.29-1.60 and 0.37-1.95, respectively. Intra-day and inter-days precision were studied by triplicate assay at three different quantities (50, 100, 200 µg mL^{-1}). Low RSD values (Table 3) indicated the method was precise.

Robustness

In all the deliberately varied chromatographic conditions (flow rate and column temperature) there was no significant change in the retention time and area observed. The low values (0.34-1.90% for piperitone and 0.22-2.11% pulegone) of the %RSD indicated the robustness of the method (Table 4).

Limit of detection and quantification

The LOD and LOQ were found as 3.5 and 11 µg mL^{-1} for piperitone; 8.0 and 20 µg mL^{-1} for pulegone respectively.

Analysis of sample

Using the novel RP-HPLC method, the piperitone and puligone in *Mentha longifolia* was quantifies successfully. The peak areas of samples were analysed to obtain the content in samples. The piperitone and puligone contents were found to be 0.088% and 0.022% w/w, respectively in *Mentha longifolia.* The method was found satisfactory and no interference was found at the retention of peaks of interest.

Table 1. Linearity of the method

Compound	Retention time ± SD (min)	Linear range (µg/mL^{-1})	Linear regression equation	Correlation coefficient (r^2)
Piperitone	7.3±0.03	10-500	y = 10530x + 53756	0.9950
Pulegone	9.90±0.03	20-500	y = 16255x + 2857	0.9990

Table 2. Accuracy of the method (n=3)

Piperitone			Pulegone		
Expected conc. (µg/mL)	Found (µg/mL)	Recovery %	Expected conc. (µg/mL)	Found (µg/mL)	Recovery %
22.4	23.4	98.29	70.4	69.89	99.28
33.6	33.9	101.39	105.6	108.47	102.71
44.8	44.2	97.69	140.8	117.54	99.83
56	55.3	99.52	176	170.70	96.99

Table 3. Precision of the method (n=3)

Analytes	Retention time (RSD) %		Peak area RSD (%)	
	Intra-day	Inter-day	Intra-day	Inter-day
Piperitone	0.77	0.88	0.44	1.80
	1.02	1.58	0.37	1.51
	0.95	0.88	1.95	1.21
Pulegone	0.29	1.60	0.44	1.64
	1.55	0.77	1.01	0.44
	0.69	0.75	1.92	0.31

Table 4.Robustness of the method by changing flow rate and column temperature (n=3)

<table>
<tr><th rowspan="2">Flow rate (mL/min)</th><th rowspan="2"></th><th colspan="2">Piperitone</th><th colspan="2">Pulegone</th></tr>
<tr><th>Retention time (RSD) %</th><th>Peak area RSD (%)</th><th>Retention time (RSD) %</th><th>Peak area RSD (%)</th></tr>
<tr><td rowspan="3"></td><td rowspan="3">0.8
1
1.2</td><td>0.72</td><td>1.90</td><td>1.20</td><td>0.66</td></tr>
<tr><td>1.4</td><td>0.40</td><td>2.11</td><td>0.49</td></tr>
<tr><td>0.66</td><td>0.58</td><td>1.52</td><td>0.52</td></tr>
<tr><td rowspan="3">Column temp. (˚C)</td><td rowspan="3">25
30
35</td><td>0.56</td><td>1.50</td><td>0.85</td><td>0.69</td></tr>
<tr><td>0.34</td><td>0.64</td><td>0.22</td><td>0.59</td></tr>
<tr><td>1.25</td><td>0.47</td><td>1.31</td><td>1.44</td></tr>
</table>

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CONCLUSION

The identification and quantification of the two compounds by liquid chromatography with UV detection in samples was successfully performed. The advantages of the method included the simplicity of the quantitative extraction and the simultaneous determination of these in a single chromatographic run. The low quantification limits pointing towards the sensitivity of the method. The validation data suggest the method was accurate and robust enough for the successful implementation of the method for the quality control of various crude and herbal formulations.

The proposed method exhibits many desirable features such as sensitivity, precision, accuracy and is thus recommended to be adopted as quality control protocol in pharmaceutical industries. As from the results obtained from the validation analysis we conclude that the proposed methodology can be suitable for the quality control and standardization of herbal formulation as well as crude drugs which contains either of these components. In summary, a rapid, robust, and precise HPLC method has been developed and validated for the determination of pulegone and piperitone simultaneously.

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