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Simultaneous Determination of (−)-Menthone and (−)-Menthol in Menthae Herba by Gas Chromatography and Principal Component Analysis

Jung-Hoon Kim, Chang-Seob Seo, and Hyeun-Kyoo Shin*

Herbal Medicine EBM Research Center, Standard Research Division,
Korea Institute of Oriental Medicine, Daejeon 305-811, Republic of Korea

Abstract – The simple and accurate method was established for the simultaneous determination of (−)-menthone and (−)-menthol in Menthae herba obtained from Korea and China. A quantitative analysis was performed with a gas chromatography-flame ionization detector and reference compounds were separated on a capillary HP-Innowax column (30 m × 0.23 mm, 0.50 μm, Agilent, MA, USA). The correlation coefficients of the compounds showed good linearity ($r^2 > 0.9997$) over the linear range. The precision, repeatability and stability showed less than 1.7% of relative standard deviation (RSD) values for two compounds. Recovery rates were within the range of 95.72 - 103.76%. The method was applied successfully to analyze 15 samples of Menthae herba and achieved sufficient and specific separation of reference compounds. The principal component analysis (PCA) exhibited the classification of 15 samples according to their locations of origin.
Keywords – Gas chromatography, Menthae herba, (−)-Menthone, (−)-Menthol, Principal component analysis

Introduction

Plants in the genus *Mentha* (Lamiaceae) are distributed worldwide and used in a variety of diverse ways in many countries. Among the genus *Mentha*, *M. arvensis* L. var. *piperascens* Malinvaud and *M. haplocalyx* Briq. are predominantly used in Korea and China respectively where they are officially classified as medicinal herbs (Pharmacopoeia of the People's Republic of China, 2005; Korean Pharmacopeia, 2007).

Menthae Herba has been used for thousands of years in Korea and China to treat numerous diseases, including low-grade fever caused by the common cold, headache, blood-shot eyes, sore throat, thrush, and rubella. The therapeutic activities of them are mainly due to $_L$-menthone and menthol consisting of essential oils [$_L$-menthone: 0.3 - 7.9%, while menthol: 77.5 - 89.3%] (Singh *et al*., 2005).

Owing to the volatile characteristics, most of the methods used to characterize menthone and menthol have been performed using gas chromatography (Ligor *et al*., 1999; Valdez *et al*., 1999; Nozal *et al*., 2002) or gas chromatography-mass spectrometry (Gherman *et al*., 2000; Lin *et al*., 2002). Previous studies have determined the chemical composition of essential oils such as menthone and menthol in *M. spicata*, *M. piperita*, *Thymus tosevii*, *T. vulgaris* and *Lavandula stoechas* ssp. *stoechas* (Gören *et al*., 2002; Sokoviæ *et al*., 2009).

In present study, a method for the simultaneous determination of (−)-menthone and (−)-menthol was established, and the quantification of these components using an internal standard (i.e., camphor) was performed. The method was successfully applied to analyze the Menthae herba samples from Korea and China. Furthermore, samples from each country were classified statistically via principal component analysis (PCA).

Materials and Methods

Reagents and plant materials – HPLC-grade *n*-hexane, chloroform, ethanol, and methanol were purchased from J.T. Baker Inc. (Phillipsburg, NJ, USA). (−)-Menthone (96%) and (−)-menthol (99.5%) were purchased from ChromaDex (Irvine, CA, USA). Camphor (96%) as an internal standard (I.S.) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Fifteen Menthae herba samples were obtained from local market, nine samples were from Korea and six samples were from China.

Chromatographic instrumentation and conditions – GC system was Shimadzu GC-17A equipped with an auto injector (AOC-20i) and a flame ionization detector

*Author for correspondence
Tel: +82-42-868-9464; E-mail: hkshin@kiom.re.kr

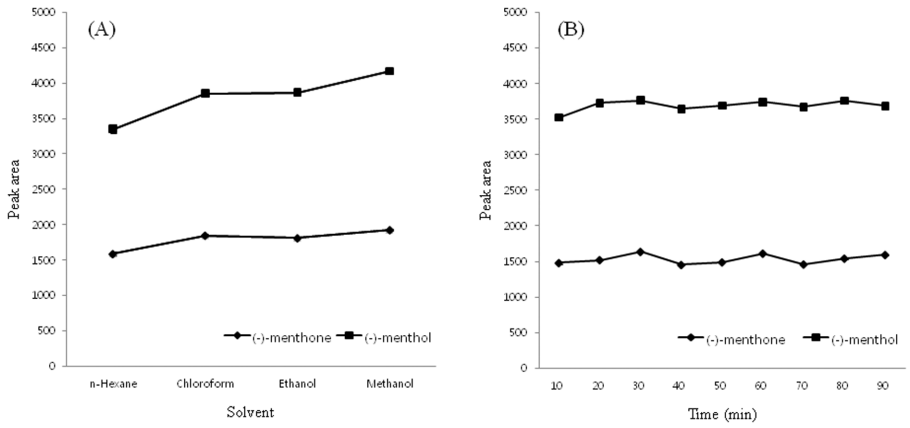

Fig. 1. Peak areas of reference compounds extracted using different solvents (A) and over duration time of extraction (B). The extraction with methanol for 30 min showed optimum yields of peak areas of reference compounds.

(CBM-102). The data acquisition system was manufactured by LC Solutions (Ver. 2.30). The capillary column was HP-Innowax (30 m × 0.23 mm, 0.50 μm, Agilent, MA, USA). N_2 was used as a carrier gas, while H_2 and compressed air were used for flame ionization detector (FID). The flow rate was 2.0 mL/min and the injection volume was 1.0 μL with a split ratio of 10 : 1. The initial column temperature started at 60 °C for 1 min, then increased at a rate of 10 °C/min to 250 °C and held at 250 °C for 10 min. The temperatures of the injection port and detector were maintained at 220 and 275 °C, respectively.

Preparation of sample solutions – Dried aerial parts of samples were pulverized through a 60 mesh sieve and accurately weighed (i.e., 1.0 g). The extraction was performed with 100 mL methanol by sonication for 30 min. The extracts were filtrated through a PTFE syringe filter (13 mm, 0.2 μm, Whatmann, Florham Park, NJ, USA) prior to GC injection.

Preparation of standard solutions – Accurately weighed standard compounds were dissolved in methanol at concentrations of 1 mg/mL. A stock solution containing the standard compounds and camphor (I.S.) was mixed and diluted to make working solutions in range of 7.81-125 μg/mL for (–)-menthone and 3.91 - 125 μg/mL for (–)-menthol, which were used to construct a calibration curve.

Linear regression, limit of detection (LOD) and limit of quantification (LOQ) – Linear regression analysis was performed by plotting the peak area ratio of each standard compound to that of the internal standard on the y-axis, versus the concentration of each component on the x-axis. The stock solutions of standard compounds were diluted to smallest concentration with methanol. The detectable smallest peaks were selected as signal, and LOD and LOQ were determined to signal-to-noise ratio of 3 and 10, respectively.

Precision and accuracy – The intra- and inter-day precisions were determined by analyzing three different concentrations of each compound (i.e., low, medium, high) spiked in sample extract. The precision was measured in five replicates of spiked samples for intra-day and five successive days for inter-day. The relative standard deviation (RSD) was measured to assess precision. The accuracy was evaluated by recovery test. The recovery test was conducted by adding three different concentrations of each known standard (i.e., low, medium, and high) to samples before extraction. The samples were extracted and analyzed using the method described above.

Results and discussion

Optimization of the extraction procedure – Different extraction solvents (i.e., *n*-hexane, chloroform, ethanol, and methanol) and extraction times (i.e., ranging from 10 to 90 min) were investigated and the yields of the reference compounds were analyzed. As shown in Fig. 1, the yields resulting from the use of methanol were higher than those resulting from the use of other solvents. Procedures lasting 30, 60, and 80 min produced similar yields. Hence, the extraction with methanol for 30 min was deemed the most suitable method of extracting the reference compounds.

Optimization of chromatographic conditions – Previous studies analyzed menthol and camphor simultaneously using wax columns (Valdez *et al*., 1999; Nozal *et al*., 2002), thus, the HP-Innowax column was used in our analysis. The column temperature was set to initiate the analysis at 60 °C and end the analysis at 250 °C. Under

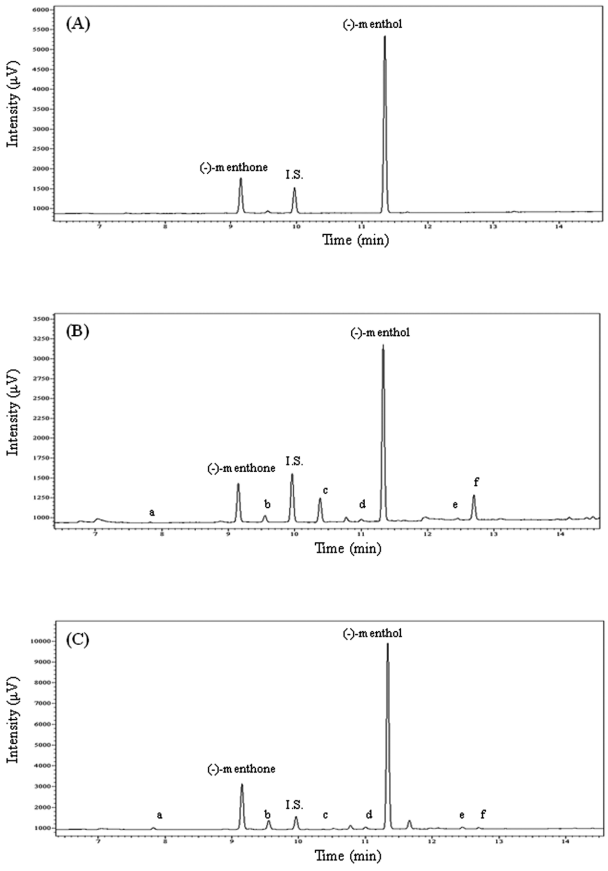

Fig. 2. The chromatograms showing baseline separation. The standard mixture (A), an Menthae Herba sample from Korea (B), an Menthae Herba sample from China (C): (I.S.), camphor; a~f, unidentified compounds.

the conditions described above, the peak resolution was sufficient for each compound and baseline separation was shown (Fig. 2A).

Linear regression, limit of detection (LOD) and limit of quantification (LOQ) – Stock solutions containing standard compounds were diluted to construct the calibration curves. At least six concentrations of solutions were analyzed with the linear range of 7.81 - 125 μg/mL for (−)-menthone and 3.91 - 125 μg/mL for (−)-menthol. Calibration curve showed good linearity ($r^2 > 0.9997$). The LOD and LOQ were 0.07 - 0.80 and 0.24 - 2.67 μg/mL, respectively (Table 1).

Precision and accuracy – The reproducibility of developed method was evaluated by the precisions of samples during intra- and inter-day. The intra- and inter-day variations of reference compounds were less than 1.5 and 0.8% of RSD values, respectively (Table 2).

To evaluate the accuracy of our method, recovery was measured by adding three different concentrations of mixed standard solutions to samples. Recovery values of the reference compounds ranged from 95.72 - 103.76%, with a RSD less than 3.0% (Table 3).

Repeatability and stability – Repeatability was determined by analyzing five sample solutions of Menthae herba. The RSD values showed good repeatability of 0.52% for (−)-menthone and 0.23% for (−)-menthol. The stabilities of the standard compounds were measured at room temperature and at 2 °C on 6 successive days, and good stabilities (RSD < 1.7%) were observed in both conditions.

Table 1. Linear regression, correlation coefficients (r^2), LOD and LOQ for the reference compounds

Compound	Linear equation	r^2	Linear range (μg/mL)	LOD[a] (μg/mL)	LOQ[b] (μg/mL)
(−)-menthone	y = 0.0108 × −0.0171	0.9999	7.81-125	0.80	2.67
(−)-menthol	y = 0.1031 × −0.0765	0.9998	3.91-125	0.07	0.24

[a] Limit of Detection.
[b] Limit of Quantification.

Table 2. Intra- and inter-day precision of the reference compounds

Compound	Spiked concentration (μg/mL)	Intra-day (n = 5)		Inter-day (n = 5)	
		Detected concentration (μg/mL)	RSD[a] (%)	Detected concentration (μg/mL)	RSD (%)
(−)-menthone	15	15.4	1.47	15.45	0.67
	30	27.88	1.18	27.88	0.19
	60	61.05	0.25	60.99	0.17
(−)-menthol	10	10.34	1.21	10.38	0.75
	20	19.18	0.38	19.09	0.4
	40	40.34	0.13	40.36	0.1

[a] Relative Standard Deviation (%) = (Standard Deviation / Mean) × 100.

Table 3. Recovery of the reference compounds

Compound	Initial concentration (μg/mL)	Spiked concentration (μg/mL)	Detected concentration (μg/mL)	Recovery[a] (%)	RSD[b] (%)[a]
(−)-menthone	70.21	15	15.56	103.76	1.78
		30	28.72	95.72	2.38
		60	63.62	105.97	1.57
(−)-menthol	49.40	10	10.26	102.61	2.25
		20	19.23	96.14	1.83
		40	41.18	102.95	0.49

[a] Recovery (%) = (Concentration detected – Concentration original) / Concentration spiked × 100
[b] Relative Standard Deviation (%) = (Standard Deviation / Mean) × 100.

Table 4. Contents (%) of reference compounds in Menthae Herba samples

Sample	(−)-Menthone (%)	(−)-Menthol (%)
k1[a]	0.62	0.48
k2	1.43	0.47
k3	0.74	0.32
k4	2.11	0.58
k5	1.67	0.33
k6	1.02	0.39
k7	0.55	0.32
k8	0.66	0.13
k9	0.51	0.42
c1[b]	3.30	1.31
c2	1.68	1.27
c3	1.51	1.05
c4	4.11	2.06
c5	2.66	2.67
c6	1.56	2.15

[a] k : Samples from Korea
[b] c : Samples from China

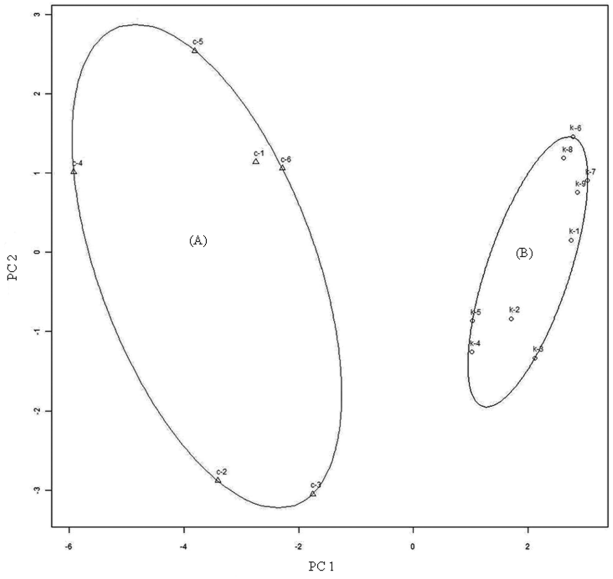

Fig. 3. Fifteen representative Menthae Herba samples on PC1 and PC2 (i.e., representing 77.8% of the total variance). c1-6, Chinese samples (A); k1-9, Korean samples (B).

Sample analysis – Fifteen samples (nine samples from Korea and six samples from China) were analyzed and the contents of the reference compounds were quantified. The variations in contents ranged from 0.5 - 2.2% for (−)-menthone and from 0.3 - 0.6% for (−)-menthol in samples from Korea. However, the variations ranged from 1.5 - 4.2% for (−)-menthone and 1.6 - 2.7% for (−)-menthol in samples from China. Although the (−)-menthone contents of some Chinese samples were similar to those of Korean samples, the contents of (−)-menthol were higher in samples from China than those from Korea (Table 4). The apparent differences in the content of each compound may reflect differences in the species of medicinal plants used at each location (e.g., *M. arvensis* var. *piperascens* in Korea, *M. haplocalyx* in China) or the different parts of the plant used for medicinal purposes (i.e., the aerial parts in Korea versus the leaves in China) (Figs. 2B and 2C).

PCA was performed to investigate the classification of the herbs. The contents of unidentified compounds which showed their own retention times on the chromatogram were analyzed by PCA along with the reference compounds. Our results revealed that the first and second principal components (i.e., PC1 and PC2) described 60.1 and 17.7%, respectively, of the variance and 77.8% of the total variance. The scatter plots were clustered into two groups, indicating that Menthae herbs were clearly classified according to their countries of origin (Fig. 3).

Conclusion

In this study, we used an GC method to simultaneously

determine two reference compounds in samples of Menthae herba. This method is simple, accurate and specific to each reference compound. Furthermore, this method showed good linearity, repeatability, precision and accuracy. Thus, the application of this method would be suitable and robust option for the quantitative analysis of Menthae herba. In addition, PCA can be used to differentiate medicinal herbs from different countries.

Acknowledgments

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References

Gherman, C., Culea, M., and Cozar, O., Comparative analysis of some active principles of herb plants by GC/MS. *Talanta* **53**, 253-262 (2000).

Gören, A., Topçu, G., Bilsel, G., Bilsel, M., Aydoğmuþ, Z., and Pezzuto, J.M., The chemical constituents and biological activity of essential oil of *Lavandula stoechas* ssp. *stoechas*. *Z. Naturforsch C*. **57**, 797-800 (2002).

Ligor, M. and Buszewski, B., Determination of menthol and menthone in food and pharmaceutical products by solid-phase microextraction-gas chromatography. *J. Chromatogr. A* **847**, 161-169 (1999).

Lin, R., Tian, J., Huang, G., Li, T., and Li, F., Analysis of menthol in three traditional Chinese medicinal herbs and their compound formulation by GC-MS. *Biomed. Chromatogr.* **16**, 229-233 (2002).

National Pharmacopoeia Commission of the People's Republic of China, Pharmacopoeia of the People's Republic of China, The Chemical Industry Press, Beijing, 261, 2005.

Nozal, M.J., Bernal, J.L., Jimenez, J.J., Gonzalez, M.J., and Higes, M., Extraction of thymol, eucalyptol, menthol, and camphor residues from honey and beeswax : Determination by gas chromatography with flame ionization detection. *J. Chromatogr. A* **954**, 207-215 (2002).

Singh, A.K., Raina, V.K., Naqvi, A.A., Patra, N.K., Kumar, B., Ram, P., and Khanuja, S.P.S., Essential oil composition and chemoarrays of menthol mint (*Mentha arvensis* L. F. *piperascens* Malinvaud ex. Holmes) cultivars. *Flavour. Frag. J.* **20**, 302-305 (2005).

Soković, M.D., Vukojević, J., Marin, P.D., Brkić, D.D., Vajs, V., and Griensven, L.J.V., Chemical composition of essential oils of *Thymus* and *Mentha* species and their antifungal activities. *Molecules* **14**, 238-249 (2009).

South Korea college of pharmacy conference, Pharmacopoeia person and society, Korean Pharmacopeia, Shinilbooks, Seoul, 1123, 2007.

Valdez, J.S., Martin, D.K., and Mayersohn, M., Sensitive and selective gas chromatographic methods for the quantitation of camphor, menthol and methyl salicylate from human plasma. *J. Chromatogr. B* **729**, 163-171 (1999).

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