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Mi Hee Park

SFC BIO

Chul Jin Kim

SFC BIO

Jin Young Lee

SFC BIO

In Seon Kim

SFC BIO

Sung-kyu Kim (✉ skkim@sfcbio.com)

SFC BIO

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Development and validation of a gas chromatography method for the determination of β -caryophyllene in clove extract and its application

Mi Hee Park¹, Chul Jin Kim¹, Jin Young Lee¹, In Seon Kim¹, Sung-kyu Kim^{1*}

¹SFC bio Co., Ltd., 119, Dandae-ro, Dongnam-gu, Cheonan-si, Chungnam 31116, Korea

Correspondence: Sung-kyu Kim, Ph.D.

Address: SFC bio Co., Ltd., 119, Dandae-ro, Dongnam-gu, Cheonan-si, Chungnam 31116, Korea

Phone: 82-41-566-7151

Fax: 82-41-566-7161

E-mail: skkim@sfcbio.com

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Abstract

The purpose of this study is to check the effectiveness of the analysis method that separates and quantifies β -caryophyllene among clove extracts and validate according to current ICH guidelines. The beta caryophyllene was active constituent of clove buds. The developed method gave a good detection response. In the specificity test, the standard solution was detected at about 17.316 minutes, and the test solution was detected at 17.317 minutes. The linearity of β -caryophyllen was confirmed, and at this time, the correlation coefficient (R^2) of the calibration curve showed a high linearity of 0.999 or more in the concentration range. The levels of LOD and LOQ were 1.28 ug/mL and 3.89 ug/mL, respectively. The accuracy was confirmed to be 101.6 ~ 102.2% and RSD 0.95 ~ 1.31%. As a result of checking the repeatability and inter-tester reproducibility to confirm the precision, the RSD was found to be 1.34 ~ 2.69%. This validated GC method was successfully applied to a soft capsule containing clove extract and other materials for clinical trials. Therefore, this method can be used as an analytical tool for quality control of various samples including clove extracts and their products for food and pharmaceutical uses.

Introduction

The preparation of functional materials and pharmacological products requires the development of good validated methods to ensure its quality, efficacy and safety within different samples. For that, the validation should be completed to confirm that an analytical method is selective, robust, accurate and reproducible at the specified range [1]. To perform the method validation, it is recommended to follow the guidelines from the regulatory agencies [2]. Mandatory Criteria for the quality of natural compounds, pharmaceuticals, foods, cosmetics, and other products are ensured by method validation [3]. Regarding development of therapeutic or functional agents from plants, a validated analytical method is required at every step. Among other things, it is necessary at the step of the quantification of the active compounds in biological components for tracking preclinical and clinical analysis [4]. Moreover, the manufacturing conditions of active pharmaceutical ingredients require proper quality control of the various ingredients involved in the synthesis [5]. Permissible limit for these materials are given in the ICH guidelines [6].

In this study, we developed and validated analysis method for the estimation of β -caryophyllene in clove extract for application to functional materials according to ICH guidelines. Actually, β -caryophyllene is present in a various plant species such as cloves, cinnamon leaves, copaiba balsam and basil, and a natural bicyclic sesquiterpene [7] It is a main ingredient in various essential oils obtained from a number of plant species such as the *Strobilanthes* (~7%), *Syzygium* (~13%) and *Betula* (~30%) species [8–10]. It has been known to have antimicrobial, anti-inflammatory, anticancer and antioxidant effects [11–14]. Clove extract oil is produced by extraction from the dried clove buds of the clove plant. Traditionally, it has been applied as a flavouring spice in foods as a fragrance [15-16]. It is also found in topical analgesics. The US Food and Drug Administration (FDA) categorizes clove oil or its components as generally recognized as safe (GRAS) for purpose of dental

cement use or food additive use [17]. In MFDS (Ministry of Food and Drug Safety), the clove buds are classified as food ingredient. Clove bud is categorized as fragrant plant in vegetable raw materials [18]. Clove represents great potential for pharmaceutical, food, cosmetic and agricultural applications [19]. Moreover, we already found that clove extract containing 98% β -caryophyllene was effective in the treatment of *H.pylori* infection and inflammatory bowel disease [20-23].

So, we will apply this material for therapeutic uses. For that, we developed the validation of methods to ensure its quality, safety and efficacy in this work. The method was effectively validated according to the contents: linearity, limit of detection and quantification, selectivity, precision and accuracy. The application of the developed method in commercial formulations containing clove extract will be very satisfactory.

Results

Method validation

The validation of the optimized method was performed in accordance with the ICH guidelines. The following parameters were considered: specificity, Linearity, Limit of detection (LOD), limit of quantification (LOQ), Accuracy, Recovery and Precision.

1) Specificity

The standard solution was detected at about 17.316 minutes, and the test solution was detected at 17.317 minutes. The chromatograms of the standard solution and test solution are as shown in Fig.1.

2) Linearity, Limit of detection (LOD) and limit of quantification (LOQ)

As for the standard solution, 25 mg of the standard was dissolved in ethanol in a 25 mL diaphragm flask, and the diaphragm was sequentially diluted with ethanol, and samples of 5.04 to 201.6 ug/mL were analyzed by instrument. As a result, the linearity of β -caryophyllen was confirmed, and at this time, the correlation coefficient (R^2) of the calibration curve showed a high linearity of 0.999 or more in the concentration range (Table 1). The detection and quantification limit is the value obtained by multiplying the standard deviation of the y-intercept by 3.3 times the standard deviation of the y-intercept by the average value of the slope using the calibration curve analyzed three times as the detection limit, and quantifying the value divided by the average value of the slope of the value multiplied by 10 times. It was set as the limit. As a result, the levels were 1.28 ug/mL and 3.89 ug/mL, respectively.

3) Accuracy and Recovery

In order to measure the accuracy of β -caryophyllene in the clove extract, the matrix effect was examined through the percentage recovered by spiking/recovery at different concentrations in the sample. A certain amount of sample was collected according to the

standard substance addition method, and the concentration of 100, 200, and 400 ug/mL of standard solution was added and adjusted to 50 mL, diluted appropriately, and the detection concentration was measured. As a result, the recovery rates at each concentration were 101.6%, 101.6%, and 102.2%, and the relative standard deviations were 1.31%, 1.04%, and 0.95% (Table 2).

4) Precision

(1) Repeatability

In order to check the repetition accuracy for the change in the sample amount, samples were taken at 0.1 g, 0.5 g, and 1.0 g, respectively, and measured repeatedly 6 times each. As a result of checking the relative standard deviation (RSD) of each concentration, it was found to be 1.34 ~ 2.69% (Table 3).

(2) Reproducibility

In order to confirm the reproducibility of the analysis of the content of β -caryophyllene in the clove extract, the analysis was performed with different analysts and analysis dates. As a result of the analysis between experiments, the content of β -caryophyllene was analyzed as an average of 967.225 mg/g, at which time the standard deviation was 6.03 mg/g and the relative standard deviation was 0.62% (Table 4).

Real sample analysis

The method was applied for real sample determination. We analyzed the β -caryophyllene in soft capsule contained clove extract, modified starch, glycerin, Carrageenan, and others. As shown in Fig.2, the β -caryophyllene was detected at 17.32min at a concentration of 238.629 mg, and this result was almost similar to the mixing ratio.

Discussion

In this study, a gas chromatography (GC) method was developed and validated for the estimation of major chemical marker, β -caryophyllene in clove extract. GC is the useful analytical technique for essential oil samples. And the ability of the GC method to effectively separate volatiles in a short analysis time is determined by several factors [24-25]. This method has been validated according to ICH guidelines for various parameters, such as specificity, limit of detection, and quantification, linearity, precision and accuracy. The validation parameters tested were found to be within acceptable limits. This method has been successfully applied to the quantification of pharmaceutical formulations.

In this test method, for the determination of β -caryophyllene in the clove extract, a standard substance and a sample were dissolved in ethanol, extracted, and analyzed by GC chromatography. As a result of GC analysis to confirm the specificity, the detection time was detected at 17 minutes for both the standard solution and the sample, and linearity was confirmed with $R^2=0.999$ or more in the range of 5.08 to 201.6 $\mu\text{g/mL}$ of the standard solution. As a result of reviewing the recovery rate by adding the standard material, the accuracy was confirmed to be 101.6 ~ 102.2% and RSD 0.95 ~ 1.31%. As a result of checking the repeatability and inter-tester reproducibility to confirm the precision, the average was 978.71 mg/g, and RSD was found to be 1.34 ~ 2.69%.

We applied this method for real sample determination. Actually, we produced the pharmaceutical materials to perform the clinical trials. In the previous study, we showed that the clove extract was effective for the eradication of *H.pylori* in mouse model. Based on the concentration of animal study, we calculated the human equivalent dose (HED), and then, the daily intake dose was determined. We manufactured the soft capsule to take twice a day, so one capsule contained about 243.0042 mg of clove extract (98% β -caryophyllene). We analyzed the β -caryophyllene concentration in the soft capsule, and the β -caryophyllene was

detected at 17.32 min at a concentration of 238.629 mg, and this result was almost similar to the mixing ratio. So, this method will be useful for the commercial formulations containing clove extract, especially for applications such as pharmaceutical drugs, functional foods or medical foods.

It is important to point out that β -caryophyllene appear to be the chemical markers in clove extract because a large diversity of studies involving β -caryophyllene have been demonstrated with their high potential as antimicrobial, anti-inflammatory, anti-allergic, insecticidal, and anti-plasmodial. Taking into account the importance of the clove extract for the development of new natural products, and its large use in functional foods and pharmaceutical and cosmetic industries, as well as the lack of validated analytical methods to accurately quantify these compounds in raw material and its products, we report a complete validated method by gas chromatography.

Methods

Instrumentation and Analytical Conditions

A gas chromatography with flame ionization detector (Agilent 8890N(G3540A)) was used for the determination of β -caryophyllene in clove extract. All the gases used in these studies were of pharmacopotential purity. The analysis conditions are as shown below.

Item	Condition
Column	HP-1 (30 m $\times$ 0.25 mm, 0.25 μ m)
Inlet temperature	240 $^{\circ}$ C
Column temperature	50 $^{\circ}$ C (5 min) $\rightarrow$ 10 $^{\circ}$ C/min $\rightarrow$ 280 $^{\circ}$ C (10 min)
Detector temperature	295 $^{\circ}$ C
Carrier gas and flow	N ₂ , 1.0 mL/min
Split ratio	20 : 1
Split ratio	20 : 1

Preparation of clove extract and test solution

Clove extract (98% β -caryophyllene) was obtained from Bordas (Sevilla, Spain). For the preparation of test solution, after weighing a sample, precisely weigh it in a 50 mL volumetric flask, add ethanol to extract it, and filter it with a 0.45 μ m nylon filter and use it as a test solution.

Preparation of standard solution

Take a standard product ((-)-trans-Caryophyllene (CAS: 87-44-5 MW: 204.35 g/mol) and dissolve it in HPLC grade ethanol to prepare it, and dilute it appropriately to use it as a

standard solution.

Production of soft capsule

We prepared the soft capsule for clinical study. In one capsule, 243.0042mg of the clove extract was contained. The capsule was produced in GMP factory (Suheung, Osong, Korea). In this study, we analyzed the β -caryophyllene concentration in the soft capsule.

Test method

1) Specificity

In order to confirm the specificity, the retention time and degree of separation in the standard and sample were checked. The standard substance and test solution (clove extract containing 98% β -caryophyllene) were analyzed by the same method, and the detected peak was confirmed to confirm that the substance was the same.

2) Linearity, Limit of detection (LOD) and limit of quantification (LOQ)

As for the standard solution, 25 mg of the standard was dissolved in ethanol in a 25 mL diaphragm flask, and the diaphragm was sequentially diluted with ethanol, and samples of 5.04 to 201.6 ug/mL were analyzed by instrument. The detection and quantification limit is the value obtained by multiplying the standard deviation of the y-intercept by 3.3 times the standard deviation of the y-intercept by the average value of the slope using the calibration curve analyzed three times as the detection limit, and quantifying the value divided by the average value of the slope of the value multiplied by 10 times.

3) Accuracy, Recovery

In order to measure the accuracy of β -caryophyllene in the clove extract, the matrix effect was examined through the percentage recovered by spiking/recovery at different concentrations in the sample. A certain amount of sample was collected according to the standard substance addition method, and the concentration of 100, 200, and 400 ug/mL of

standard solution was added and adjusted to 50 mL, diluted appropriately, and the detection concentration was measured.

4) Precision

In order to check the repetition accuracy for the change in the sample amount, samples were taken at 0.1 g, 0.5 g, and 1.0 g, respectively, and measured repeatedly 6 times each. In order to confirm the reproducibility of the analysis of the content of β -caryophyllene in the clove extract, the analysis was performed with different analysts and analysis dates.

References

- [1] González, A.G. & Herrador, M.A. A practical guide to analytical method validation, including measurement uncertainty and accuracy profiles. *Trends Anal. Chem.* 26, 227–238 (2007).
- [2] Geneva. International Conference on Harmonization (ICH) of Technical Requirements for registration of Pharmaceuticals for Human Use Topic Q2 (R1): Validation of Analytical Procedures: Text and Methodology (2005).
- [3] João Paulo B Sousa, Ana P S Brancalion, Ariana B Souza, Izabel C C Turatti, Sérgio R Ambrósio, Niede A J C Furtado, Norberto P Lopes & Jairo K Bastos. Validation of a gas chromatographic method to quantify sesquiterpenes in copaiba oils. *J Pharm Biomed Anal.* 54, 653-659 (2011) .
- [4] SK Manirul Haque & Elaref, S. Ratemi Drug Development and Analysis Review. *Pharmaceutical Chemistry Journal* 50, 837–850 (2017) .
- [5] Petrelli Fabio, Caraffa Alessandro, Scuri Stefania, Grappasonni Iolanda, Magrini Elena, & Cocchini Aldo The requirements for manufacturing highly active or sensitising drugs comparing Good Manufacturing Practices. *Acta Biomed.* 90, 288–299 (2019) .
- [6] Olena Golembiovska, Oleksii Voskoboinik, Galina Berest, Sergiy Kovalenko & Liliya Logoyda. Method development and validation for the determination of residual solvents in quinabut API by using gas chromatography. *Message 2. Pharmacia* 68, 53-59 (2021).
- [7] Opdyke, D. L. (1973) Monographs on fragrance raw materials. *Food Cosmet. Toxicol.* 11, 1011–1081.
- [8] Agarwal, R.B. & Rangari, V.D. Phytochemical investigation and evaluation of anti-

inflammatory and anti-arthritic activities of essential oil of *Strobilanthus ixiocephala* Benth. Indian J. Exp. Biol. 41, 890–894 (2003) .

[9] Demirci, B., Baser, K.H., Demirci, F. & Hamann, M.T. New caryophyllene derivatives from *Betula litwinowii*. J. Nat. Prod. 63, 902–904 (2000).

[10] Prashar, A., Locke, I.C. & Evans, C.S. Cytotoxicity of clove (*Syzygium aromaticum*) oil and its major components to human skin cells. Cell Prolif. 39, 241–248 (2006) .

[11] Pichette, A., Larouche, P.L., Lebrun, M. & Legault, J. Composition and antibacterial activity of *Abies balsamea* essential oil. Phytother. Res. 20, 371–373 (2006).

[12] Kubo, I., Chaudhuri, S.K, Kubo, Y., Sanchez, Y., Ogura, T., Saito, T., Ishikawa, H. & Haraguchi, H. Cytotoxic and antioxidative sesquiterpenoids from *Heterotheca inuloides*. Planta Med. 62, 427–430 (1996) .

[13] Cho, J.Y., Chang, H.J., Lee, S.K., Kim, H.J., Hwang, J.K. & Chun, H.S. Amelioration of dextran sulfate sodium-induced colitis in mice by oral administration of beta-caryophyllene, a sesquiterpene. Life Sci. 80, 932–939 (2007) .

[14] Singh, G., Marimuthu, P., De Heluani, C.S. & Catalan, C. Antioxidant and biocidal activities of *Carum nigrum* (seed) essential oil, oleoresin, and their selected components. J. Agric. Food Chem. 54, 174–181 (2006) .

[15] "Clove". Drugs.com. (2018)

[16] Balch, Phyllis, Balch & James Prescription for Nutritional Healing. 3rd ed., Avery Publishing 94 (2000) .

[17] Jasna Ivanovic, Suzana Dimitrijevic Brankovic, Dusan Misic, Mihailo Ristic & Irena Zizovic. Evaluation and improvement of antioxidant and antibacterial activities of

supercritical extracts from clove buds. *Journal of Functional Foods* 5,416-423 (2013).

[18] Food Code and Food Additive Code in Ministry of Food and Drug Safety

[19] Diego Francisco Cortés-Rojas, Claudia Regina Fernandesde Souza & Wanderley PereiraOliveira. Clove (*Syzygium aromaticum*): a precious spice. *Asian Pacific Journal of Tropical Biomedicine*. 4, 90-96 (2014) .

[20] Jung, D.H., Park, M.H., Kim, C.J., Lee, J.Y., Keum, C.Y., Kim, I.S., Yun, C.H., Kim, S.K., Kim, W.H. & Lee, Y.C. Effect of β -caryophyllene from Cloves Extract on *Helicobacter pylori* Eradication in Mouse Model. *Nutrients* 12, 1000 (2000) .

[21] Woo, H.J., Yang, J.Y., Lee, M.H., Kim, H.W., Kwon, H.J., Park, M., Kim, S.K., Park, S.Y., Kim, S.H. & Kim, J.B. Inhibitory Effects of β -Caryophyllene on *Helicobacter pylori* Infection In Vitro and In Vivo. *Int. J. Mol. Sci.* .21, 1008 (2020).

[22] Cho, J.Y., Chang, H.J., Lee, S.K., Kim, H.J., Hwang, J.K. & Chun, H.S. Amelioration of dextran sulfate sodium-induced colitis in mice by oral administration of beta-caryophyllene, a sesquiterpene. *Life Sci.* 80, 932-939 (2007) .

[23] Cho, J.Y., Kim, H.Y., Kim, S.K., Park, H.Y., Lee, H.J. & Chun, H.S. β -Caryophyllene attenuates dextran sulfate sodium-induced colitis in mice via modulation of gene expression associated mainly with colon inflammation. *Toxicol. Rep.* 2,1039-1045 (2015) .

[24] Marriot, P.J., Shellie, R. & Cornwell, C. Gas chromatographic technologies for the analysis of essential oils. *J. Chromatogr. A*. 936, 1-22 (2001) .

[25] Sanpeng Fan, Jin Chang, Yufeng Zong, Gaosheng Hu & Jingming Jia. GC-MS Analysis of the Composition of the Essential Oil from *Dendranthema indicum* Var. *Aromaticum* Using Three Extraction Methods and Two Columns. *Molecules*. 23, 576 (2018) .

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Author contributions

PMH wrote the paper and conducted the analysis of the data. KCJ contributed to set the analysis methods, LJY and KIS performed the GC experiment. KSK contributed to research planning, in charge of overall research and development of soft capsule product.

Competing interests

The authors declare no competing interests

Figure and Figure legends

Fig. 1.

A. Test solution

B. Standard solution

Fig. 1. β -caryophyllene chromatogram of standard solution and test solution.

The chromatogram data represents the β -caryophyllene in clove extract (test solution) and standard solution ((-)-trans-Caryophyllene (CAS: 87-44-5 MW: 204.35 g/mol). β -caryophyllene was detected at about 17.317 minutes in test solution, and at 17.316 minutes in the standard solution,

Fig. 2.

Fig. 2. β -caryophyllene chromatogram of soft capsule

β -caryophyllene was analyzed the in soft capsule contained clove extract, modified starch, glycerin, Carrageenan, and others. The β -caryophyllene was detected at 17.32min at a concentration of 238.629 mg, and this result was almost similar to the mixing ratio.

Tables

Table 1. Linearity, Limit of detection (LOD) and limit of quantification (LOQ)

Number of repetitions	1		2		3	
	Conc. (ug/mL)	Area	Conc. (ug/mL)	Area	Conc. (ug/mL)	Area
1	5.04	6.905	5.04	7.161	5.04	7.632
2	10.08	13.936	10.08	14.044	10.08	13.998
3	25.2	36.226	25.2	36.418	25.2	37.072
4	50.4	65.874	50.4	68.683	50.4	70.121
5	100.8	131.287	100.8	129.593	100.8	131.312
6	201.6	256.655	201.6	262.917	201.6	275.587
Inclination	1.27962		1.29536		1.35371	
y-intercept	1.40588		1.36925		0.50727	
R ²	0.99979		0.99966		0.99923	
Calibration curve
Slope mean (s)	1.31		SD of y-intercept(δ)		0.51	
Limit of detection	1.28					
Limit of quantificati on	3.89					

Table 2. Accuracy of β -Caryophyllene

Treatment	Spiked Conc.(ug/mL)		
	100	200	400
1	1062.900	1195.654	1394.463
2	1082.257	1175.211	1383.116
3	1093.577	1166.552	1406.975
4	1067.140	1178.607	1376.881
Measured mean (mg/g)	1076.468	1179.006	1390.359
%RSD	1.31	1.04	0.95
Recovery mean(%)	101.6	101.6	102.2

Table 3. Repeatability of β -Caryophyllene

Treatment	Sample Content (g)		
	0.1 g	0.5 g	1.0 g
1	1026.648	950.692	969.532
2	950.247	953.141	948.116
3	993.056	973.061	981.012
4	996.005	968.798	967.862
5	1018.699	963.468	969.341
6	1005.163	990.472	985.471
Measured mean (mg/g)	998.303	966.605	970.222
%RSD	2.69	1.51	1.34

Table 4. Reproducibility of β -Caryophyllene

	Test date and analyst			
	2020.10.20., analyst A		2020.10.23., analyst B	
	0.5 g	1.0 g	0.5 g	1.0 g
1	977.332	994.886	967.114	984.609
2	980.525	975.808	969.481	965.449
3	957.202	958.808	947.049	948.277
4	989.845	958.043	979.689	947.894
5	968.440	959.856	958.089	949.447
6	965.053	983.083	955.024	972.406
Average (mg/g)	973.066	971.747	962.741	961.347
%RSD	0.62			

Figures

A. Test solution

B. Standard solution

Figure 1

β -caryophyllene chromatogram of standard solution and test solution. The chromatogram data represents the β -caryophyllene in clove extract (test solution) and standard solution ((-)-trans-Caryophyllene (CAS: 87-44-5 MW: 204.35 g/mol). β -caryophyllene was detected at about 17.317 minutes in test solution, and at 17.316 minutes in the standard solution,

Figure 2

β -caryophyllene chromatogram of soft capsule. β -caryophyllene was analyzed the in soft capsule contained clove extract, modified starch, glycerin, Carrageenan, and others. The β -caryophyllene was detected at 17.32min at a concentration of 238.629 mg, and this result was almost similar to the mixing ratio.