# Enhancement of teprenone isomer separation by subcritical fluid chromatography using porous graphitic carbon column

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## Abstract

Teprenone is a therapeutic anti-ulcer agent developed in Japan. As described in the Japanese Pharmacopoeia (JP) 17th Edition, gas chromatography/hydrogen flame ionization detection (GC/FID) and high-performance liquid chromatography/ultraviolet detection (HPLC/UV) have been used for the assay of active pharmaceutical ingredients (APIs) and teprenone capsules, respectively. The critical aspect of the assay is a separation of the structural isomers (mono-cis and all-trans) of teprenone. Herein, we propose an improved quantitative method for the quality control of teprenone in APIs and capsules via subcritical fluid chromatography/photo diode array detection (SubFC/PDA) using a porous graphitic carbon column. SubFC conditions, i.e., type and content of the organic modifier in the mobile phase, column temperature, injection volume, and flow rate, were optimized. The developed SubFC/PDA method was validated according to ICH guidelines Q2(R1) in terms of accuracy, precision (repeatability and intermediate precision), specificity, linearity, quantification range, robustness, and stability. Comparison of SubFC/PDA method with the GC/FID or HPLC/UV method (described in JP) revealed that the SubFC/PDA method gave better resolution and run time than the JP methods. The developed SubFC/PDA method is expected to be useful for pharmaceutical analysis or quality control of teprenone isomers.

**Keywords:** Teprenone, Subcritical fluid chromatography, Mono-cis, All-trans, Graphitic carbon column, Japanese Pharmacopoeia

## Introduction

Teprenone, an acyclic polyisoprenoid compound, and is sometimes called tetraprenylacetone or geranylgeranylacetone (Murakami et al. 1981), is a therapeutic agent developed in Japan for the treatment of gastric ulcers and gastritis. It protects gastric mucosal lesions by increasing the amount of gastric mucus in the injured area of the patient's stomach without affecting physiological functions, such as gastric juice secretion or stomach exercise. This involves increasing the number of important macromolecule proteins and phospholipids as a defense factor in gastric mucosa and gastric mucus by enhancing the biosynthesis of glycolipids in microsomes of gastric mucosal cells. Teprenone has been reported to protect against ulcers induced by hydrochloric acid, aspirin, and ethanol, and to improve the poor proliferative capacity of gastric mucosal proliferation cells in hydrocortisone-induced ulcers (Arakawa et al. 1988; Murakami et al. 1982). It maintained the homeostasis of gastric mucosal proliferation cells to promote the healing of mucosal injury, and enhanced the biosynthesis of prostaglandin E2 in the gastric body and esophageal mucosa of normal rats. Teprenone

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promotes acetic acid-induced chronic gastric ulcer healing in rats (Kobayashi et al. 2001) and is now widely used clinically because of its low drug-drug interaction, unlike antacids containing metal ions (Saruta et al. 2010).

Teprenone is a mixture of (5*E*, 9*E*, 13*E*)-geranylgeranylacetone (hereinafter referred to as "all-trans" form) and (5*Z*, 9*E*, 13*E*)-geranylgeranylacetone (hereinafter referred to as "mono-cis" form). The APIs of teprenone should contain the mono-cis (Fig. 1A) and all-trans (Fig. 1B) forms at a ratio of approximately 2:3, and constitute 97.0–101.0% of teprenone.

Quantitation of teprenone is required for quality control of active pharmaceutical ingredients (APIs) and capsule preparation. In the pharmaceutical industry, quality control of teprenone has been performed by GC and HPLC according to the assay method in Japanese Pharmacopoeia (JP) 17th Edition. However, separation efficiency between teprenone isomers is poor in HPLC, and analysis time is too long in GC. Gas chromatography/hydrogen flame ionization detection (GC/FID) using packed silica-gel column and high-performance liquid chromatography/ultraviolet detection (HPLC/UV) using octadecylsilanized silica gel column have been applied for the assay of APIs and teprenone capsules, respectively (The Ministry of Health 2016).

Previously reported analytical studies for APIs and teprenone capsules were also based on the GC/FID (Guang 2009) or HPLC/UV (Xiao-jun 2007) methods described in the JP. The bioanalytical methods for human pharmacokinetics have been developed using high-performance liquid chromatography/fluorescence detection (HPLC/FLD) with fluorescent derivatization of teprenone (Seki et al. 1988), gas chromatography–mass spectrometry (GC–MS) (Tanaka et al. 1982), and liquid chromatography–mass spectrometry (LC–MS) (Ding et al. 2007).

However, these previous reported methods including JP methods using HPLC/UV were not able to completely separate the mono-cis and all-trans forms of teprenone. The reported method using GC/FID has a disadvantage

of a long analysis time (~20 min) for separation of teprenone isomers. Therefore, it was not satisfactory as an analytical method used for quality control of teprenone APIs and capsules. To improve this, subcritical fluid chromatography (SubFC) and a porous graphitic carbon (PGC) column were introduced in our study. The combination of SubFC and PGC columns was expected to separate stereo isomers of teprenone effectively with good resolution within a short run time and showed good performance as expected. West et al. analyzed substituted aromatic isomers (o-, m-, p-xylene, etc.) with PGC column in SFC. In this paper, very similar positional isomers were separated within approximately 6 min (West and Lesellier 2005b). Gyllenhaal et al. separated selectively metoprolol from related amino alcohols using PGC column in SFC. This paper showed separation of closely structurally related substances (Gyllenhaal and Karlsson 2010).

In our study, mono-cis and all-trans forms of teprenone in capsules were separated by a SubFC/photo diode array detection (PDA) method using a PGC column to overcome and improve the disadvantages of the conventional LC and GC methods, and the quantitative analysis method of teprenone was developed and validated. SFC or SubFC has been a powerful analytical technique to separate the structural isomers within short run time that uses supercritical (or subcritical) fluid and is orthogonal to reversed-phase chromatography. The most commonly used supercritical (or subcritical) fluid is $CO_2$ and requires temperature and pressure conditions above those of the thermodynamic critical point as a phase, not as liquid or gas. The critical point of $CO_2$ is at a temperature of 31 °C and pressure of 73.8 bar. The supercritical (or subcritical) fluid has properties intermediate between those of liquid and gas. It has lower viscosity than that of liquid and higher solubility and density than those of gas. Because of these properties, high flow rates can be applied, enabling fast and highly efficient analysis.
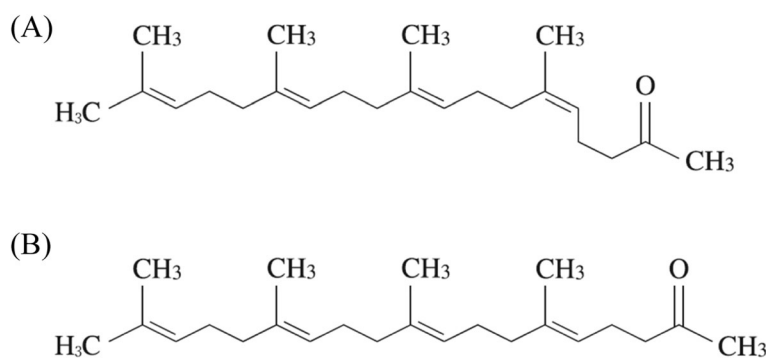


**Fig. 1** Chemical structures of (**A**) mono-cis teprenone and (**B**) all-trans teprenone

The PGC stationary phase used herein, which consists of hexagonal sheets of carbon atoms (Knox et al. 1986), has a different mechanism compared to the $C_{18}$ stationary phase. Use of the PGC column increased retention of analytes, including aromatic compounds, by dispersion and strong π-π interactions, and the analyte's electron-withdrawing or donating properties (Rinne et al. 2006; West and Lesellier 2005a; West and Lesellier 2005b). In addition, the PGC column is also known to be excellent for the separation of positional and structural isomers due to the unique surface properties of the graphitic carbon (Gundersen 2001). The planar surface of PGC contributes to the separation of structurally different analytes, and the edge of the graphite sheet is energetically homogeneous, which is also good for the separation of ionic compounds (Wan et al. 1995).

To the best of our knowledge, this is the first SubFC-based analytical method developed for separation of teprenone isomers. This method showed improved resolution between the teprenone isomers and reduced the analysis time compared to previous methods.

## Materials and methods
### Chemicals and reagents
The teprenone standard was provided by Hanseo Chemical Co. Ltd. (Pyeongtaek, Republic of Korea). HPLC-grade solvents (methanol, ethanol, isopropanol, and acetonitrile) were purchased from J&W Honeywell (Phillipsburg, NJ, USA). Pressurized carbon dioxide gas (> 99.999%) was provided by KS Tech Co., Ltd. (Anseong, Republic of Korea). Teprenone capsule (50 mg/capsule, With CapTM) was provided by Samjinpharm. Co. Ltd (Seoul, Republic of Korea).

### SubFC conditions
The SubFC system used was equipped with a $CO_2$ delivery unit (LC-30ADSF), a back-pressure control unit (SFC-30A), a modifier pump (LC-20ADXR), a column oven (CTO-20AC), and a photodiode array detector (SPD-M20A), all obtained from Shimadzu (Tokyo, Japan). The analytical column was Hypercarb PGC column (100 × 4.6 mm, 5 μm; Thermo Scientific, Waltham, MA, USA) and was maintained at 40 °C. The mobile phase was composed of subcritical fluid $CO_2$ and ethanol (70/30, v/v) at a 4.0 mL/min flow rate. The injection volume was 10 μL, and the detection wavelength was 210 nm. The back-pressure of the SubFC system was maintained at 10 MPa. The data acquisition program used the LabSolution (Ver. 5.91) software from Shimadzu.

### Preparation of standard solutions and samples
The standard stock solution of teprenone was prepared at a concentration of 5 mg/mL by dissolving in ethanol.

The stock solution was diluted with ethanol to prepare working solutions of different concentrations (0.40, 0.45, 0.50, 0.55, and 0.60 mg/mL) for constructing the calibration curve.

The capsule sample was prepared by weighing out the teprenone standard powder corresponding to one capsule and adding ethanol to obtain a volume of 100 mL.

### Optimization of SubFC conditions
For analysis of mono-cis and all-trans teprenone isomers with fast separation and high efficiency, the SubFC conditions, such as the type and content of organic modifier, column temperature, injection volume, and flow rate, were optimized.

The organic modifiers commonly used in SubFC are acetonitrile, methanol, ethanol, and isopropanol; thus, these solvents were compared. The content of the selected organic modifier was optimized by varying to 10, 20, 30, 40, and 50%. In addition, because subcritical fluid is affected by temperature, the effect of column temperature was investigated by comparing temperatures of 35, 40, 45, 50, and 55 °C. Injection volumes of 5, 10, and 20 μL and flow rate of 2, 3, 4, and 5 mL/min were compared.

The optimization experiment was performed with the teprenone standard working solution (0.5 mg/mL). The peak parameters for optimal SubFC conditions were evaluated in terms of capacity factor (k'), retention time (RT), theoretical plate number (N), tailing factor ($T_f$), and resolution (Rs).

### Method validation
The developed method was validated as per ICH guideline Q2(R1) in terms of accuracy, precision (repeatability and intermediate precision), specificity, linearity, quantification range, and robustness (ICH 1994), along with stability.

#### Specificity
The specificity was confirmed by comparing the chromatograms of ethanol, excipient (placebo) of one blank capsule, and excipient spiked with teprenone isomers. The blank excipient and the excipient spiked with teprenone isomers were prepared by sample preparation method. We assessed whether interference appeared at the retention time of the teprenone isomers.

#### Linearity and range
The standard working solutions of teprenone isomers were prepared at different concentrations (0.4, 0.45, 0.5, 0.55, and 0.6 mg/mL) in ethanol by diluting the standard stock solution (5 mg/mL in ethanol). These concentrations are 80–120% of the labeled amount of teprenone in drug preparation. The working solutions in the calibration curve were analyzed by repeating three times

under the optimized SubFC condition, and calibration curves were constructed for the linearity test over the concentration range 0.4–0.6 mg/mL.

### Accuracy

Accuracy was evaluated in terms of recovery by addition of known quantities of the analyte to quantified drug product, according to the ICH guideline. Teprenone standard solutions equivalent to 5, 10, and 15% of QC sample (0.5 mg/mL) were added to quantified capsule samples. Three samples with added teprenone standard were prepared at each concentration and were quantified. Accuracy was calculated by comparing the theoretical and measured concentrations of teprenone isomers at each concentration.

### Precision

In the ICH guideline, precision includes repeatability and intermediate precision. The repeatability test was performed by injecting six replicates at 0.5 mg/mL of standard solution, which was 100% concentration of the dosing preparation. We evaluated the repeatability by RSD of retention time and peak area of teprenone isomers.

The intermediate precision test was performed on both intra-day ($n = 6$) and inter-day ($n = 3$) basis. Intraday precision was evaluated as follows: one teprenone capsule was weighed precisely and dissolved in ethanol to a final volume of 100 mL, from which six test solutions were prepared. The teprenone isomers were quantified using the linear calibration curve constructed by a developed method during a day. Inter-day precision was similarly assessed, with three test solutions over 3 days.

### Robustness

In the ICH guideline, robustness is defined as the reliability of an analysis with respect to deliberate variations in method parameters. We verified that there is no significant effect on the analysis results, even when a slight change is made in the developed analytical method. The conditions of flow rate, organic modifier (ethanol) concentration in the mobile phase, and column oven temperature were changed by ±10% from the optimized value for the evaluation of robustness. The applied conditions for robustness test were as follows: flow rate 3.6, 4.0, and 4.4 mL/min; organic modifier concentration 27, 30, and 33%; column oven temperature 36, 40, and 44 °C.

### Stability

Since the sample remains in solution for several hours in the laboratory prior to analysis, it is necessary to examine whether the stability of the sample solution is maintained in the laboratory environment during the time required for the test.

We evaluated the stability of the teprenone solution in a room temperature for 14 h, by comparing the initial peak area of teprenone isomers and the post-elapsed peak area.

## Results and discussion
### Optimization of SubFC conditions
#### Selection of organic modifier

The type and content of the organic modifier in the mobile phase influence the retention time, resolution, and selectivity of the analytes in SubFC. The subcritical fluid $CO_2$ and the organic modifier act as non-polar and polar solvents, respectively, in SubFC.
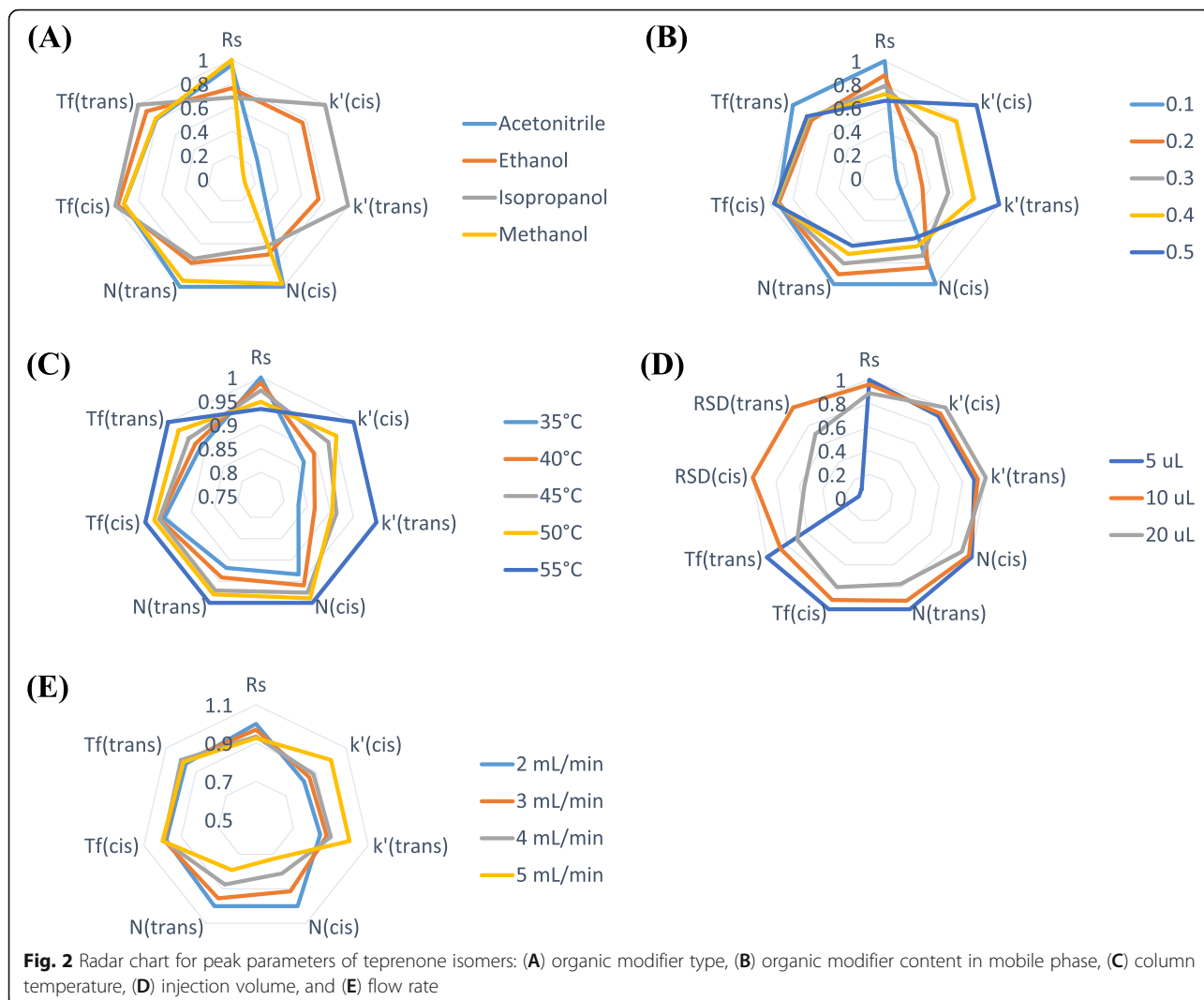
Acetonitrile, methanol, ethanol, and isopropanol were tested as organic modifiers for teprenone analysis. A 50/50 (v/v) ratio of $CO_2$ to organic modifier in the mobile phase was applied for the selection test. The other conditions (column temperature, injection volume, and flow rate) of the SubFC system were maintained at 40 °C, 5 µL, and 3 mL/min, respectively.

The organic modifiers were evaluated in terms of resolution, retention time (capacity factor), theoretical plate number, and tailing factor. These results are plotted as a radar chart (Fig. 2), in which the best value of each peak parameter was set at 100% and the rest of the values were expressed as percentages. The ideal value of each peak parameter was that which gave the highest resolution and theoretical plate number, and the lowest capacity factor and tailing factor.

The ascending order of retention time for the last analyte peak was as follows: isopropanol, ethanol, acetonitrile, and methanol. Relatively non-polar solvents, i.e., isopropanol and ethanol, showed fast elution of teprenone, while methanol showed the slowest elution. For methanol, the resolution between cis- and trans-teprenone was the best (Rs: 7.3), but the retention time of teprenone was too long (RT: cis, 21.8 min; trans, 32.7 min). Considering the influence of background noise at low wavelength (210 nm), acetonitrile was a suitable organic modifier. However, ethanol gave short analysis run time (RT: cis, 3.6 min; trans, 5.1 min) and good tailing factor ($T_f$: cis, 1.14; trans, 1.26), along with reasonable resolution (Rs 5.5), compared to acetonitrile. In addition, ethanol is the most environment-friendly solvent, having the lowest toxicity among the four solvents. Thus, ethanol was selected as the organic modifier. The chromatograms for the organic modifier test are shown in Fig. 3A.

#### Optimization of organic modifier content in mobile phase

The elution in SFC (or SubFC) works like normal-phase chromatography. The organic modifier acts as a polar solvent in the SFC (or SubFC) system. The higher the content of the organic modifier, the faster the elution is obtained from the SFC (or SubFC). The modifiers were

**Fig. 2** Radar chart for peak parameters of teprenone isomers: (**A**) organic modifier type, (**B**) organic modifier content in mobile phase, (**C**) column temperature, (**D**) injection volume, and (**E**) flow rate
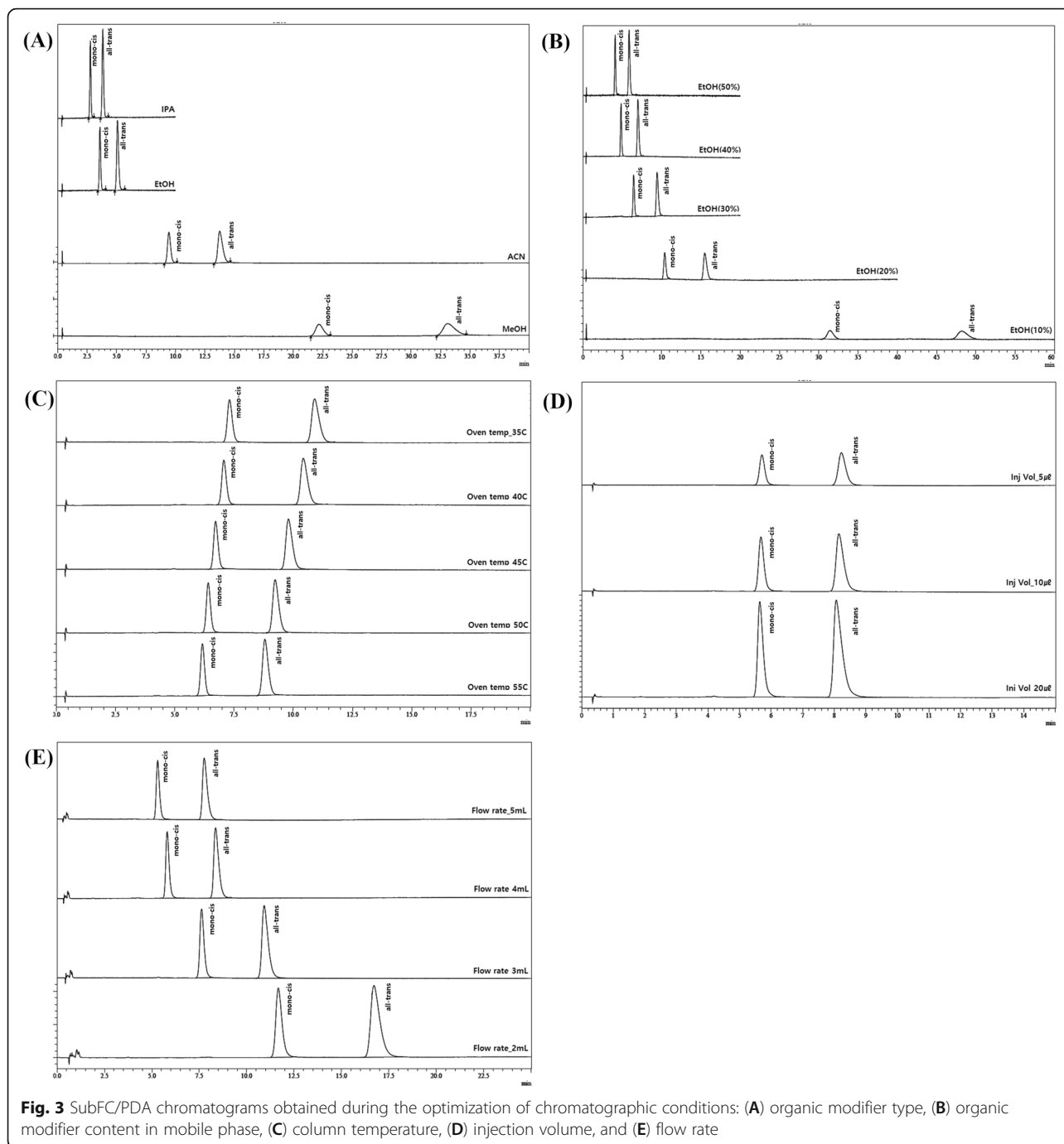
added to shorten analysis time, reduce peak tailing, and improve peak resolution, but it is true that the presence of a modifier increases the critical point. That is, higher temperatures and/or pressures are required to have a $CO_2$-based mobile phase in the supercritical stage. However, due to the continuum of properties when moving from the supercritical to the subcritical phase, temperature and pressure conditions can be kept constant throughout the experiment. The difference between supercritical and subcritical condition is whether the temperature and pressure of the mobile phase have reached the critical point.

In this study, the content of ethanol was adjusted to shorten the retention time as much as possible at an appropriate resolution. For this reason, it was carried out under subcritical conditions, not supercritical conditions due to the high content of ethanol. If the organic modifier content is too high, $CO_2$ can be worked as subcritical fluid and the system pressure may become unstable.

The effect of ethanol content was evaluated by varying the content to 10, 20, 30, 40, and 50%. The other conditions were kept constant, as in the selection of organic modifier (column temperature, 40 °C; injection volume, 5 μL; flow rate, 3 mL/min).

With increasing ethanol content, capacity factor (k'), resolution (Rs), and theoretical plate number (N) were found to decrease. The tailing factor ($T_f$) showed no remarkable change, except that of trans-teprenone at 10% ethanol content (Fig. 2B). The decrease in theoretical plate number with increasing ethanol content is attributed to the subcritical fluid gradually becoming liquid-like with increasing viscosity. In the radar chart, the Rs and N values were inversely related to the capacity factor when the type and content of organic modifier and the flow rate were compared (Fig. 2A, B, E). Finally, ethanol content of 30% was chosen, at which the subcritical fluid was stable and reasonable Rs and N values were obtained for teprenone isomers, along with separation

**Fig. 3** SubFC/PDA chromatograms obtained during the optimization of chromatographic conditions: (**A**) organic modifier type, (**B**) organic modifier content in mobile phase, (**C**) column temperature, (**D**) injection volume, and (**E**) flow rate

within 10 min. The chromatograms of the organic modifier content test are shown in Fig. 3B.

### Optimization of column temperature

Different column temperatures (35, 40, 45, 50, and 55 °C) were tested by applying 30% ethanol as the optimized organic modifier. The injection volume and flow rate were maintained at 5 μL and 3 mL/min, respectively.

The experimental results showed that with increasing column temperature, the peak parameters were improved, except resolution, unlike the other SubFC conditions. However, there were no remarkable differences in peak parameters at different column temperatures. In the radar chart, the range of peak parameters was 75–100% (Fig. 2C). The selected column temperature was 40 °C in consideration of resolution and deterioration of

column at high temperature. The chromatograms for the column temperature test are shown in Fig. 3C.

### Optimization of injection volume
The injection volume affects the sensitivity and reproducibility of the peaks. The peak parameters and repeatability of peak area were evaluated at different injection volumes. In the radar chart, the repeatability (relative standard deviation (RSD)) of trans- and cis-teprenone was added. The injection volumes of 5, 10, and 20 μL were compared by applying 30% ethanol as the optimized organic modifier. The column temperature and flow rate were maintained at 40 °C and 4 mL/min, respectively.

With decreasing injection volume, N, Rs, and $T_f$ values of teprenone isomers were improved. The standard working solutions of teprenone isomers were analyzed six times to evaluate the repeatability of the peak area; the RSD of the peak area was remarkably different according to the injection volume. The best reproducible peak area was obtained for an injection volume of 10 μL (Fig. 2D); therefore, 10 μL was selected as the injection volume due to appropriate resolution and reproducibility. The chromatograms for the injection volume test are shown in Fig. 3D.

### Optimization of flow rate
Different flow rates of the mobile phase (2, 3, 4, and 5 mL/min) were compared with 30% ethanol. The teprenone working solution was analyzed with three times repetition at a column temperature of 40 °C and an injection volume of 10 μL. With increasing flow rate, capacity factor was improved, and resolution and theoretical plate number were decreased. No remarkable difference in tailing factor was observed for different flow rates. The selected flow rate was 4 mL/min, considering run time within 10 min and appropriate peak parameters. The chromatograms for the flow rate test are shown in Fig. 3E.

## Method validation
### Specificity
The excipient of one capsule (blank) and the excipient spiked with 50 mg teprenone standard were dissolved in 100 mL of ethanol and analyzed to evaluate the specificity of the method. The excipients in teprenone capsule are composed of cornstarch, lactose hydrate, polyethylene glycol 6000, talc, silicon dioxide, and dl-α-tocopherol. Most of the excipient components did not have chromophore. We confirmed that no interference was present at the retention time of teprenone isomers in the chromatograms of ethanol and excipient (blank). The chromatograms for specificity are shown in Fig. 4A–C.

The expected elution order was confirmed through the contents of the Japanese and Korean Pharmacopoeia and the previous GC and LC analytical results. That is, as explained in the Japanese Pharmacopoeia (17th Edition) and the Korean Pharmacopoeia (12th Edition), "Teprenone is comprised of mono-cis and all-trans isomer, with their ratio being about 2:3". Even in the numerous analysis results in our study, the isomer area ratio was always 2/3 for the early retention time peak (mono-cis)/ the late retention time peak (all-trans). This was the same as the elution order in HPLC or GC.

### Linearity and range
The teprenone standard working solutions were prepared at concentrations of 80–120% (0.4–0.6 mg/mL) in pharmaceutical preparation and were analyzed three times. The correlation coefficient ($r^2$) exceeded 0.999 at the calibration range 0.4–0.6 mg/mL, which indicated favorable linearity. The precision ($n = 3$) of the calibration curve slope was within 0.5% as the relative standard deviation (RSD, %).

### Accuracy
Accuracy was evaluated in terms of recovery by addition of teprenone standard equivalent to 5, 10, and 15% of QC sample (0.5 mg/mL) to the quantified drug product. The average recovery was 99.94–100.39%, and RSD was 0.5–0.8%. These results of recovery close to 100% and RSD of less than 1% prove that the developed method was accurate (Table 1).

### Precision
The repeatability test was performed by analyzing six replicates at 0.5 mg/mL of standard solution and evaluated in terms of RSD of retention time and peak area. The RSD of retention time and peak area was 0.07–0.10% and 0.35–0.55%, respectively. Thus, the peaks of cis- and trans-teprenone showed good repeatability of the retention time and peak area.

The intermediate precision test was evaluated by assay results on intra- ($n = 6$) and inter-day ($n = 3$). The results of intra- and inter-day precision test showed RSD of 0.4% and 0.3–1.4%, respectively. These RSD values of approximately 1% or less confirmed the precision of the developed method and the SubFC system (Table 2).

### Limit of quantitation (LOQ) and limit of detection (LOD)
The calibration curve samples were analyzed three times for calculation of the LOQ and LOD. The LOQ and LOD were calculated by the following equation. $\sigma$ is the standard deviation of intercept. $S$ is the mean value of slope.

$$\text{LOQ} = 10 \times \sigma/S; \text{LOD} = 3.3 \times \sigma/S$$

In this developed method, the LOQ and LOD were 25.8 and 8.5 μg/mL, respectively. In the previous reports,

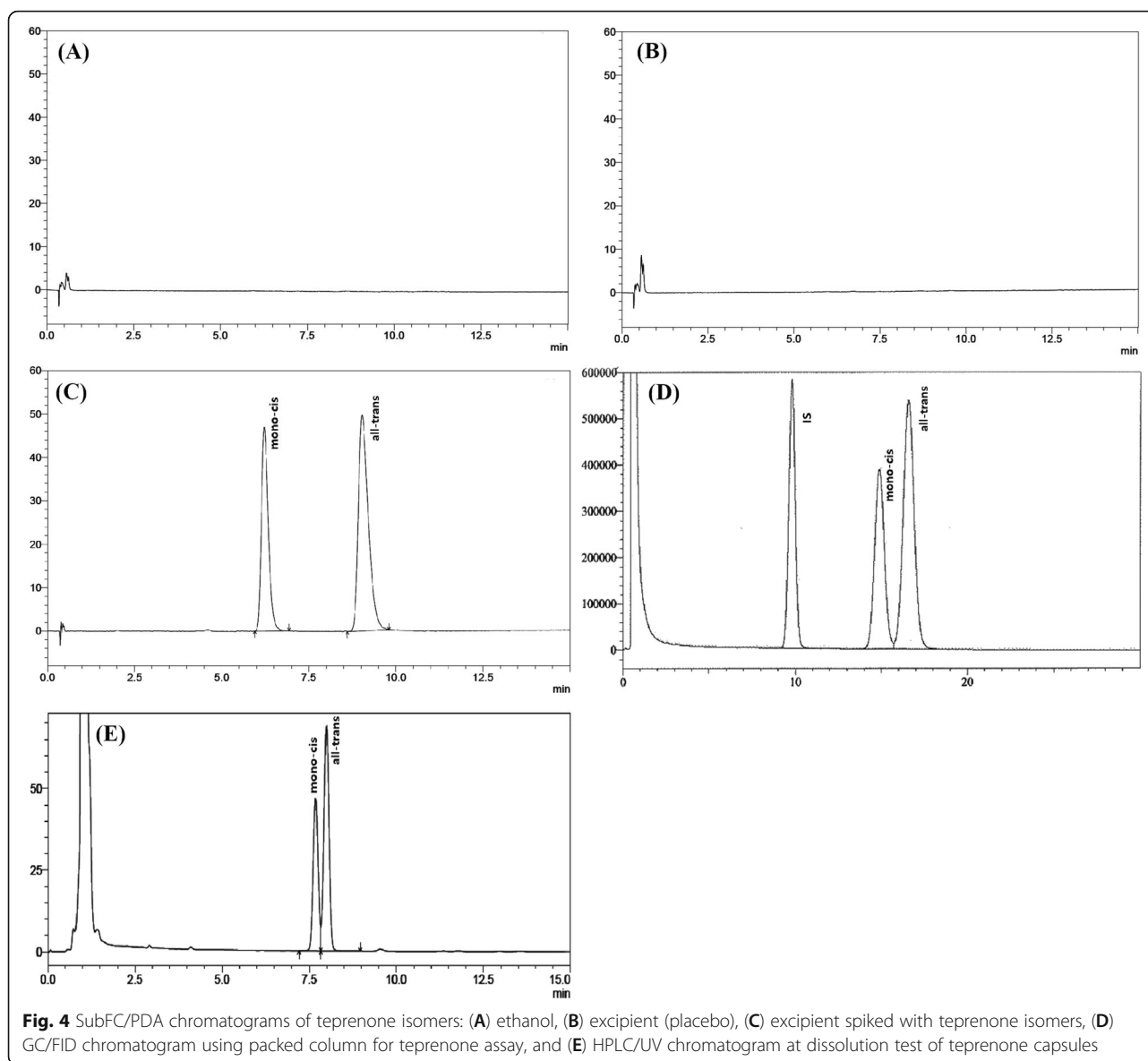**Fig. 4** SubFC/PDA chromatograms of teprenone isomers: (**A**) ethanol, (**B**) excipient (placebo), (**C**) excipient spiked with teprenone isomers, (**D**) GC/FID chromatogram using packed column for teprenone assay, and (**E**) HPLC/UV chromatogram at dissolution test of teprenone capsules

the LOQ of teprenone was 100 μg/mL by GC/FID (Guang 2009) and 0.05 μg/mL by HPLC/UV (Xiao-jun 2007). Although the LOQ (25.8 μg/mL) in this method is lower than that of the HPLC/UV method (Xiao-jun 2007), it is considered to be sufficient to measure the nominal concentration of 500 μg/mL in the teprenone capsule. In addition, it is not clear whether mono-cis and all-trans forms were completely separated in the previously reported HPLC/UV method (Xiao-jun 2007).

### Robustness
The robustness test was performed by varying flow rate, content of organic modifier in mobile phase, and column oven temperature by ±10% from optimized value. The applied conditions were as follows: flow rates 3.6, 4.0, and 4.4 mL/min; organic modifier contents 27, 30, and

33%; column oven temperatures 36, 40, and 44 °C. The N, $T_f$, and Rs of cis- and trans-teprenone were evaluated by varying these analytical conditions; no remarkable differences in peak parameters were observed. Thus, teprenone isomers were analyzed with a good peak shape and resolution. The RSDs of the N, $T_f$, and Rs values were 2.8–4.6%, 0.2–2.2%, and 0.2–2.6%, respectively.

### Stability
The prepared solution stability of teprenone isomers was evaluated at room temperature for 14 h during analysis. There was almost no difference between the initial peak area of teprenone isomers and the post-elapsed peak area, with an RSD of 0.33–0.60%. This confirms that the teprenone solution was stable during analysis.

**Table 1** Accuracy determination by method validation ($n = 3$)

| Added conc. ratio | Measured conc. in capsule (µg/mL) | Added conc. (µg/mL) | Measured total conc. (µg/mL) | Recovery Mean (%) (95% CI*) | RSD (%) |
|---|---|---|---|---|---|
| 5% | 495.8 | 24.4 | 524.4 | 100.1 ± 0.7 | 0.7 |
|  | 495.6 |  | 519.5 |  |  |
|  | 499.0 |  | 520.9 |  |  |
| 10% | 495.8 | 48.8 | 543.0 | 99.9 ± 0.6 | 0.5 |
|  | 495.6 |  | 547.3 |  |  |
|  | 499.0 |  | 545.6 |  |  |
| 15% | 495.8 | 73.2 | 575.0 | 100.4 ± 0.9 | 0.8 |
|  | 495.6 |  | 572.4 |  |  |
|  | 499.0 |  | 569.4 |  |  |

*CI confidence interval

## Comparison of SubFC/PDA, HPLC/UV, and GC/FID

The API of teprenone was analyzed by the GC/FID and HPLC/UV methods (described in the JP) for comparing the SubFC/PDA, HPLC/UV, and GC/FID methods. In the JP methods, a glass column with an inside diameter of 3 mm and length of 2 m, packed with 149–177 µm silica-gel coated in 5% with polyethylene glycol 2-nitroterephthalate is used for gas chromatography. Herein, Uniport HP 80/100 mesh (4 mm I.D. × 2.1 m; 5% FFAP) column was used. The GC/FID method using the packed column for the JP methods required a long run time of ~20 min to separate the teprenone isomers. In HPLC/UV method in the JP, a stainless-steel column 4.6 mm in inside diameter and 15 cm in length, packed with octadecylsilanized silica gel (5 µm of particle size) is used. Herein, ACE5 $C_{18}$ column (4.6 mm I.D. × 150 mm, 5 µm) with 40°C of column temperature was used. The mobile phase was a mixture of acetonitrile and water (87/13, v/v) as in the JP with 1.6 mL/min of flow rate.

In the GC/FID method, the resolution between the cis- and trans-teprenone was approximately 1.5, indicating narrow separation (Fig. 4D). In the assay using the HPLC/UV method described in the JP, the teprenone isomers were slightly separated within 10 min, but there was no baseline separation (Fig. 4E).

The SubFC/PDA method developed herein had a resolution of 6.3 for teprenone isomers within 10 min of run time (Fig. 4C), which is improved compared to those of

GC/FID or HPLC/UV method. In addition, di-n-butyl phthalate, used as the internal standard in the JP methods, is an endocrine disruptor, which can be dangerous to researchers. However, the SubFC/PDA method showed excellent accuracy and precision in method validation, even though no internal standard was used.

Although SFC (or SubFC) has disadvantages in cost and complexity compared to HPLC, it is worthwhile as it has a different separation mechanism and orthogonality than HPLC and GC. The initial price of SFC (or SubFC) equipment is rather expensive; however, it has obvious advantages in terms of maintenance costs (solvent price) and environmental aspects. In addition, it is very beneficial to the safety of analytical researchers as it uses only $CO_2$ and ethanol, without the use of harmful organic solvents for the preparation of standards and samples, and analysis procedures.

## Conclusion

A very fast and highly selective SubFC/PDA method was developed and validated for the separation of mono-cis and all-trans teprenone isomers in pharmaceutical preparation. The separation of teprenone isomers was performed on a porous graphitic carbon column. The SubFC conditions were evaluated and optimized for high-efficiency analysis. The SubFC parameters were optimized for the type and content of organic modifier, column temperature, injection volume, and flow rate,

**Table 2** Intermediate precision by method validation

| Validation | | Concentration (µg/mL) | Measured conc. (µg/mL) (95% CI*) | Precision (RSD, %) | Accuracy (%) (95% CI*) |
|---|---|---|---|---|---|
| Intra-day ($n = 6$) | | 500.0 | 494.8 ± 1.5 | 0.4 | 99.0 ± 0.3 |
| Inter-day ($n = 3$) | Day 1 | 500.0 | 495.7 ± 3.4 | 0.6 | 99.1 ± 0.6 |
|  | Day 2 | 500.0 | 496.5 ± 1.8 | 0.3 | 99.2 ± 0.3 |
|  | Day 3 | 500.0 | 494.7 ± 7.7 | 1.4 | 98.9 ± 1.5 |

*CI confidence interval

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which were ethanol, 30%, 40 ℃, 10 μL, and 4 mL/min, respectively.

These parameters were evaluated using the peak parameters of teprenone isomers, such as capacity factor (k'), retention time (RT), theoretical plate number (N), tailing factor ($T_f$), and resolution (Rs). The developed method was validated in terms of accuracy, precision (repeatability and intermediate precision), specificity, linearity, range, and robustness, according to ICH guideline Q2(R1). In addition, the stability of teprenone was evaluated during 14 h at room temperature. When the SubFC/PDA method described herein and the GC/FID or HPLC/UV methods described in the JP were compared, the SubFC/PDA method showed improved resolution of teprenone isomers and shortened run time.

Although a large number of actual teprenone capsules were not analyzed in this study, it is considered that it can be sufficiently applied to quality control of actual products through method validation. In addition, the developed SubFC/PDA analysis method is expected to be reflected in the official compendium and applied to the industrial site.

### Declarations

#### Competing interests
The authors declare that they have no competing interests.

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