Regular Article*Highlighted Paper selected by Editor-in-Chief***Assessment of Repeatability in Supercritical Fluid Chromatography with Electrochemical Detection Based on the ISO 11843 Part 7**Kazuhiro Yamamoto,^a Akira Kotani,^{*,a} Yuzuru Hayashi,^b and Hideki Hakamata^a^a*School of Pharmacy, Tokyo University of Pharmacy and Life Sciences; 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan; and* ^b*Institute for FUMI Theory; 3-3-15 Inaridai, Sakura, Chiba 285-0864, Japan.*

Received September 3, 2018; accepted October 7, 2018

The present study proposes a method for the assessment of repeatability in supercritical fluid chromatography with electrochemical detection (SFC-ECD), based on the ISO 11843 part 7 (ISO 11843-7:2018) which can theoretically provide detection limits and standard deviation (S.D.) through the stochastic properties of baseline noise without repetitive measurements of real samples. On the baseline noise of SFC-ECD, large-amplitude and periodic noises with less than 0.05 Hz were observed, and the power spectrum of the baseline noise showed $1/f$ fluctuation (f = frequency). It was found that the present power spectrum analysis, according to the law of error propagation, can provide suitable noise parameters to calculate S.D. of baseline noise and a relative S.D. (RSD) of peak area by ISO 11843-7. The chromatographic determinations of α -, β -, γ - and δ -tocopherol have been taken as examples. In the present SFC-ECD, the RSDs of peak areas for α -, β -, γ - and δ -tocopherol obtained by ISO 11843-7 were within 95% confidence intervals of the RSD of them obtained by repetitive measurements ($n = 6$). Thus, we found that ISO 11843-7 is applicable to the assessment of repeatability in SFC-ECD for determining tocopherols without repetitive measurements.

Key words electrochemical detection; ISO 11843-7; supercritical fluid chromatography; power spectra analysis; baseline noise

Introduction

Supercritical fluid chromatography (SFC) is a chromatographic technique as well as HPLC and GC, and supercritical carbon dioxide is used as a mobile phase for SFC, because it has moderate values of critical pressure and temperature (7.3 MPa and 31°C).¹⁾ Typical applications are similar to a normal-phase chromatographic technique, but SFC is attractive as an environmentally conscious technology because the use of organic solvents as a mobile phase can be reduced.²⁾ SFC can be achieved quickly and with high-resolution separation when compared with HPLC separations because the diffusion of solutes in the supercritical fluids is about 10 times greater than that in liquids.²⁾ Thus, SFC is now commonly and widely used to perform chiral and/or achiral separations and purifications for the analysis of agrichemicals,^{3–5)} drugs,^{6,7)} foods,^{8–10)} flavors,¹¹⁾ fossil fuels,¹²⁾ and natural products.¹³⁾ In the analysis of the above-mentioned fields, the development of a suitable SFC method with satisfactory precision is significant to the obtaining of reliable results. However, it is difficult to develop an optimal SFC system compared with an HPLC system by following reasons: (1) Construction of an SFC system is complicated because a pump for modifier solvent delivery and a back-pressure regulator to maintain the desired pressure in the system are required in addition to a system of flow analysis such as HPLC. (2) Baseline noises in an SFC system would be variable compared with these in an HPLC system, because the stability of SFC flow is affected by a slight change of pressure and temperature in the SFC system. By these reasons, an SFC system would have many opportunities to gain various noise sources caused by the instruments and chromatographic conditions in comparison with an HPLC system. Thus, to provide SFC as a quantitative method with satisfactory precision, an assessment of repeatability in SFC system is necessary during

construction of the SFC system and optimization of SFC conditions.

The importance of validation of analytical methods has been described in various official documents. For example, the guidelines of the International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH-Q2) is a representative document for analytical method validation in the development of medicines.¹⁴⁾ And, the pharmacopoeias in regions such as the United States, Europe and Japan *etc.* also describe analytical method validation.^{15–17)} In these documents, the assessment of repeatability is recommended by multiple injections of a standard solution to obtain a relative standard deviation (RSD) of peak areas and/or heights of analyte from chromatograms, however it requires funds to measure the same sample at several times.

In the ISO 11843 part 7 (ISO 11843-7:2018, “Methodology based on stochastic properties of instrumental noise”), stochastic theories, called the Function of Mutual Information (FUMI) theory, are introduced to estimate detection limits in spectrometric and chromatographic analyses. In the case of chromatographic analysis, the standard deviation (S.D.) of blank areas and the RSD of peak areas are obtained based on the stochastic properties of baseline noise and signal data on a chromatogram. In ISO 11843-7, to obtain the S.D. and the RSD without repetitive measurements of real samples, it has utilized chromatographic baseline noise in most of the HPLC and GC systems that exhibited flicker noise (or $1/f$ fluctuation),^{18–23)} and chromatographic baseline noise is approximated by a mixed well-defined stochastic process of white noise and Markov process using power spectrum analysis.²⁴⁾ If the baseline noise in SFC with electrochemical detection (SFC-ECD) corresponds to the stochastic process, the RSD of the peak area can be obtained without repetitive measurements by

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ISO 11843-7. However, it has not been clarified whether the baseline noise in SFC-ECD can also be dealt with in a similar way when repeatability assessments in HPLC methods are performed.²⁵⁾

In this study, we firstly investigate the characteristics of baseline noise in SFC-ECD by power spectrum analysis. Secondly, we have attempted to prove that ISO 11843-7 is applicable to assess repeatability in SFC-ECD.

Experimental

Chemicals α -, β -, γ - and δ -tocopherol for analytical standard substance, methanol, and ammonium acetate were all purchased from FUJIFILM Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Apparatus and Chromatographic Conditions The present SFC-ECD system, essentially the same as that reported previously,²⁶⁾ consisted of two pumps (PU-2080-CO₂ Plus and PU-2080 Plus, JASCO, Tokyo Japan), a stainless steel heating coil, a sample injector (7725i, Rheodyne, Cotati, CA, U.S.A.) fitted with a 5 μ L injection loop, a silica gel column (Spherisorb Silica, 150 $\times$ 4.6 mm, i.d., 5 μ m, Waters, Milford, MA, U.S.A.) which was maintained at 40°C in a column oven (CO-2065, JASCO), a self-made flow-through electrochemical cell,²⁶⁾ a potentiostat (1112, Huso Electrochemical System, Kanagawa, Japan), a back-pressure regulator (BP-2080, JASCO), and a recorder (TR-W1000, KEYENCE, Osaka, Japan).

Liquefied carbon dioxide (>99.99%, Tomoe Shokai, Tokyo, Japan) and methanol containing 1.0 mol/L ammonium acetate were used as a mobile phase and a modifier solvent, respectively. The mixture of the mobile phase and modifier solvent (98:2, v/v) was made to flow at 1.5 mL/min. The applied potential for monitoring tocopherols was set at +0.8 V. The back-pressure of the SFC-ECD system was kept at 15 MPa.

Data Analysis The digital data of chromatograms were recorded at a sampling interval of 0.02 s/point. The power spectrum analysis and the series of calculation based on the ISO 11843-7 were performed to utilize computer software (TOCO, Institute for FUMI Theory, Chiba, Japan).

Results and Discussion

Power Spectra Analysis to Obtain Baseline Noise Parameters in SFC-ECD System With a motion of the back-pressure regulator, flow-through electrolysis in the present system was performed to monitor baseline noise under a supercritical pressure. As shown in Fig. 1, periodic noises with less than 0.05 Hz were observed in baseline noise under supercritical pressure. These large-amplitude and periodic noises were caused by the motion of the back-pressure regulator, which periodically opened and closed the outlet of the flow line to maintain constant supercritical pressure in the SFC system. In the ISO 11843-7, in order to provide an RSD of the peak area from stochastic properties of baseline noise and signal in a chromatogram, the time variation in the baseline noise was approximated by a mixed stochastic process of white noise and the Markov process. White noise is a time-independent process with one parameter, which is the S.D. of white noise ($\tilde{w}$), whereas the Markov process has a time correlation with two parameters, which are the S.D. ($\tilde{m}$) and retention parameters (ρ) of the Markov process. For determining the three parameters, $\tilde{w}$, $\tilde{m}$, and ρ , the consecutive baseline noise data of

2ⁿ (2 to the *n*th power) points were transformed into the real power spectrum by the Fourier transform, and then the theoretical power spectrum ($P(f)$, Eq. 1) was least-squares fitted to the observed power spectrum²²⁻²⁵⁾:

$$P(f) = \frac{\tilde{m}^2}{1 - \rho^2} \times \frac{2\alpha}{\alpha^2 + 4\pi^2 f^2} + \tilde{w}^2 \quad (1)$$

where f is the frequency, $\alpha = (1 - \rho)/\Delta t$, and Δt is the sampling interval.

Fig. 1. Baseline Noise Obtained by SFC-ECD

Fig. 2. Power Spectra Analysis for (A) 256, (B) 512, (C) 1024, (D) 2048, (E) 4096, and (F) 8192 Points of Baseline Noise

The zigzag line indicates the real power spectrum, which was obtained from the consecutive digital data of baseline noise transferred by the Fourier transforms. The smooth solid line indicates the best fit of the theoretical power spectrum based on the FUMI theory ($P(f)$, Eq. 1) with the real power spectrum.

When the $P(f)$ overlapped the figured real power spectrum in the wide frequency range, adequate noise parameters were obtained to provide an RSD of the peak area by power spectrum analysis based on the ISO 11843-7. The real power spectrum, which was obtained from the baseline noise of 256 points in Fig. 1 by the Fourier transform, is represented as the zigzag line in Fig. 2A, and the best fit of the $P(f)$ by least-squares fitting is represented as the smooth line in Fig. 2A. The real power spectra less than 0.1 Hz are not figured, and the $P(f)$ did not overlap with the real power spectrum ranging from 0.2 to 0.8 Hz. Thus, from the results of this fitting, it was found that adequate noise parameters would not be obtained from the power spectrum analysis using 256 points of baseline noise.

To figure the real power spectrum less than 0.1 Hz, consecutive digital data of 1024, 2048, 4096 and 8192 points of baseline noise in Fig. 1 were transferred to power spectra by the Fourier transform, and the $P(f)$ were obtained by least-squares fitting as shown in Figs. 2B–E. Considering each fit between $P(f)$ and real power spectrum less than 0.1 Hz in Fig. 2, the better fit of the six was the results from baseline noise of 8192 points as shown in Fig. 2E. In Fig. 2E, the $P(f)$ were

not fitted with the real power spectra at more than 0.1 Hz. The power spectrum has the common natural phenomena feature where the power spectrum densities at low frequencies are larger than those at high frequencies.^{22–25}) It is often called $1/f$ fluctuation. And, based on the Eq. 1, power spectrum densities at each frequency in $P(f)$ are represented by terms containing S.D. such as $\tilde{w}$ and $\tilde{m}$. Thus, the mismatch between the $P(f)$ and the real power spectrum could be ignored according to the law of error propagation because power spectrum densities less than 0.1 Hz are remarkably large compared with those more than 0.1 Hz. In the case of HPLC with electrochemical detection (HPLC-ECD), a real power spectrum of baseline noise overlapped with the $P(f)$ in the wide frequency ranging from 0.005 Hz to 2.5 Hz.²⁷) Thus, the suitable noise parameters were obtained from the $P(f)$. However, in the case of SFC-ECD, there is the mismatch between the real power spectrum and the $P(f)$. Therefore, the above mentioned additional method based on the law of error propagation is utilized to obtain the suitable noise parameters.

Moreover, to estimate the correlation between the $P(f)$ and real power spectra, power spectrum density at each frequency in the $P(f)$ and real power spectra obtained from 256, 512,

Fig. 3. Comparison of Power Spectrum Densities on Real (x-Axis) and Theoretical (y-Axis) Power Spectra Obtained from (A) 256, (B) 512, (C) 1024, (D) 2048, (E) 4096, and (F) 8192 Points of Baseline Noise

Correlation coefficients (r) of each regression expression are shown in the figure.

Fig. 4. Chromatogram of 25 $\mu\text{mol/L}$ Tocopherol Standard Mixture Obtained by SFC-ECD

SFC-ECD conditions were as follows: mobile phase, carbon dioxide; modifier solvent, methanol containing 1.0 mol/L ammonium acetate; total flow rate, 1.5 mL/min; ratio of mobile phase and modifier solvent, 98:2 (v/v); applied potential, +0.8 V; back-pressure, 15 MPa. Peaks: a, α -tocopherol; b, β -tocopherol; c, γ -tocopherol, and d, δ -tocopherol.

1024, 2048, 4096 and 8192 points of baseline noises were compared, and those results are shown in Fig. 3. The correlation coefficient (r) was 0.99 when power spectrum densities were compared between the real power spectrum and the $P(f)$ obtained from 8192 points, and they were the best results of the six correlations. It was shown that the 8192 points of baseline noise in SFC-ECD could be approximated by a mix of stochastic process of white noise and the Markov process. From these findings, it was determined that adequate noise parameters can be obtained to provide an RSD of peak area by the power spectrum analysis of baseline noise. In the case of SFC-ECD, it was found that baseline noise monitoring for about 3 min (8192 points) was required to parameterize periodic noises with less than 0.05 Hz when the sampling interval was set at 0.02 s/point.

Estimation of S.D. of Baseline Noise and RSD of Peak Area Provided from ISO 11843-7 We next examined whether S.D. caused by the baseline noise (S_B), when obtained by the use of three noise parameters from the present power spectrum analysis, was suitable. In the ISO 11843-7, S_B in the integration domain (k_e) was obtained by Eq. 2 which consisted of three noise parameters, $\tilde{w}$, $\tilde{m}$, and ρ , as follows²²⁻²⁵:

$$S_B^2 = k_e \tilde{w}^2 + \frac{\tilde{m}^2}{(1-\rho)^2} \left(k_e - 2\rho \frac{1-\rho^{k_e}}{1-\rho} + \rho^2 \frac{1-\rho^{2k_e}}{1-\rho^2} \right) + \beta^2 \tilde{w}^2 + \tilde{m}^2 \left[\beta^2 \frac{1-\rho^{2k_e}}{1-\rho^2} - 2\beta \sum_{i=1}^{k_e} \left(\rho^{k_e-i} \cdot \frac{1-\rho^{k_e+1-i}}{1-\rho} \right) \right] \quad (2)$$

where $\beta = (k_e + 1)/2$.

An SCF-ECD system for determining tocopherols was an example. In Fig. 4, a typical chromatogram of a standard mixture of tocopherol is shown by the SFC-ECD system. The chromatographic peaks of α -, β -, γ - and δ -tocopherol were observed at 3.4, 4.8, 5.2 and 6.4 min, respectively. S_B obtained by the present power spectra using 256, 512, 1024, 2048, 4096 and 8192 points of baseline noise before appearance of α -tocopherol peak were 0.015, 0.013, 0.023, 0.029, 0.12 and 0.22, respectively. Because the periodic noises with less than

Fig. 5. Precision Profiles for the Determination of (A) α -Tocopherol, (B) β -Tocopherol, (C) γ -Tocopherol, and (D) δ -Tocopherol by SFC-ECD

The solid lines mean RSDs of the peak area were obtained by ISO 11843-7. The closed circles mean RSDs of the peak area obtained by six repetitive measurements at each concentration. The error bars show 95% confidence intervals for RSDs of the peak area obtained by six repetitive measurements.

0.05 Hz were not analyzed in the power spectrum obtained from 256, 512, 1024 and 2048 points of baseline noise, these S_B could be underestimated in comparison with suitable S_B . Indeed, S_B obtained from 4096 and 8192 points of baseline noises were larger than those from 256, 512, 1024 and 2048 points of baseline noise. Moreover, as shown in Fig. 3, the r between the $P(f)$ and the real power spectrum obtained from 8192 points of baseline noise was better than that from 4096 points of baseline noise. Thus, it was shown that suitable S_B could be obtained to provide an RSD of the peak area in SFC-ECD by the power spectrum analysis using 8192 points of baseline noise.

The evaluation of repeatability based on ISO 11843-7 was performed to examine whether S_B obtained by the present power spectrum analysis can be applied to provide an RSD of the peak area in SFC-ECD. In the ISO 11843-7, an RSD of the peak area of the analyte, which has a peak area (A), was obtained by Eq. 3 which consisted of S_B as follows²²⁻²⁵:

$$\text{RSD}^2 = \frac{S_B^2}{A^2} + I^2 \quad (3)$$

where I is the RSD of the volume error of the sample injector. According to the specification sheet of the sample injector, the RSD of the volume error of the sample injector is 0.1%. This value was utilized as I in the present study.

The RSD of the peak area by ISO 11843-7 was compared with that by repetitive measurements to verify suitable noise parameters obtained by the present power spectrum analysis. As shown in Fig. 5, the RSD of the peak area over a wide concentration range for tocopherols was obtained by ISO 11843-7 using only 25 $\mu\text{mol/L}$ of each tocopherol for each chromatogram. And the RSD of peak area by ISO 11843-7 was within 95% of the confidence interval for RSD of the peak area by repetitive measurements at 5, 10 and 25 $\mu\text{mol/L}$ tocopherols ($n = 6$ each). Meanwhile, when three noise parameters obtained by the $P(f)$ from 512, 1024, 2048 and 4096 data points were assigned into Eq. 2, the RSD of the peak area by ISO 11843-7 was not within 95% of the confidence interval for

RSD of the peak area by repetitive measurements. From the results of these precision profiles for determining tocopherols, we verified that ISO 11843-7 can be applied for the evaluation of repeatability in SFC-ECD when noise parameters were obtained from 8192 data points of baseline noise. An RSD of peak area by ISO 11843-7 is more reliable compared with six repetitive measurements because the confidence interval of the RSD by ISO 11843-7 is equal to the S.D. error from 40 repetitive measurements.²⁸⁾ Furthermore, by ISO 11843-7, both experimental time and chemicals can be saved to estimate the repeatability in chromatographic methods. These advantages are also valid to the evaluation of repeatability in an SFC-ECD system. In this study, we demonstrate that the application of ISO 11843-7 is useful to performing a method of validation for SFC-ECD.

Conclusion

This paper is the first report that ISO 11843-7 has been applied to estimate the repeatability in SFC-ECD. Although chromatographic baseline in SFC-ECD involved large-amplitude and periodic baseline noises, it was demonstrated that suitable noise parameters can be obtained to provide RSD of peak area by the present spectra analysis using the baseline noises of 8192 points. In an SFC-ECD system, the fitting between the $P(f)$ and the real power spectrum derived from a main noise source of chromatographic baseline was significant to the obtaining of suitable noise parameters of baseline and precision profiles of an analyte. In conclusion, it was found that ISO 11843-7 contributes as a great chemometric tool to perform repeatability assessment in an SFC-ECD system.

Acknowledgment We are grateful to Dr. Fumiyo Kusu, Professor Emeritus of Analytical Chemistry, Tokyo University of Pharmacy and Life Sciences, for her encouragement to the authors throughout this study.

Conflict of Interest The authors declare no conflict of interest.

References

- West C., *Anal. Bioanal. Chem.*, **410**, 6441–6457 (2018).
- Lesellier E., West C., *J. Chromatogr. A*, **1382**, 2–46 (2015).
- Tao Y., Zheng Z., Yu Y., Xu J., Liu X., Wu X., Dong F., Zheng Y., *Food Chem.*, **241**, 32–39 (2018).
- Li R., Chen Z., Dong F., Xu J., Liu X., Wu X., Pan X., Tao Y., Zheng Y., *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.*, **1081–1082**, 25–32 (2018).
- Cheng Y., Zheng Y., Dong F., Li J., Zhang Y., Sun S., Li N., Cui X., Wang Y., Pan X., Zhang W., *J. Agric. Food Chem.*, **65**, 234–243 (2017).
- Desfontaine V., Guillaume D., Francotte E., Nováková L., *J. Pharm. Biomed. Anal.*, **113**, 56–71 (2015).
- Lemasson E., Bertin S., West C., *J. Sep. Sci.*, **39**, 212–233 (2016).
- Zhou Q., Gao B., Zhang X., Xu Y., Shi H., Yu L., *Food Chem.*, **143**, 199–204 (2014).
- Tu A., Ma Q., Bai H., Du Z., *Food Chem.*, **221**, 555–567 (2017).
- Qi N., Gong X., Feng C., Wang X., Xu Y., Lin L., *Food Chem.*, **207**, 157–161 (2016).
- Schaffrath M., Weidmann V., Maison W., *J. Chromatogr. A*, **1363**, 270–277 (2014).
- Thiébaud D., *J. Chromatogr. A*, **1252**, 177–188 (2012).
- Eisath N., Sturm S., Stuppner H., *Planta Med.*, **84**, 361–371 (2018).
- The International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use, “ICH Harmonised Tripartite Guideline Validation of analytical procedures: text and methodology Q2 (R1),” 2005.
- The United States Pharmacopeial Convention, “The United States Pharmacopeia 41th ed.,” 2017.
- The Council of Europe, “European Pharmacopoeia 9th ed.,” 2018.
- The Ministry of Health, Labour and Welfare, “The Japanese Pharmacopoeia 17th ed.,” 2016.
- Lub T. T., Smith H. C., Poppe H., *J. Chromatogr. A*, **149**, 721–733 (1978).
- Felinger A., Vigh E., Gelencsér A., *J. Chromatogr. A*, **839**, 129–139 (1999).
- Felinger A., Guiochon G., *J. Chromatogr. A*, **913**, 221–231 (2001).
- Ingle J. D. Jr., Crouch S. R., “Spectrometrical analysis,” Prentice Hall, Englewood Cliffs, NJ, 1998.
- Hayashi Y., Matsuda R., *Anal. Chem.*, **66**, 2874–2881 (1994).
- Matsuda R., Hayashi Y., Sasaki K., Saito Y., Iwaki K., Harakawa H., Satoh M., Ishizuki Y., Kato T., *Anal. Chem.*, **70**, 319–327 (1998).
- The International Organization for Standardization, “ISO 11843 Part 7 Methodology based on stochastic properties of instrument noise,” 2018.
- Kotani A., Tsutsumi R., Shoji A., Hayashi Y., Kusu F., Yamamoto K., Hakamata H., *J. Chromatogr. A*, **1454**, 26–31 (2016).
- Yamamoto K., Kotani A., Hakamata H., *Anal. Methods*, **10**, 4414–4418 (2018).
- Kotani A., Yuan Y., Yang B., Hayashi Y., Matsuda R., Kusu F., *Anal. Sci.*, **25**, 925–929 (2009).
- Hayashi Y., Matsuda R., Poe P. B., *Analyst*, **121**, 591–599 (1996).