Faster results with SFC for the analysis of choline and acetylcholine in rat cerebrospinal fluid samples

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Supercritical Fluid Chromatography (SFC)

A supercritical fluid is a state of substance wherein the temperature and pressure are both above their critical points (Figure 1). Supercritical fluids deliver better solvating power than gases, and are more diffusive and have lower viscosities than liquids.

Chemical substances have their specific critical points, and the especially low critical point of carbon dioxide (critical temperature: 31.1°C, critical pressure: 7.38 MPa) makes the gas easy to handle. As it is non-flammable, inert, non-toxic and from an economic point of view low-cost, it has been used widely in industrial processes such as decaffeination of coffee beans, the extraction of hops, and isolation of many flavour compounds. Supercritical fluids are also applied in analytical fields, including being the major mobile phase component in supercritical fluid chromatography (SFC) and the serving as the extraction solvent in supercritical fluid extraction (SFE).

As the name 'Supercritical Fluid Chromatography' or SFC indicates, it is a chromatographic separation technique much like high-performance liquid chromatography (HPLC) where the mobile phase consists mostly of supercritical CO_2. The CO_2 is kept under pressure so it does not expand to become a gas. The HPLC requires a so-called back pressure regulator to maintain pressure above the critical limit, from the time the CO_2 is pressurised by the pump, until it has been successfully detected. Once introduced into the chromatographic pumping system the CO_2 is no longer a true liquid; the characteristics are somewhere between a gas and a liquid with the favourable attributes of both being combined in one the newly converted species

Although supercritical carbon dioxide has a similar hydrophobicity as hexane, this property alone is often insufficient for the elution of target compounds from a column. These target compounds can be eluted by adding an organic solvent, called a modifier, to modify the polarity of the mobile phase. Organic solvents that are compatible with carbon dioxide, such as methanol, ethanol, isopropyl alcohol, and acetonitrile, are used as modifiers. Organic solvents with an added acid (e.g., formic acid or acetic acid), salt (e.g., ammonium formate or ammonium acetate), or base (e.g., diethylamine) are also used as modifiers for the analysis of highly polar compounds.

Because of the properties of supercritical ¬fluids, including low viscosities and high diffusivities, SFC delivers less of a pressure drop across the chromatographic column than conventional HPLC. Recent advances in commercially available SFC systems and the packed columns they utilise have made it possible for practitioners of HPLC to become proficient with SFC in their laboratories. Although the hydrophobicity of supercritical carbon dioxide is similar to hexane, the

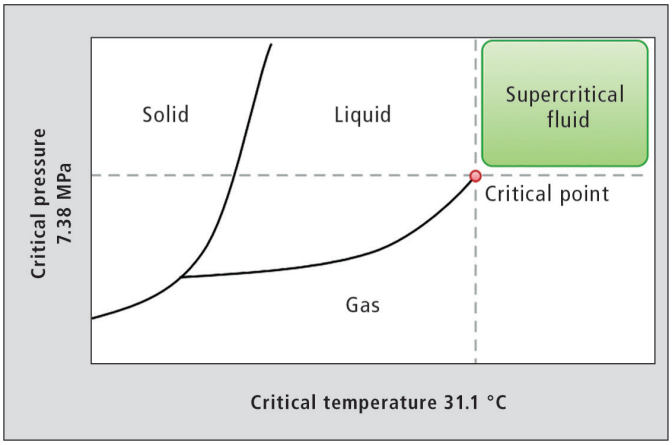

Figure 1: Phase diagram of carbon dioxide.

elutropic strength of the pressurised CO_2 alone is often insufficient for the elution of target compounds from a column. These target compounds can be eluted by adding an organic solvent, called a modifier, to increase the polarity of the mobile phase. For carbon dioxide, organic solvents such as methanol, ethanol, isopropyl alcohol and acetonitrile are compatible. Organic solvents with an added acid (e.g. formic acid or acetic acid), salt (e.g. ammonium formate or ammonium acetate), or base (e.g. diethylamine) are also used as modifiers for the analysis of highly polar compounds [1,2].

Application of analysis of choline and acetylcholine in rat cerebrospinal fluid samples using a SFC-MS/MS system

Choline, a structural element of cell membranes, and acetylcholine, a well-known neurotransmitter, are both compounds frequently encountered in the field of bioanalysis. Since acetylcholine is biosynthesised in the body from choline, it is possible to estimate the quality of internal activity by monitoring both compounds. This application example focuses on SFC analysis of these compounds in a rat cerebrospinal fluid sample by direct injection of the cerebrospinal fluid to the Nexera UC SFC-MS/MS system (Shimadzu) [3]. This research is also done because acetylcholine plays a decisive role in the autonomic nervous system and at the interface between motor nerves and skeletal muscles [4].

Also introduced in this research is the automatic extraction and analysis of a cerebrospinal fluid sample impregnated into filter paper, in consideration of convenience and durability for storage and transport, using the Nexera UC online SFE-SFC-MS/MS system.

SFC-MS/MS analysis

A cyano (CN) column provided the desired selectivity to separate choline and acetylcholine in SFC-MS/MS analysis. A three point calibration curve was created from the average of six peak area replicates for 10, 100 and 1000 µg/L standards. Acceptable linearity was obtained and the quantitation limit (LOQ, ASTM method) was determined to be 30 µg/L for choline and 10 µg/L for acetylcholine. Table 1 lists the conditions of SFC-MS/MS analysis. Figure 2 shows the structural formula of choline and acetylcholine while Figure 3 displays the calibration curves obtained.

Table 1: SFC-MS/MS analytical conditions column.

Column	Intersil CN-3, 250 mm L. x 4.6 mm I.D., 5 µm
Mobile phase	A) Supercritical fluid of CO_2 B) Modifier: Methanol containing 20 mmol/L ammonium formate / water = 95/5 (v/v)
Time program	B Conc. 10 % (0. min) → 25 % (10 min) → 50 % (10.1 - 12 min) → 10 % (12.1 - 15 min)
Flow rate	2.5 mL/min
Column temp.	40 °C
Injection vol.	1 µL
BPR pressure	10 Mpa
BPR temp.	50 °C
Detector	LCMS-8050 (ESI, MRM mode)
Make-up	Methanol
Make-up flow rate	0.2 mL/min
MRM transitions	(+) m/z 104.1 > 60.1 (for choline) (+) m/z 146.1 > 87.1 (for acetylcholine)

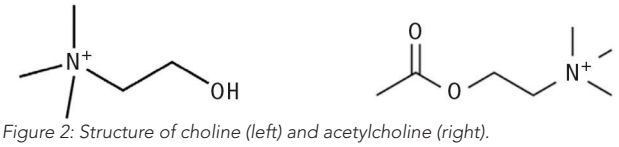

Figure 2: Structure of choline (left) and acetylcholine (right).

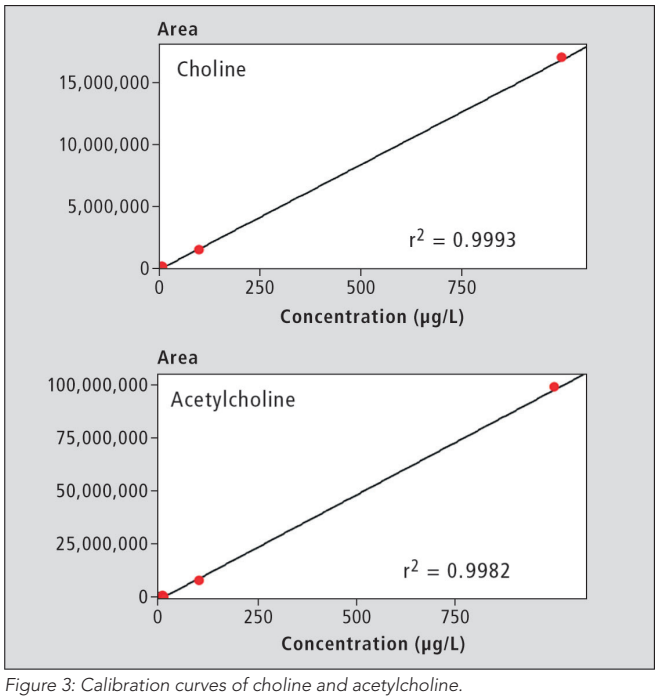

Figure 3: Calibration curves of choline and acetylcholine.

Retention time and peak area repeatability of six replicates at each concentration of 10, 100 and 1000 µg/L were confirmed at calibration curve creation, and the results are summarised in Table 2. Linearity (r^2) was found to be 0.9993 for choline and 0.9982 for acetylcholine. Figure 4 shows the MRM chromatograms for 100 µg/L.

Table 2: Repeatability of choline and acetylcholine standards (n = 6).

		Retention time (%RSD)	Peak area (%RSD)
Choline	10 µg/L	0.22	7.5
Choline	100 µg/L	0.05	1.7
Choline	1,000 µg/L	0.07	2.2
Acetylcholine	10 µg/L	0.07	5.7
Acetylcholine	100 µg/L	0.06	4.2
Acetylcholine	1,000 µg/L	0.07	6.0

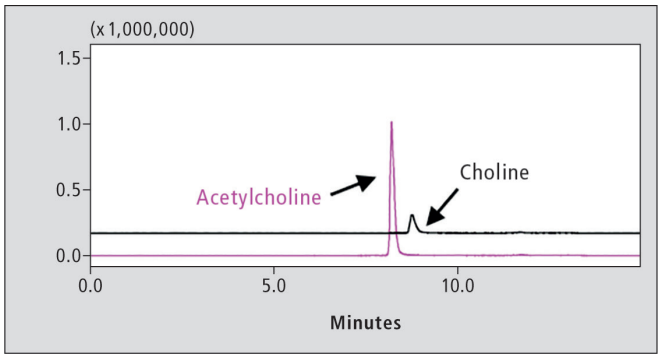

Figure 4: Choline and acetylcholine standards (100 µg/L).

Upon determining the analytical method was suitable for analysis, the next step was employing the micro dialysis method in which biological compounds are sampled continuously from an awake animal via the semipermeable membrane of a minute dialytic probe connected to a pump. Cerebrospinal fluid was sampled from a rat and delivered directly to SFC analysis. The injection volume of cerebrospinal fluid was set to 1 µL due to concerns regarding the miscibility between the aqueous sample and low polar supercritical carbon dioxide serving as the main component of the mobile phase.

With respect to acetylcholine, the LOQ determined according to the ASTM method was approximately 10 µg/L. Since the calculated concentration was less than the LOQ, only qualitative peak identification was performed. As listed in Table 3, retention time and peak area repeatability were favourable for the six repeated analyses of choline. Figure 5 shows the chromatograms resulting from SFC analysis of the cerebrospinal fluid sample.

Table 3: Choline quantitative value in rat cerebrospinal fluid sample and repeatability (n = 6).

	Retention time (%RSD)	Peak area (%RSD)
Choline (concentration 229.6 µg/L)	0.10	3.1

Online SFE-SFC-MS/MS Analysis

As a next step, a sample was prepared by impregnating cerebrospinal fluid sample into filter paper and drying the paper. SFE-SFC-MS/MS analysis was then performed on the sample. The convenience of this method is gaining attention not only because of the ease of sample handling but also because of improved miscibility concerns between a mobile phase of low polarity supercritical carbon dioxide and an aqueous solvent containing a biological sample. Table 4 lists the conditions used in the online SFE-SFC-MS/MS analysis.

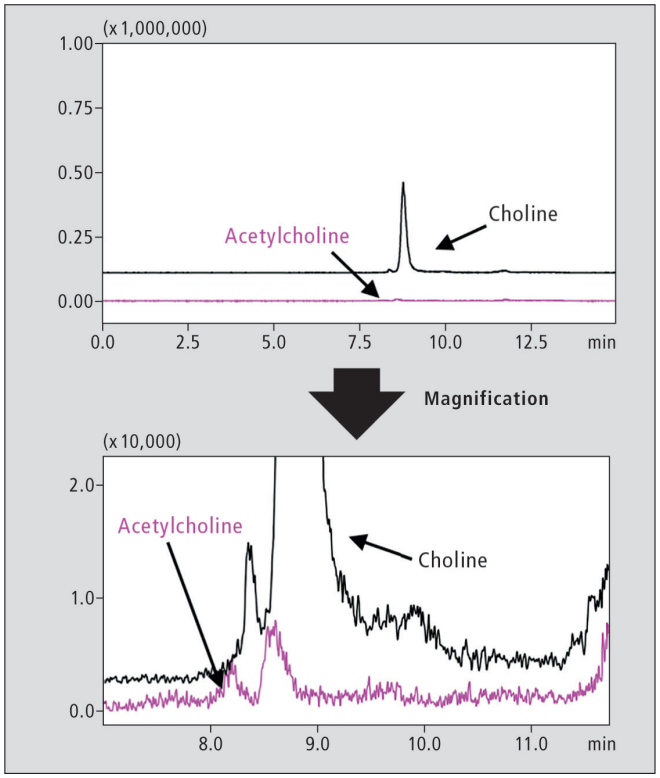

Figure 5: SFC analysis of choline and acetylcholine in a cerebrospinal fluid sample.

Table 4: Online SFE-SFC-MS/MS conditions.

Vessel	0.2 mL (1 µL of sample was added to filter paper)
Extractant	A) Supercritical fluid of CO_2 B) Methanol containing 20 mmol/L ammonium formate / water = 95/5 (v/v) / A/B = 9/1 (v/v)
Flow rate	2.5 mL/min
Extraction time	Static (0 - 3 min) – Dynamic (3 - 6 min) – Static (6 - 8 min) – Dynamic (8 - 11 min) – Static (11 - 13 min) – Dynamic (13 - 16 min)
BPR pressure	10 Mpa
Extraction temp.	60 °C
Time program	B Conc. 10 % (16 min) → 25 % (26 min) → 50 % (26.1 - 28 min) → 10 % (28.1 - 31 min)

* SFC-MS/MS conditions are identical to table 1 except for the time program

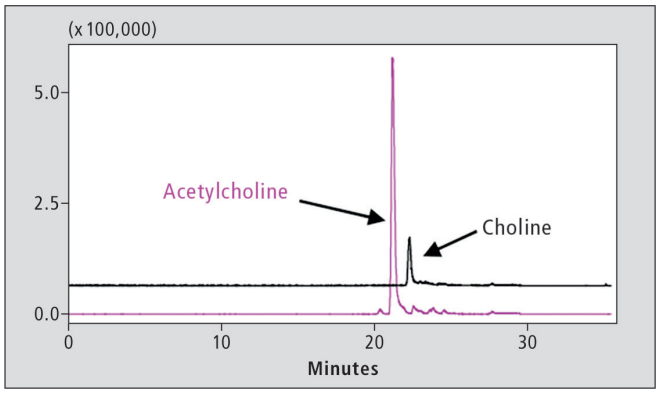

Figure 6: Results from online SFE-SFC-MS/MS analysis with dropping 1 µL of 100 µg/L standard solution onto filter paper.

Figure 6. shows the result obtained from online SFE-SFC-MS/MS analysis of a sample created by dropping 1 µL of 100 µg/L standard solution onto filter paper (GA-200 by ADVANTEC).

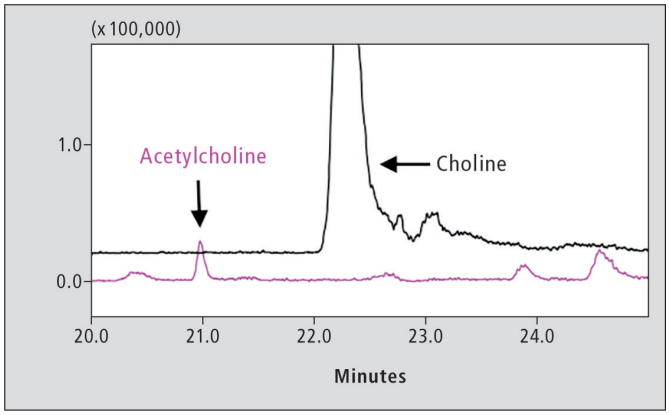

Figure 7: Result of processing the rat cerebrospinal fluid sample with online SFE-SFC-MS/MS analysis.

Figure 7 shows the result obtained by processing the rat cerebrospinal fluid sample in the same manner. The peak obtained for acetylcholine was small like the SFC analysis result, however, since the baseline noise level was improved in comparison, improved LOQ was obtained. Because the S/N value of corresponding peak to acetylcholine was more than 15 based on the baseline noise determined by ASTM method, a simple quantitative calculation was made based on the 100 µg/L standard data in the same way as the more concentrated choline. The obtained choline concentration of 297 µg/L was close to the SFC result and suggested that extraction in online SFE was performed efficiently. For acetylcholine, a calculation result of 1.7 µg/L was obtained from the peak area.

Summary and conclusion

The article provides a general overview of the advantages provided by SFC over traditional HPLC. SFC can be performed at higher flow rates with lower pressure drop created across the column for increased sample throughput. Based on the SFC application an automatic extraction and analysis of a cerebrospinal fluid sample impregnated into filter paper, in consideration of convenience and durability for storage and transport, using the Nexera UC online SFE-SFC-MS/MS system was performed.

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