C219-E001C

SHIMADZU
Excellence in Science

Shimadzu Analysis Guidebook

Pharmaceutical Analyses

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1. General Pharmaceuticals

1.1 Analysis of Cold Medicine - GC

■Explanation

This data introduces direct analysis of a cold medicine without derivatization.

■Pretreatment

Cold medicine is dissolved in methanol, and 1 µL of this solution is injected.

■Analytical Conditions

Column	: DB-1 (30 m × 0.25 mm I.D. df = 0.25 µm)
Column Temp.	: 250 °C
Injector Temp.	: 300 °C
Detector Temp.	: 300 °C (FID)
Carrier Gas	: He 0.5 mL/min
Injection Method	: Split Injection
Split Ratio	: 1 : 100

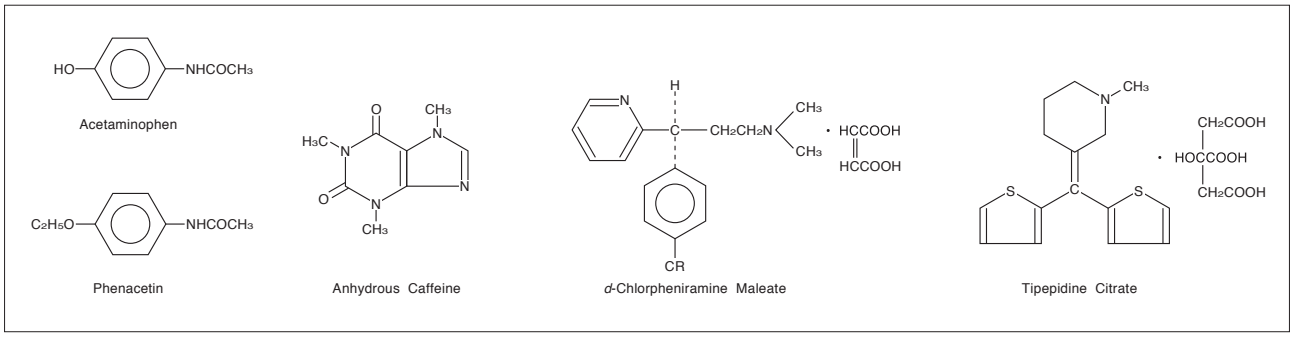


Fig. 1.1.1 Structural Formula of Acetaminophen, Phenacetin, Anhydrous Caffeine, *d*-Chlorpheniramine Maleate and Tipepidine Citrate

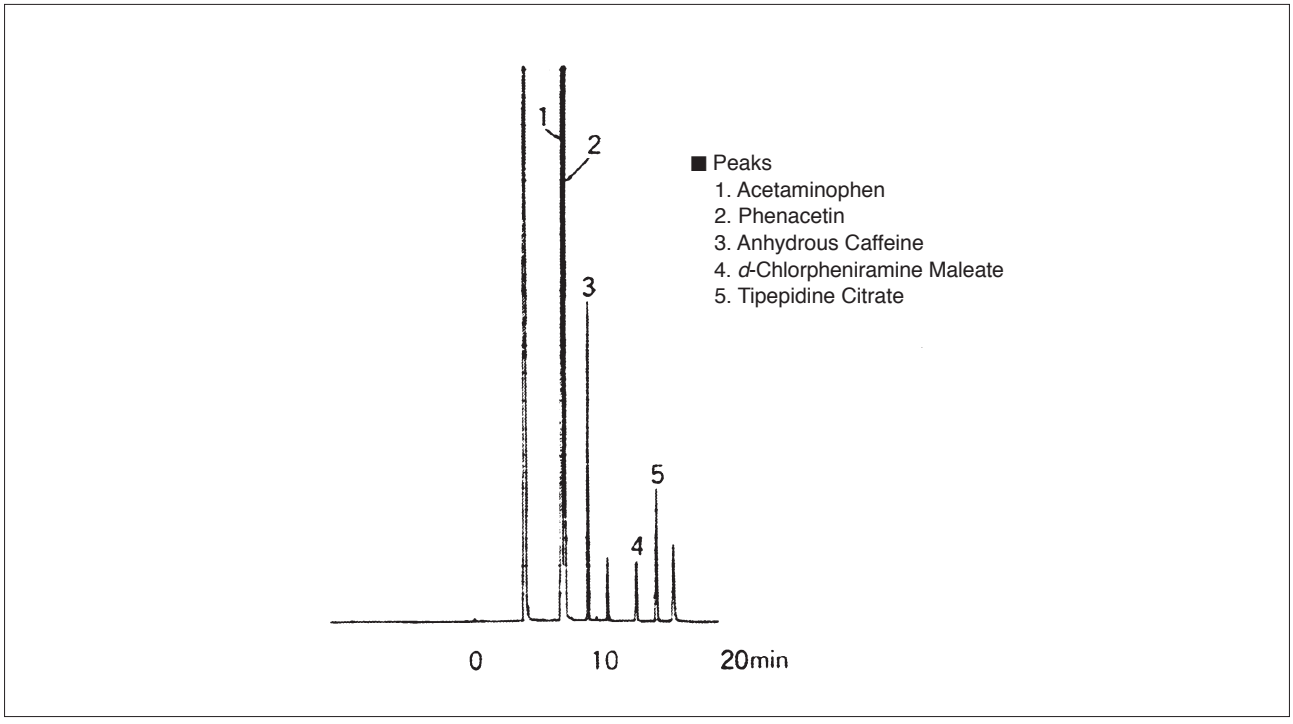


Fig. 1.1.2 Chromatogram of Cold Medicine

1.2 High Speed Analysis of Cold Medicine (1) - LC

■Explanation

Commercial cold medicines are formulated with a variety of ingredients such as antipyretics and analgesics to reduce fever and soothe pain, antihistamines to suppress allergy symptoms such as runny nose and sneezing, antitussives and expectorants to relieve cough and phlegm ymptoms, caffeine to relieve headache, as well as vitamins and herbal extracts. Here, we focus on the active ingredients contained in commercial cold medicine, and introduce a high speed analysis of these ingredients.

■Analysis of Standard Solution

A standard mixture of cold medicine ingredients was prepared (dissolving them in mobile phase A / acetonitrile = 1/1 (v/v), 100 mg/L each), and then analyzed. The mixture consisted of thiamine, acetoaminophen, caffeine, riboflavin, hesperidin, ethenzamide, chlorpheniramine, ambroxol, noscapine, isopropamide, isopropylantipyrine, dextromethorphan, glycyrrhizin, bromhexine, clemastine and ibuprofen. Fig. 1.2.1 shows the chromatograms at two different wavelengths obtained using photodiode array detection.

■Analytical Conditions

Instrument	: Prominence UFLCXR System
Column	: Shim-pack XR-ODS (50 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase	: A : 20 mmol/L Sodium Phosphate Buffer (pH 2.5) containing 100 mmol/L Sodium Perchlorate B : Mobile phase A / Acetonitrile (3/7, v/v) Gradient Elution Method
Time Program	: B 15 % (0min) →45 % (1min) →90 % (2 min - 2.2 min) → 100 % (2.21 min - 2.6 min) →15 % (2.61 min - 3.2 min)
Flowrate	: 1.4 mL/min
Column Temp.	: 40 °C
Injection Volume	: 1 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 220 nm and 250 nm
Flow Cell	: Semi-micro Cell

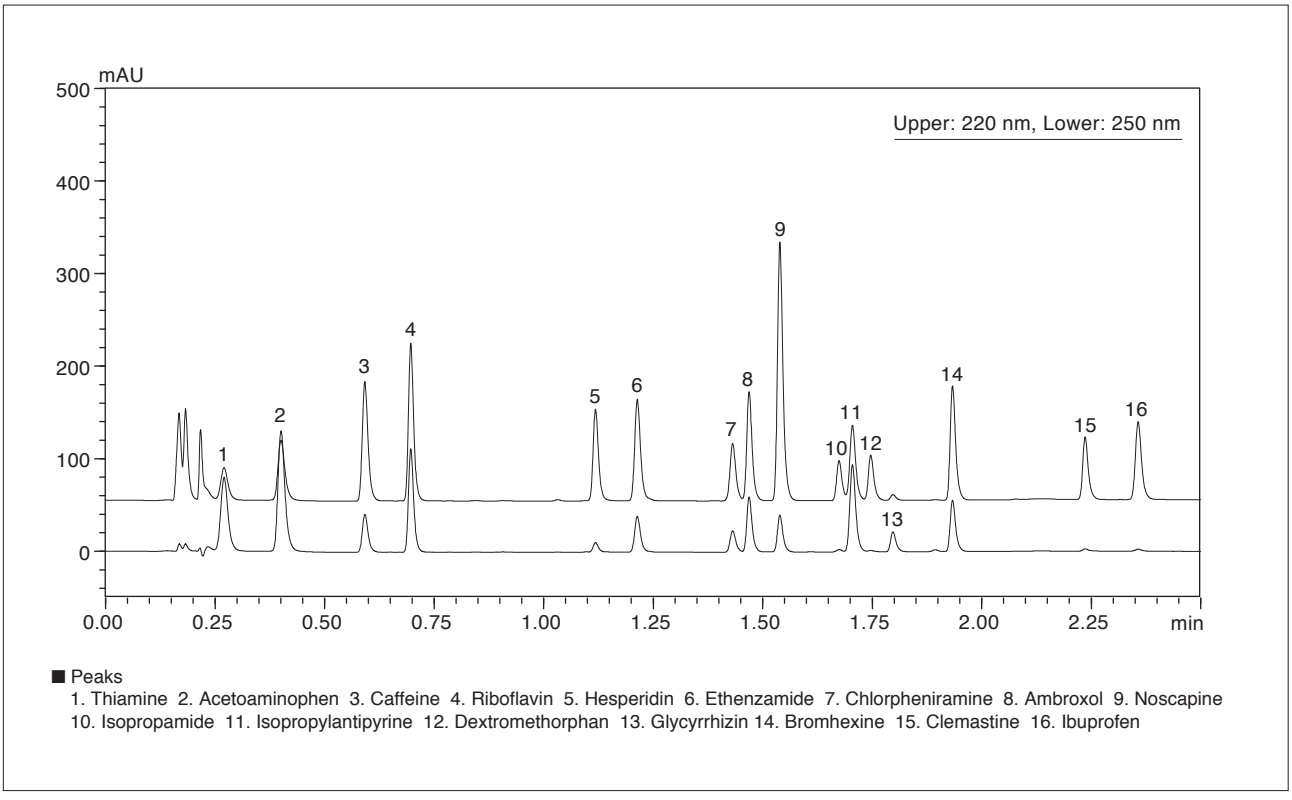


Fig. 1.2.1 Chromatograms of a Standard Mixture of 16 Active Ingredients

1.2 High Speed Analysis of Cold Medicine (2) - LC

■Analysis of Over-the-Counter (OTC) Cold Medicines

The contents of a capsule of cold medicine was dissolved in 100 mL of mobile phase A/acetonitrile = 1/1 (v/v), or in the case of tablet form, a single tablet was dissolved in 50 mL of the above mobile phase acetonitrile solution. The resultant solution was then passed through a 0.22 µm pore membrane filter, and then analyzed.

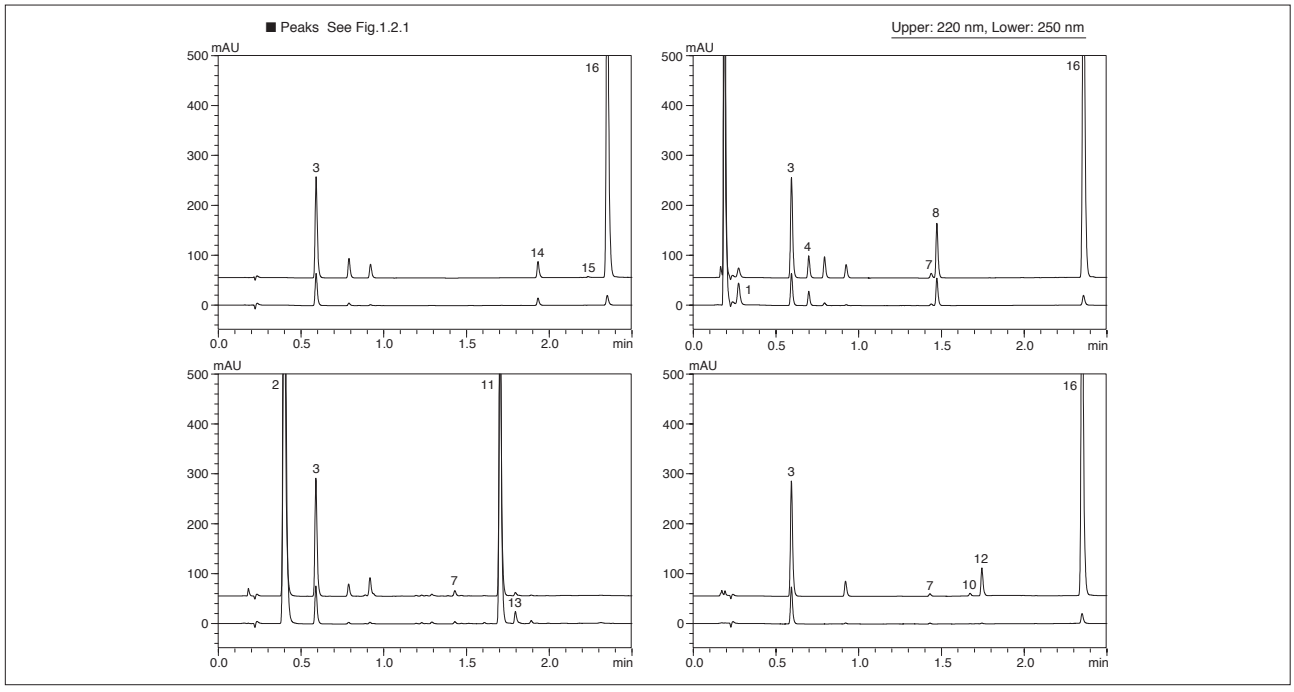


Fig. 1.2.2 Chromatograms of 4 OTC Cold Medicines

■Ultra High Speed Analysis

Fig.1.2.3 shows the result of 5 serial ultra-high-speed analyses of OTC cold medicine. In ultra-high-speed analysis, the autosampler cycle time becomes a critical factor. The injection speed of the Prominence UFLC/UFLCXR's autosampler is an ultra-fast 10 seconds*, and the total time for all 5 analyses took no more 5 minutes.

(* Under shimadzu specified conditions)

■Analytical Conditions

Column	: Shim-pack XR-ODS (30 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase	: The same as previous page
Time Program	: B 10 % (0 min) →70 % (0.4 min) → 10% (0.41 min-0.65 min)
Flowrate	: 2.0 mL/min
Column Temp.	: 40 °C
Injection Volume	: 2 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 210 nm
Flow Cell	: Semi-micro Cell

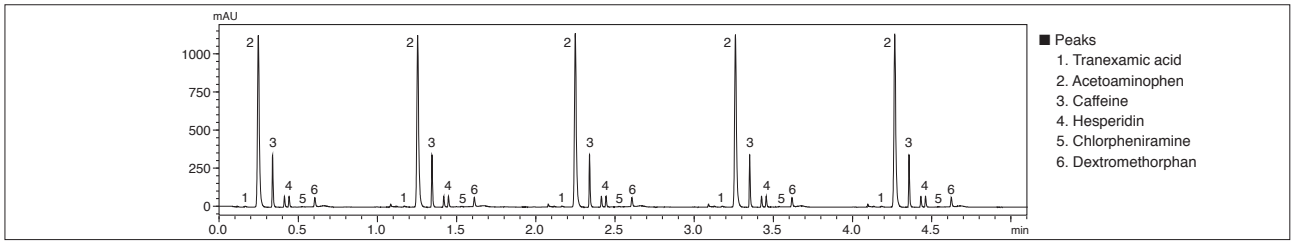


Fig. 1.2.3 Chromatogram of 5 Serial Ultra-High-Speed Analyses

1.3 Ultra Fast Analysis of Combination Cold Remedy (1) - LC/MS

■Explanation

Now that ultra fast LC can provide analyses with peak widths in fractions of a second, it is necessary to have detectors that can keep pace with the sharp peaks generated by this technique. The LCMS-2020 is a mass spectrometer with "UFswitching" that allows high speed positive / negative ionization mode switching (15 msec positive / negative ionization switching), and "UFscanning" that allows high-speed scan measurement (max. 15,000 u/sec scan speed) to fully support the progression to LC ultra fast analysis. Here we present an example of ultra fast analysis of a combination pharmaceutical cold remedy using the Prominence UFLCXR ultra fast, high-resolution LC system and the LCMS-2020.

■Analysis of Combination Cold Remedy

Sample preparation consists of crushing a tablet of combination cold remedy A and dissolving 10 mg of the sample in 10 mL of purified water. The solution was filtered and then analyzed by LC/MS. An electrospray source (ESI) was used for ionization, and simultaneous positive/negative ion analysis was used to maximize detection of different compounds. The total ion chromatogram (TIC) and mass chromatograms of combination cold remedy A are shown in Fig. 1.3.1, with the structural formulas of the 7 confirmed ingredients shown in Fig. 1.3.2. Using a mobile phase flow rate of 1.8 mL/min, analysis was performed in just 1.5 minutes, including the time required for column equilibration. The entire mobile phase flow was directed into the MS without splitting. Fig. 1.3.3 shows the mass spectra of all ingredients. A protonated molecule was found for every ingredient in positive ion mode. In addition, a deprotonated molecule was also confirmed for 2. acetaminophen in the negative ion mode.

■Analytical Conditions

Column	: Advanced Materials Technology HALO C18 (50 mmL. × 3.0 mm I.D., 2.7 µm)
Mobile Phase A	: 5 mmoL/L Ammonium Formate and 5 mmoL/L Formic Acid - Water
Mobile Phase B	: Acetonitrile
	Gradient Elution Method
Time Program	: B 7 % (0 min) → 45 % (1 min) → 7 % (1.01 min - 1.5 min)
Flowrate	: 1.8 mL/min
Column Temp.	: 60 °C
Injection Volume	: 1 µL
Probe Voltage	: 4.5 kV/-3.5 kV
	(ESI-Positive Mode/Negative Mode)
DL Temp.	: 250 °C
BH Temp.	: 200 °C
Nebulizing Gas Flow	: 1.5 L/min
Drying Gas Flow	: 20 L/min
DL,Q-array Voltage	: Default Values
Scan Range	: *m/z* 100 - 700 (60 msec/Scan)

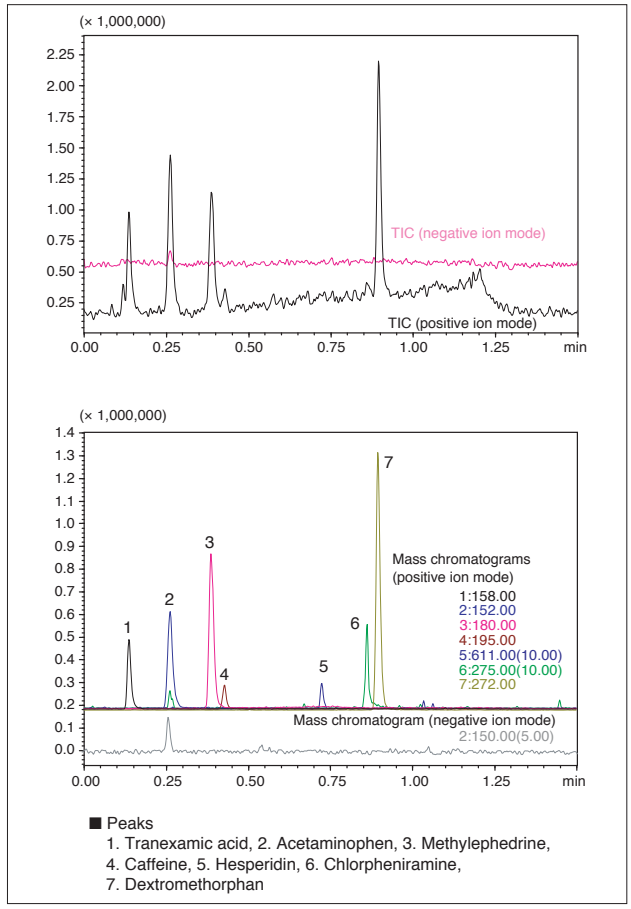


Fig. 1.3.1 TIC (upper) and Mass Chromatograms (lower) of Combination Cold Remedy A

1.3 Ultra Fast Analysis of Combination Cold Remedy (2) - LC/MS

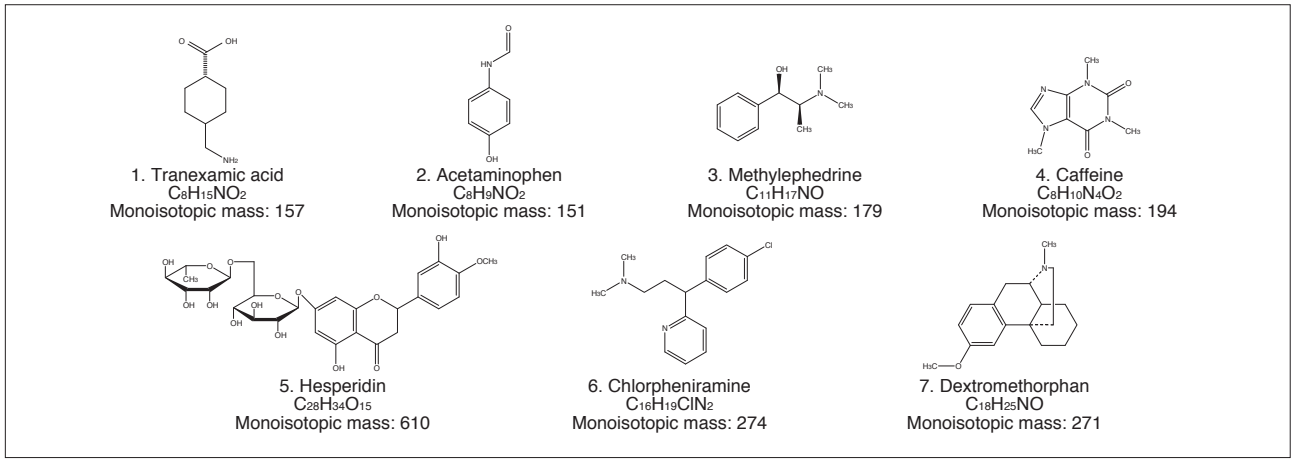


Fig.1.3.2 Structural Formulas of Ingredients in Combination Cold Remedy A

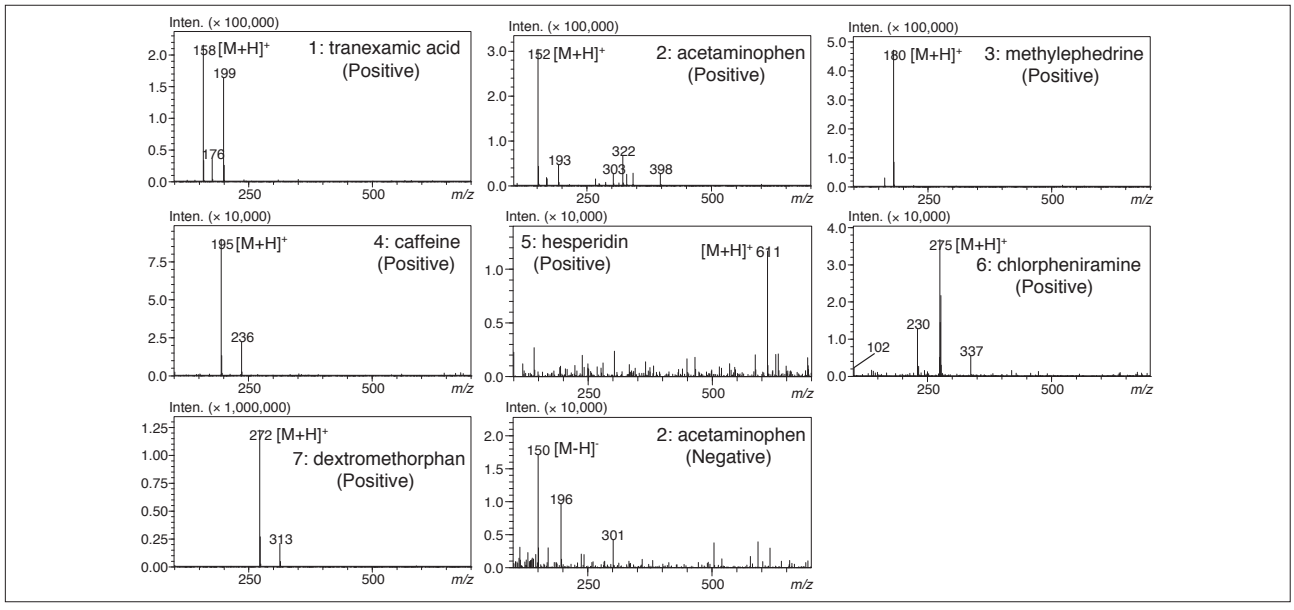


Fig. 1.3.3 Mass Spectra of Ingredients in Combination Cold Remedy A

■Ultra Fast Mass Analysis

In this analysis, measurement was conducted with a scan speed of 15,000 u/sec. With the mass range of *m/z* 100-700 for the acquisition, each scan required just 60 msec (Fig.1.3.4). This fast sampling time assures that a sufficient number of scans are acquired for the sharp peaks generated using the Prominence UFLCXR. The LCMS-2020 clearly supports ultra fast analysis together with the Prominence UFLCXR ultra fast, high-resolution LC system. This combination provides excellent, reproducible results for 1.5-min fast analysis of combination cold remedy. Even for the fastest peaks, spectral quality is maintained and reliable for correct confirmation of each constituent.

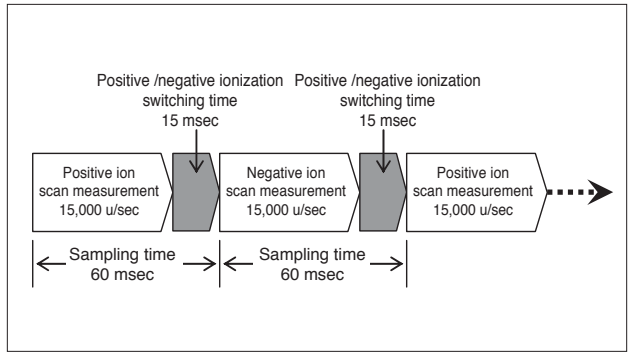


Fig. 1.3.4 Sampling Time and Positive/Negative Ionization Switching Time

1.4 Analysis of Sedative Sleeping Drug and Intravenous Anesthetic - GC

■Explanation

This data introduces direct analysis of a sedative sleeping drug and intravenous anesthetic without derivatization.

■Pretreatment

Sedative sleeping drug and intravenous anesthetic are dissolved in methanol, and 1 µL of this solution is injected.

■Analytical Conditions

Column	: DB-1 (30 m × 0.25 mm I.D. df = 0.25 µm)
Column Temp.	: 210 °C
Injector Temp.	: 300 °C
Detector Temp.	: 300 °C (FID)
Carrier Gas	: He 0.5 mL/min
Injection Method	: Split Injection
Split Ratio	: 1 : 100

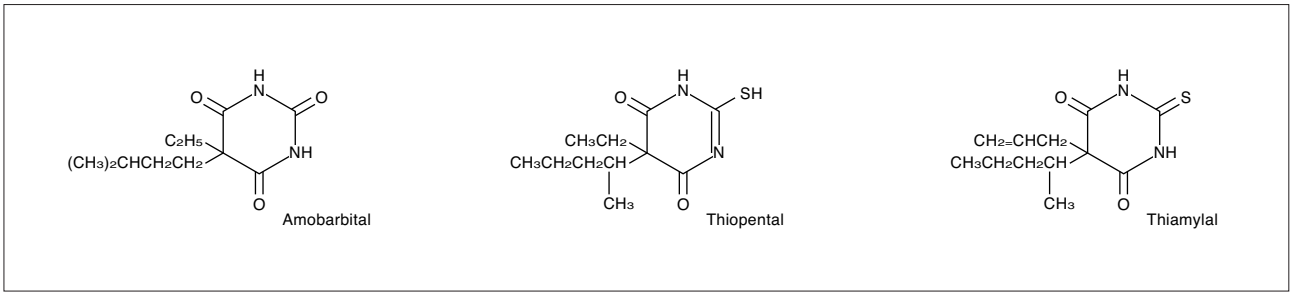


Fig. 1.4.1 Structural Formula of Amobarbital, Thiopental and Thiamylal

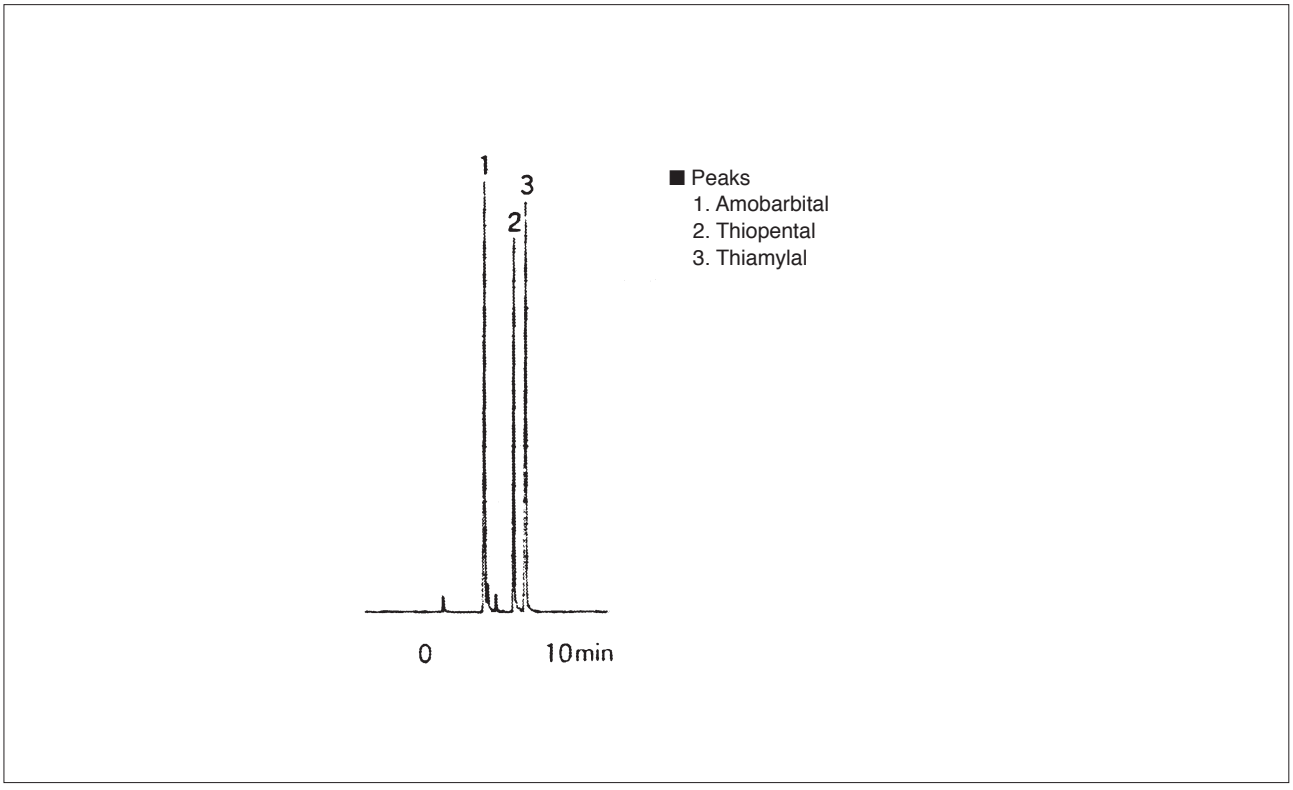


Fig. 1.4.2 Sedative Sleeping Drug and Intravenous Anesthetic

1.5 Headspace Analysis of Volatile Components in Pharmaceuticals - GC

■Explanation

This data introduces analysis of two types of antiphlogistic pain relief ointment using the headspace gas chromatography method.

■Pretreatment

The sample for analysis (pharmaceutical) is enclosed in a vial, is warmed for a set time at a constant temperature, and the headspace gas analyzed.

■Analytical Conditions

Column	: ULBON HR-20M (25 m × 0.32 mm I.D. df = 0.25 µm)
Column Temp.	: 200 °C
Injector Temp.	: 230 °C
Detector Temp.	: 230 °C (FID)
Carrier Gas	: He 1.2 mL/min
Injection Method	: Split Injection
Split Ratio	: 1 : 14
Sample Quantity	: 20 mg
Sample Thermostatting	: 150 °C, 60 min
Headspace Injection Volume	: 0.8 mL

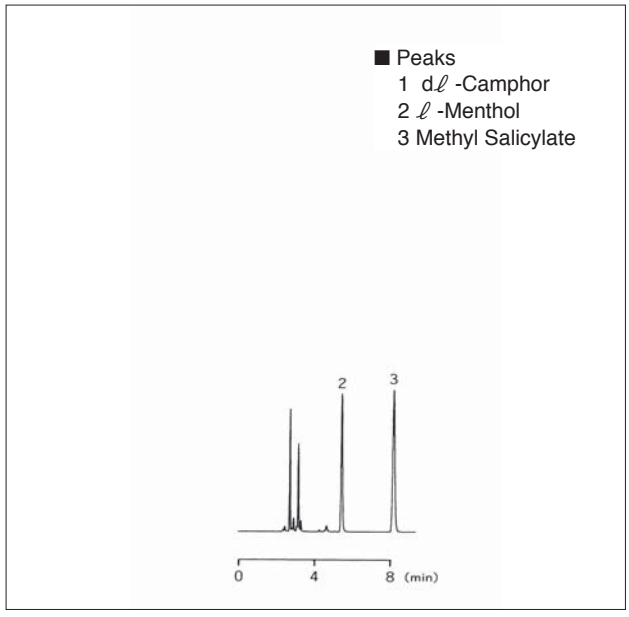


Fig. 1.5.1 Headspace Gas Chromatogram of Antiphlogistic Pain Relief Ointment A

■Analytical Conditions

Column	: CBP20 (25 m × 0.53 mm I.D. df = 1.0 µm)
Column Temp.	: 50 °C (5 min) -10 °C/min -180 °C
Injector Temp.	: 220 °C
Detector Temp.	: 220 °C (FID)
Carrier Gas	: He 5.3 mL/min
Injection Method	: Split Injection
Split Ratio	: 1 : 4
Sample Quantity	: 0.3 g
Sample Thermostatting	: 80 °C, 40 min
Headspace Injection Volume	: 0.8 mL

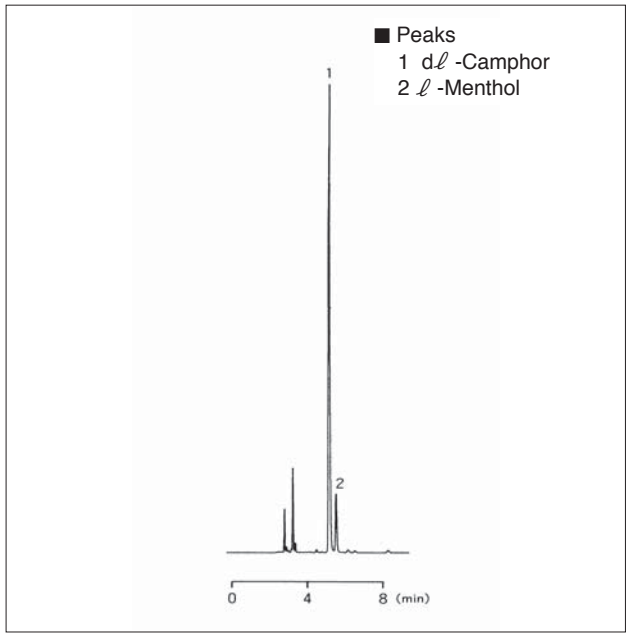


Fig. 1.5.2 Headspace Gas Chromatogram of Antiphlogistic Pain Relief Ointment B

1.6 Analysis of Antispasmodic Drug - GC

■Explanation

This data introduces direct analysis of an antispasmodic drug without derivatization.

■Pretreatment

1 g of antispasmodic drug is dissolved in 10 mL of methanol, and 1 µL of this solution is injected.

■Analytical Conditions

Column	: DB-1 (30 m × 0.25 mm I.D. df = 0.25 µm)
Column Temp.	: 250 °C
Injector Temp.	: 300 °C
Detector Temp.	: 300 °C (FID)
Carrier Gas	: He 0.5 mL/min
Injection	: Split Injection
Split Ratio	: 1 : 100

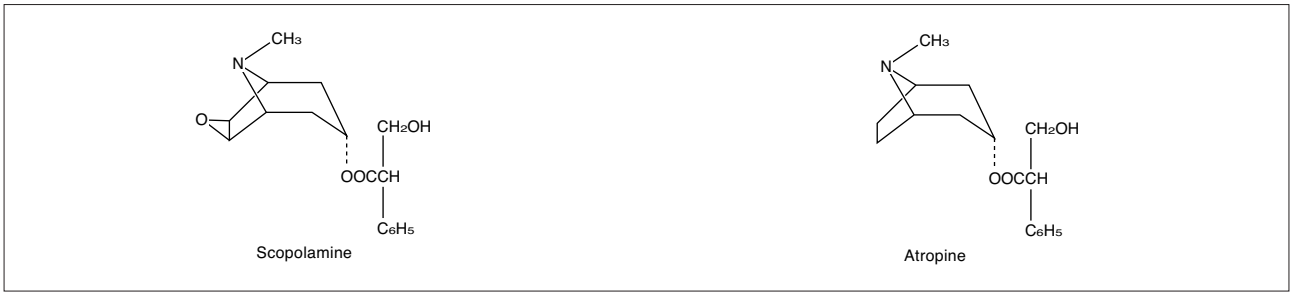


Fig. 1.6.1 Structural Formula of Scopolamine and Atropine

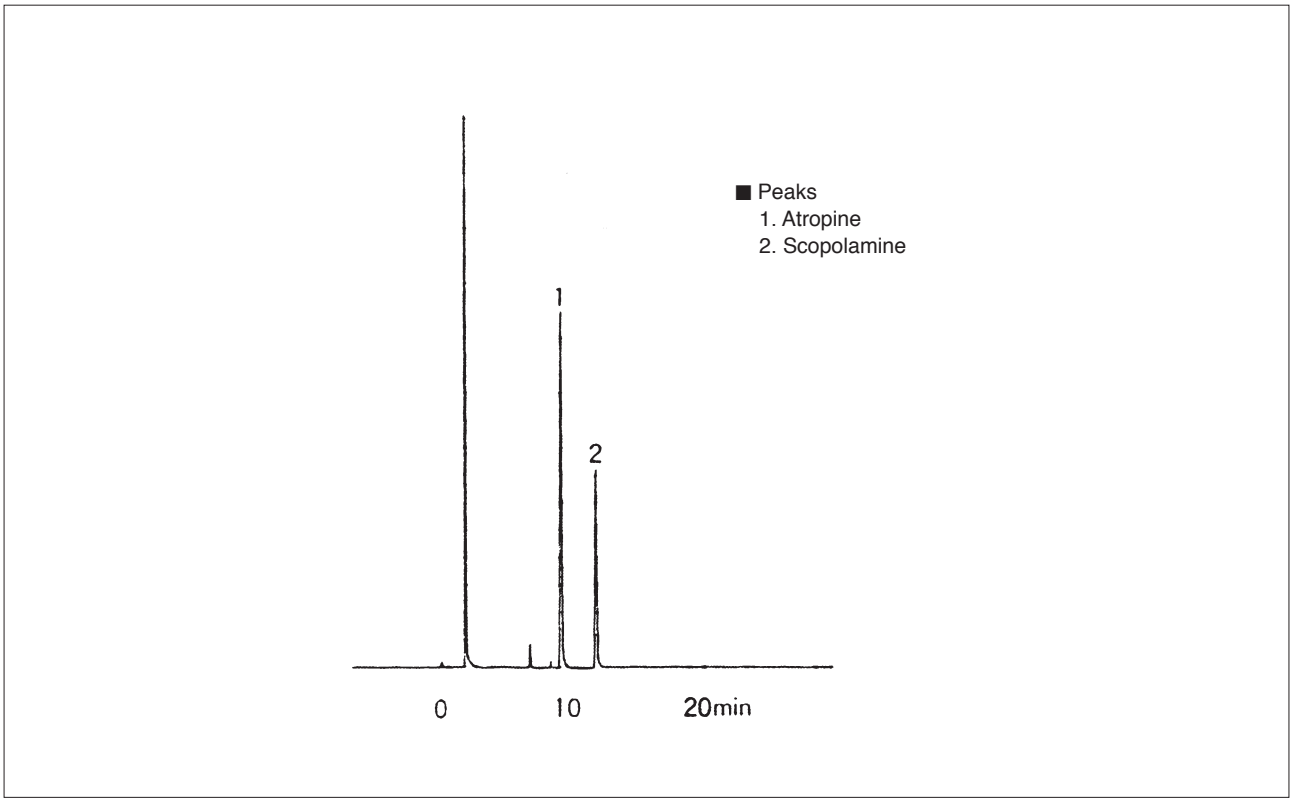


Fig. 1.6.2 Chromatogram of Antispasmodic Drug

1.7 High Speed Analysis of Non-steroidal Anti-inflammatory Drugs (1) - LC

■Explanation

Non-steroidal anti-inflammatory drugs (NSAIDs) are drugs that display as their main action, analgesic, antipyretic, and anti-inflammatory effects. However, since they also display other effects such as platelet aggregation inhibition activity and uricosuric activity, they are considered to have wide applicability in disease treatment. Here we introduce an example of high-speed analysis of non-steroidal anti-inflammatory drugs using the Prominence UFLC ultra-high-speed LC system with the Shim-pack XR-Phenyl columns.

■Analysis of Standard Solution

Fig. 1.7.1 shows the structures of 6 non-steroidal anti-inflammatory drugs (piroxicam, ketoprofen, diflunisal, ibuprofen, diclofenac and indomethacin). Fig. 1.7.2 shows the results of analysis of a standard mixture of these 6 compounds (10 mg/L each, dissolved in acetonitrile and brought to volume using purified water), using the Shim-pack XR-Phenyl column (upper) and the Shim-pack XR-ODS (lower) columns. "The Shim-pack XR-Phenyl is a column in which the phenyl group linked to the silica backbone has been modified for reversed-phase chromatography. Separation is basically accomplished through hydrophobic interaction, but interaction with the phenyl group is also thought to occur depending on the type of sample, so selectivity that differs from ODS columns is often displayed.

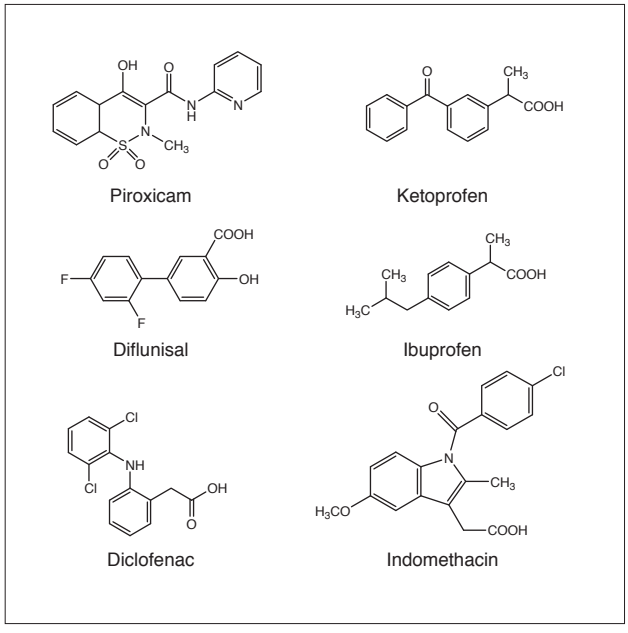


Fig. 1.7.1 Structures of 6 Non-steroidal Anti-inflammatory Drugs

■Analytical Conditions

Column	: Shim-pack XR-Phenyl (50 mmL. × 3.0 mm I.D., 2.2 µm) Shim-pack XR-ODS (50 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase	: A : 20 mmol/L Potassium Phosphate Buffer (pH 2.5) B : Acetonitrile A/B = 62/38 (v/v)
Flowrate	: 1.5 mL/min
Column Temp.	: 40 °C
Injection Volume	: 5 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 220 nm
Flow Cell	: Semi-micro Cell

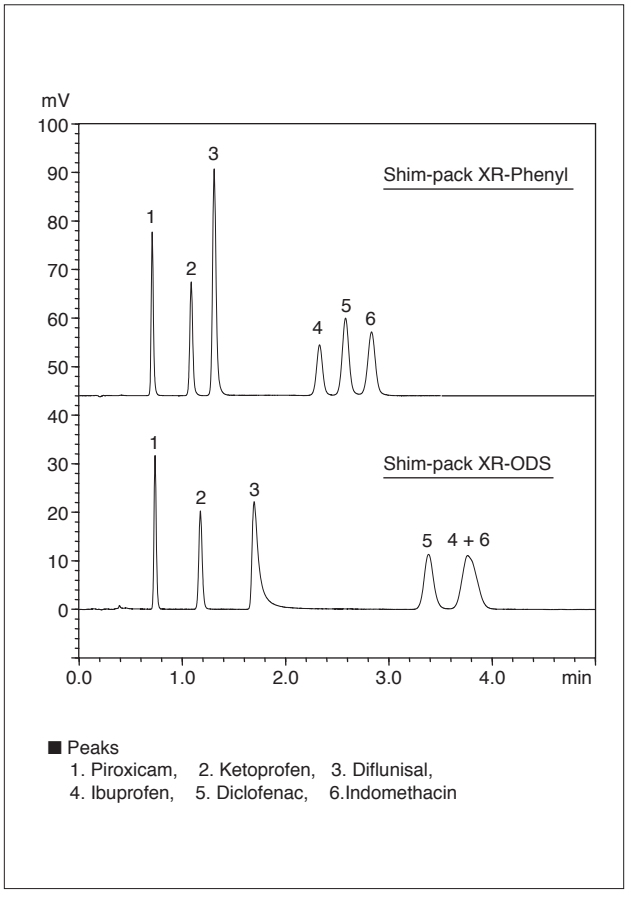


Fig. 1.7.2 Chromatograms of a Standard Mixture of 6 Non-steroidal Anti-inflammatory Drugs
- Comparison of Results with Shim-pack XR-Phenyl and Shim-pack XR-ODS Columns

1.7 High Speed Analysis of Non-steroidal Anti-inflammatory Drugs (2) - LC

■Analysis of Over-the-Counter (OTC) Cold Medicines

Two OTC medicines were prepared as shown in Fig. 1.7.3, and the results of their analyses using the Shim-pack XR Phenyl column are shown in Fig. 1.7.4 and Fig. 1.7.5.

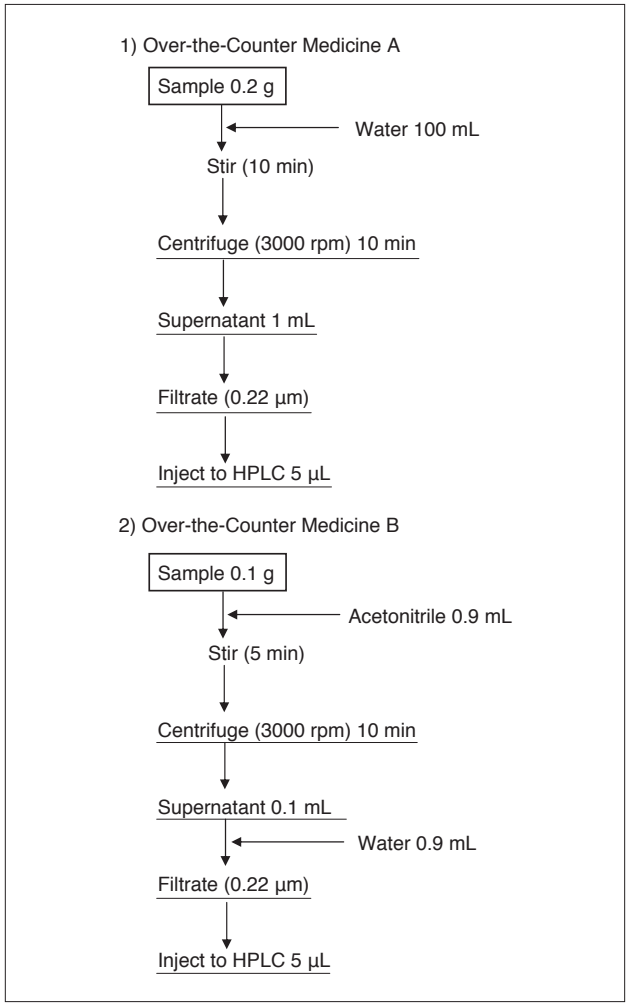


Fig. 1.7.3 Sample Preparation

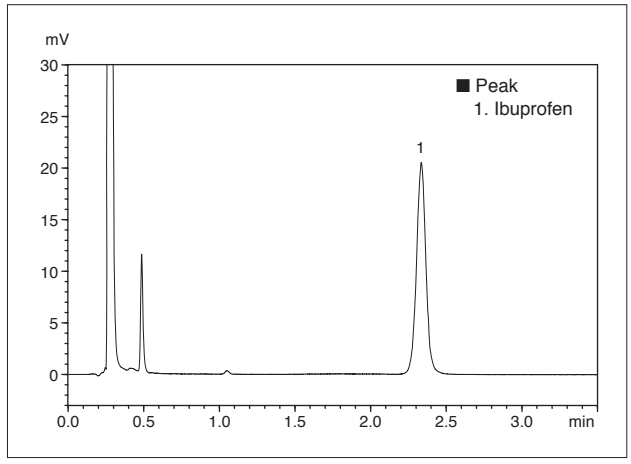


Fig. 1.7.4 Chromatogram of OTC Medicine A

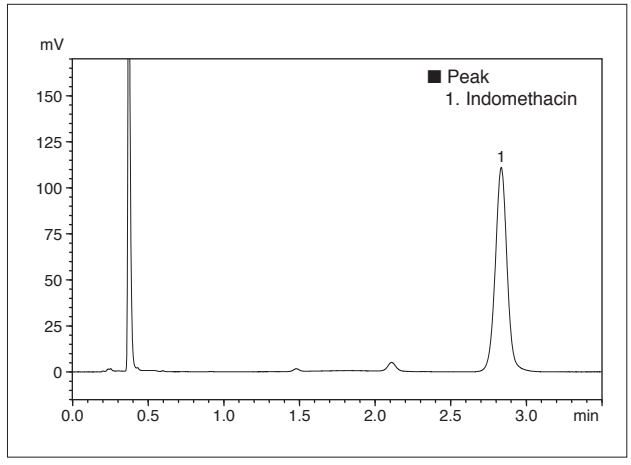


Fig. 1.7.5 Chromatogram of OTC Medicine B

1.8 Analysis of Steroidal Anti-Inflammatory Agents - LC/MS

■Explanation

Steroidal anti-inflammatory agents (adrenocortical hormones) display extremely high anti-inflammatory efficacy, but due to their known side-effects it can be difficult to appropriately adjust their dosage. Moreover, due to reports of the detection of these steroidal anti-inflammatory agents in imported products labeled as "health foods", it is important to monitor these drugs. Here we introduce an example of LC/MS analysis of steroidal anti-inflammatory agents. As steroidal anti-inflammatory agents have a low polarity, the positive ion atmospheric pressure chemical ionization method (APCI-Positive) was used. Although a methanol mobile phase generally provides greater ionization efficiency with APCI than an acetonitrile mobile phase, the acetonitrile mobile phase was selected for this analysis to achieve better separation of dexamethasone and betamethasone, which have the same molecular weight. Fig. 1.8.2 shows a SIM chromatogram of steroidal anti-inflammatory agents.

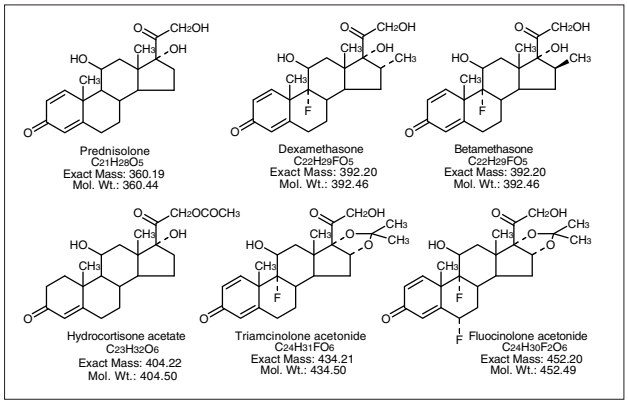


Fig. 1.8.1 Six Structures of Specific Steroidal Anti-inflammatory Agents

■Analytical Conditions

Column : Phenomenex Synergi MAX-RP (150 mmL. × 2.0 mm I.D.)
Mobile Phase : 0.1 % Formic Acid-Water /Acetonitrile (70:30)
Flowrate : 0.3 mL/min
Column Temp. : 40˚C
Injection Volume : 2 μL
Probe Voltage : +4.5 kV (APCI-Positive Mode)
Nebulizer Gas Flow : 2.5 L/min
Drying Gas Pressure : 0.04 MPa
Probe Temp. : 350 ˚C
CDL Temp. : 200 ˚C
Block Heater Temp. : 200 ˚C
CDL & Q-array Voltage : Default Values
Interval : 0.8 sec/ 16 chs
Monitor Ions : *m/z* 402.1,<u>361.1</u>,343.1,325.1 for Prednisolone
m/z 434.1, <u>393.1</u> 373.1 355.1 for Betamethasone & Dexamethasone
m/z 476.1, <u>435.1</u>, 415.1 for Triamcinolone acetonide
m/z 494.1, <u>453.1</u>, 413.1 for Fluocinolone acetonide
m/z 446.1, <u>405.1</u> for Hydrocortisone acetate

In addition to the protonated molecules $(M+H)^+$ observed in these mass spectra, fragment ions $(M+H-H_2O)^+$ and $(M+H-2H_2O)^+$ from which 1 to 2 water molecules have been lost due to the presence of hydroxyl groups in the structure, and in addition, mobile phase- (acetonitrile) adduct protonated molecules $(M+H+CH_3CN)^+$ seen with low polarity compounds are also observed. In this analysis, all of the principal ions were monitored, the protonated molecules underlined in the Analytical Conditions are used as quantitation ions, and the other ions are used as reference ions.

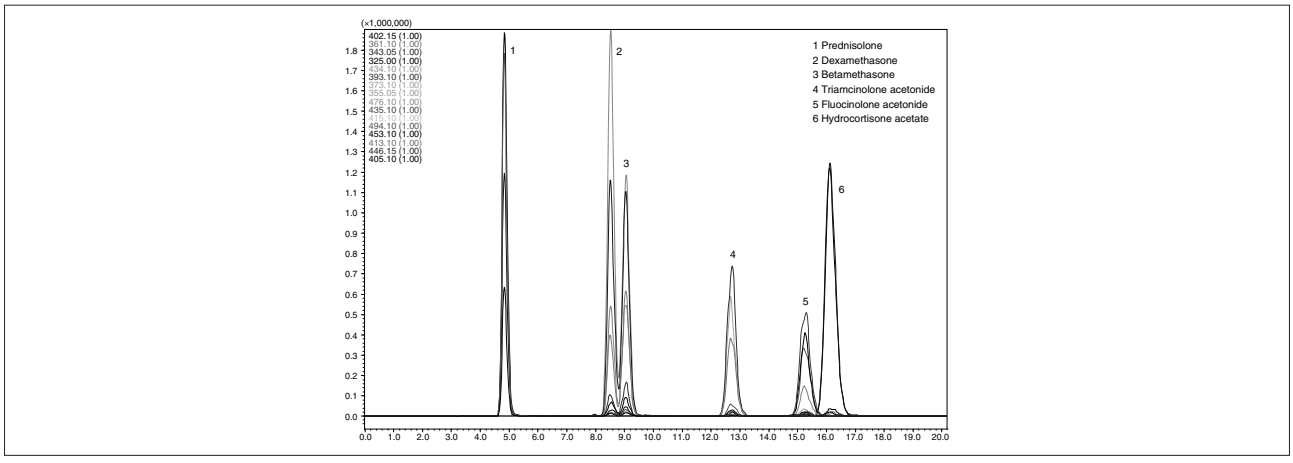


Fig. 1.8.2 Selected Ion Monitoring (SIM) Chromatograms of Six Specific Steroidal Anti-inflammatory Agents

1.9 High Speed Analysis of Chlorhexidine in Ointment - LC

■Explanation

Chlorhexidine is commonly used as an antiseptic agent in the form of a gluconate compound.The antibacterial properties of chlorhexidine gluconate are clearly demonstrated through its use as an effective skin disinfectant, oral rinse, etc. Here we present the analysis of chlorhexidine in an antiseptic ointment for surface injuries.

■Sample Preparation

The sample was prepared by diluting with mobile phase to a concentration of 10 mg/mL, followed by filtering through a 0.45 μm membrane filter.

■Analytical Conditions

Instrument	: Prominence UFLC System
Column	: Shim-pack XR-ODS (75 mmL. × 3.0 mm I.D.)
Mobile Phase	: A : 100 mmol/L Sodium Perchlorate in 10 mmol/L Sodium Phosphate Buffer (pH 2.6) B : Acetonitrile A/B = 3/2 (v/v)
Flowrate	: 1.0 mL/min
Column Temp.	: 40 °C
Injection Volume	: 4 μL
Detection	: UV Absorbance Detector at 260 nm
Flow Cell	: Semi-micro Cell

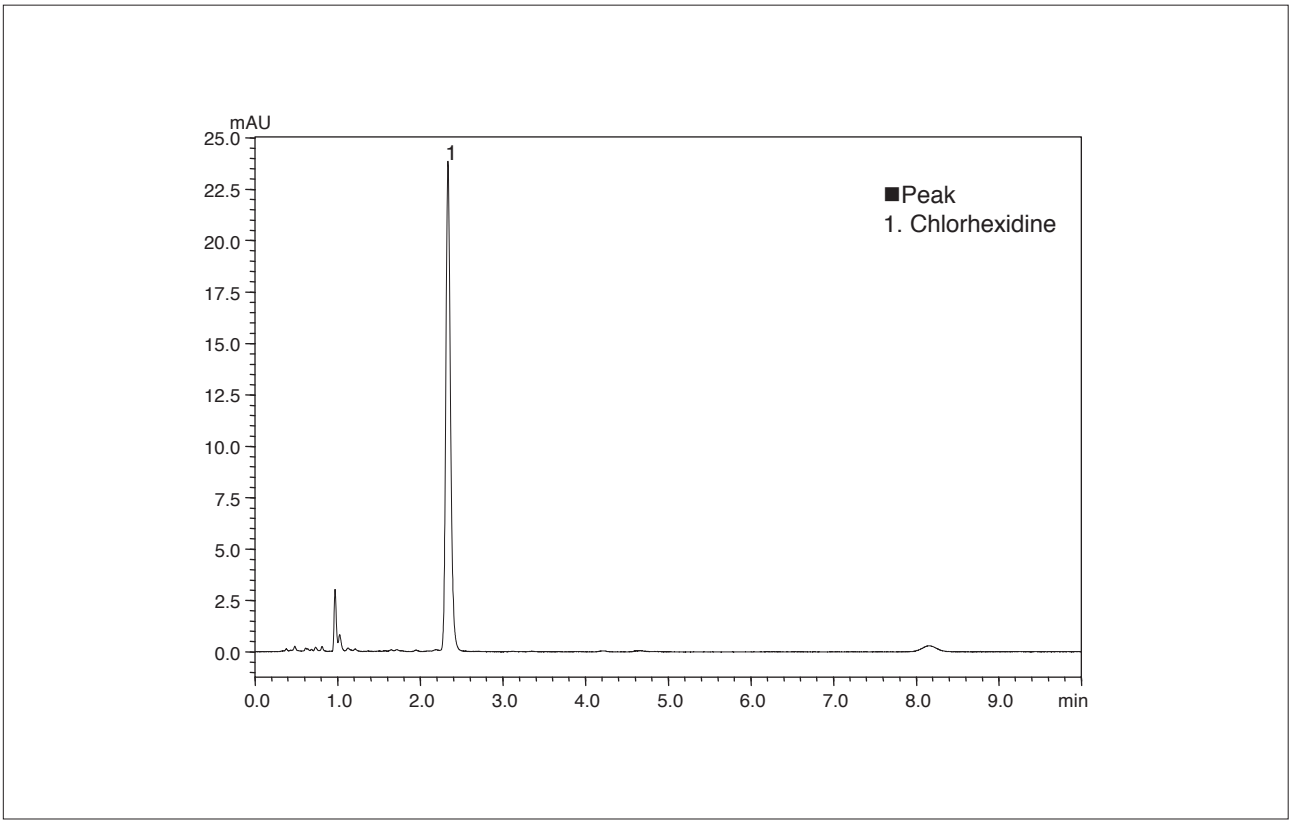


Fig. 1.9.1 Analysis of Chlorhexidine in Surface Injury Ointment

1.10 Analysis of Suppository - LC

●Analysis of Ephedrine and Procaine in Suppository

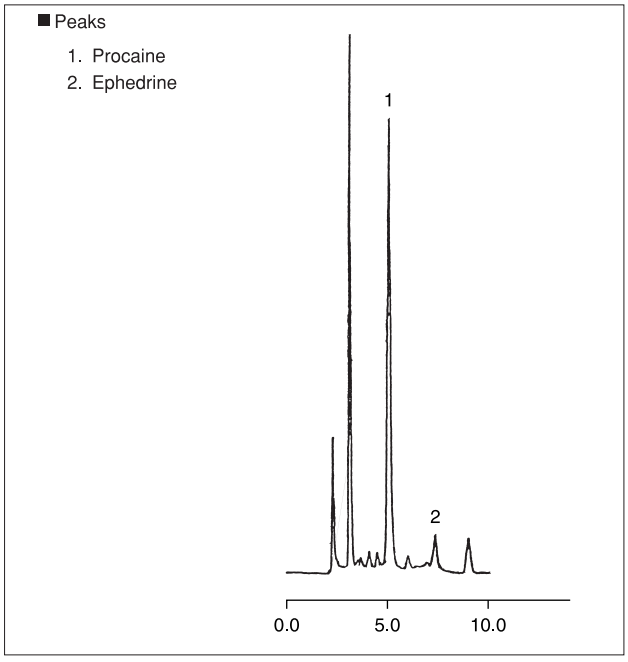


Fig. 1.10.1 Analysis of Ephedrine and Procaine in Suppository

■Analytical Conditions

Column : STR ODS-M
(150 mmL. × 4.6 mm I.D.)
Mobile Phase : A: 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 100 mmol/L $NaClO_4$
B: Methanol
A/B = 4/1 (v/v)
Flowrate : 0.8 mL/min
Column Temp. : 50 ˚C
Detection : UV Absorbance Detector at 210 nm

■Pretreatment

1. Add 20 mL of solution (0.1 N Perchloric Acid aq solution/Methanol = 1/1(v/v)) to sample (500 mg)
2. Ultrasonication for 5 min.
3. Heating and Shaking (at 60 ˚C for 10 min.) in water bath
4. Filtration with membrane filter (0.45 µm) after cooling
5. Inject 5 µL of filtrate

●Analysis of Ethyl Aminobenzoate in Suppository

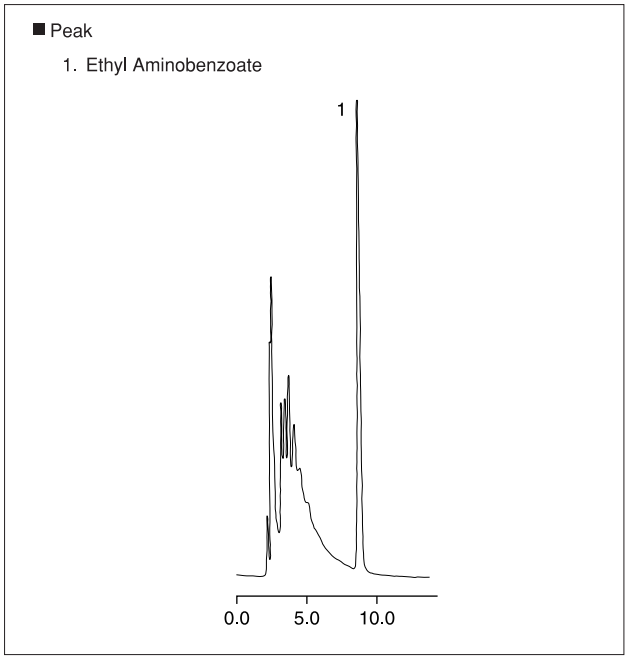


Fig. 1.10.2 Analysis of Ethyl Aminobenzoate in Suppository

■Analytical Conditions

Column : STR ODS-M
(150 mmL. × 4.6 mm I.D.)
Mobile Phase : A: 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 100 mmol/L $NaClO_4$
B: Methanol
A/B = 3/2 (v/v)
Flowrate : 0.8 mL/min
Column Temp. : 50 ˚C
Detection : UV Absorbance Detector at 210 nm

■Pretreatment

1. Add 20 mL of solution (0.1 N Perchloric Acid aq solution/Methanol = 1/1(v/v)) to sample (500 mg)
2. Ultrasonication for 5 min.
3. Heating and Shaking (at 60 ˚C for 10 min.) in water bath
4. Filtration with membrane filter (0.45 µm) after cooling
5. Inject 5 µL of filtrate

1.11 High Sensitivity Analysis of a Minor Impurity in Pharmaceuticals Using Co-Sense for Impurities System (1) - LC

■Explanation

The presence of impurities in commercial products can seriously affect their quality, resulting in more stringent requirements for impurities management. When an impurity that is present at trace levels becomes the target of quantitation, it may not be detectable due to insufficient sensitivity with the existing analytical conditions. This makes it necessary to determine the analytical conditions that will provide the required higher sensitivity. One approach to this is to implement sample pretreatment that includes the extraction and concentration of the analyte. Here, we introduce an example in which the Co-Sense for Impurities System is applied to a drug impurity analysis, making it possible to conduct measurement quickly and easily through automation of the sample preparation.

■Principle of Co-Sense for Impurities System

The Co-Sense for Impurities System is a unique system that utilizes HPLC column-switching and 2-dimensional separation technology. Fig. 1.11.1 shows the flow lines.

Step 1: Fractionation

- When the autosampler injects the sample, separation begins using Column Ⅰ. The flowpath is from Detector A→ValveA→ then discharge to waste bottle.
- By inputting the elution interval (Start Time – End Time) of the target impurity peak (and neighboring peaks) in the software beforehand, flow from Valve A is switched to redirect the eluent to Column Ⅱ where it is trapped as a separate fraction during that time interval.

Step 2: Concentration

- The impurity peak (and neighboring peaks) fraction proceeds toward Column Ⅱ after passing through Valve B.
- At a time point T prior to arriving at Column Ⅱ, a large volume of mobile phase is added to the flow via Pump Ⅱ. This solvent should be a mobile phase (for example, aqueous mobile phase in the case of reverse phase analysis) that will strengthen the retention of the impurity peak on Column Ⅱ. Thus, the target impurity peak (and neighboring peaks) are trapped and concentrated in Column Ⅱ.

Step 3: Quantitation

- When trapping of the impurity is completed, Pump Ⅱ stops pumping mobile phase, the flow line of Valve B is switched, and Pump Ⅲ begins pumping mobile phase through Column Ⅱ. The impurity peak (and neighboring peaks) are eluted from Column Ⅱ,and proceed in the direction of Column Ⅲ.
- Finally, after the impurity and its neighboring peaks are further separated by Column Ⅲ, Detector B detects the target impurity with high sensitivity.

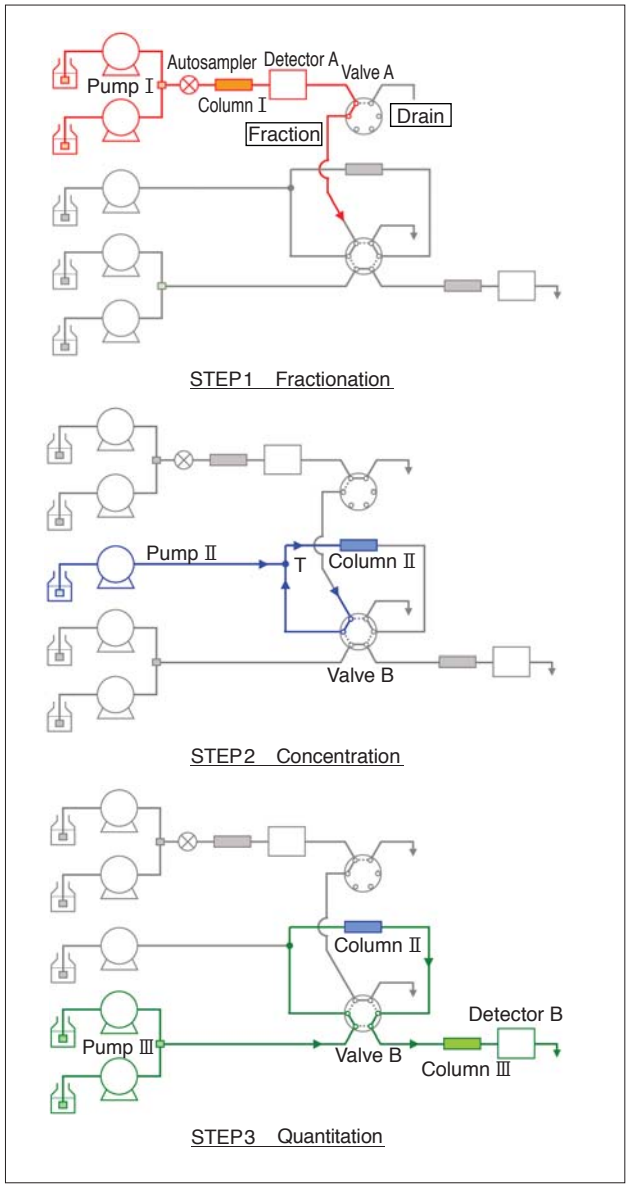


Fig. 1.11.1 Flow Diagram of Co-Sense for Impurities System

1.11 High Sensitivity Analysis of a Minor Impurity in Pharmaceuticals Using Co-Sense for Impurities System (2) - LC

■Analysis of Impurities in Imipramine Hydrochloride

Better detection sensitivity can be achieved by effectively conducting online trap oncentration of a target fraction using Co-Sense for Impurities. Here we introduce an application example of the analysis of impurities in imipramine hydrochloride. Fig. 1.11.2 shows a chromatogram that includes an impurity of imipramine hydrochloride. The impurity peak (approximately 0.0002 % of the peak area of the principal substance, peak height about 0.015 mAU), with the approximate retention time of 13.5, was selected as the target. Fig. 1.11.3 shows that chromatogram. The target impurity peak height obtained with Co-Sense for Impurities was about 0.56 mAU, and it was detected with a sensitivity about 37 times that compared to the original, non-concentrated peak in Fig. 1.11.2. In addition, excellent peak area repeatability of about 1.1 %RSD was obtained based on 6 consecutive repeat analyses.

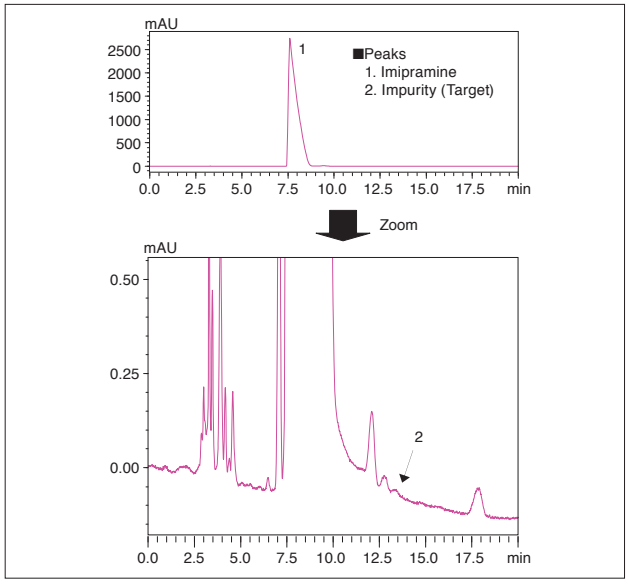


Fig. 1.11.2 Chromatogram of Imipramine Hydrochloride (5 mg/mL)

■Analytical Conditions

[Column Ⅰ] : Shim-pack VP-ODS
(250 mmL. × 10 mm I.D., 4.2 µm)
Mobile Phase : A : 0.1 % Perchloric Acid aq.Solution
B : Acetonitrile
A/B = 60/40 (v/v)
Flowrate : 4 mL/min
Column Temp. : 40 °C
Injection Volume : 50 µL
Detection : UV Absorbance Detector SPD-20A at 269 nm
Flow Cell : Conventional Cell

[Column Ⅱ] : Shim-pack GVP-ODS
(10 mmL. × 4.6 mm I.D., 4.2 µm)
Mobile Phase : 50 mmol/L Ammonium Acetate
Flowrate : 10 mL/min (12.5-14 min)

[Column Ⅲ] : Shim-pack XR-ODS Ⅱ
(100 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase : A : 0.1 % Formic Acid aq. Solution
B : Acetonitrile
A/B = 69/31 (v/v)
Flowrate : 0.35 mL/min
Detection : UV Absorbance Detector SPD-20A at 269 nm
Flow Cell : Conventional Cell

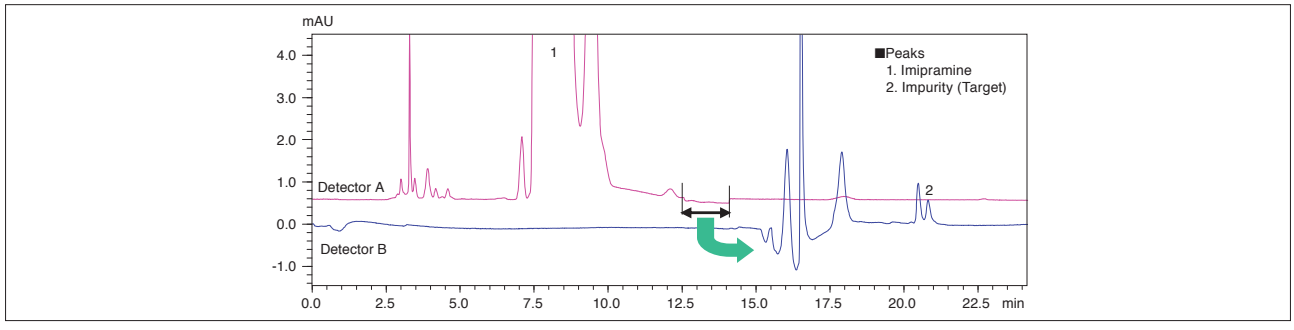


Fig. 1.11.3 Chromatograms of Impurities in Imipramine Hydrochloride (5 mg/mL) by Co-Sense for Impurities

1.12 Analysis of Sulfonic Acid Esters (1) - GC/MS

■Explanation

Methanesulfonic acid (mesylate), benzenesulfonic acid (besilate), and *p*-toluenesulfonic acid (tosylate), chemicals used in the process of synthesizing active pharmaceutical ingredients, are likely to generate sulfonic acid ester (Fig. 1.12.1) as a reaction byproduct. These compounds are known as potential genotoxic impurities (PGI) and are a significant cause for concern among pharmaceutical manufacturers. Here we introduce the analysis of sulfonic acid esters utilizing the GCMS-QP2010 Ultra.

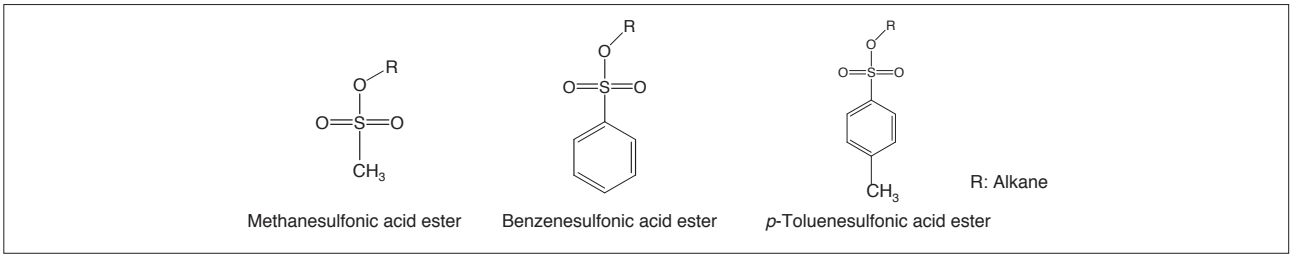


Fig. 1.12.1 Structural Formulas for Sulfonic Acid Esters

■Analytical Conditions

FASST (Fast Automated Scan/SIM Type), which is capable of simultaneous Scan and SIM measurements, was used as the measurement mode. The SIM measurement monitoring *m/z* values are shown in Table 1.12.1.

Instrument : GCMS-QP2010 Ultra
Column : Rtx-200 (30 m × 0.25 mm I.D. df = 0.25 µm)
Glass Insert : Split insert containing wool

[GC]

Injector Temp. : 280 °C
Column Temp. : 70 °C (2 min) →15 °C/min →320 °C (3 min)
Carrier Gas : He
Control Mode : Linear velocity (40 cm/sec)
Purge Flowrate : 3.0 mL/min
Injection Method : Split Injection
Split Ratio : 1 : 20
Injection Volume : 1.0 µL

[MS]

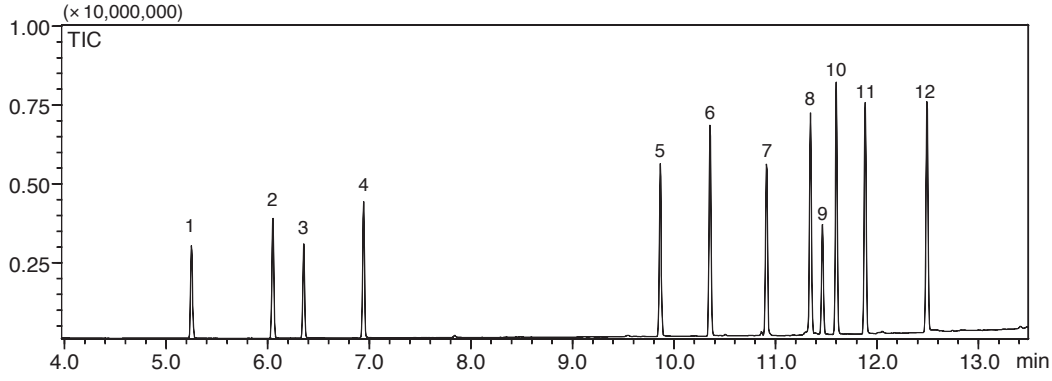
Interface Temp. : 280 °C
Ion Source Temp. : 230 °C
Solvent Elution Time : 1.5 min
Tuning Mode : High sensitivity
Measurement Mode : FASST (simultaneous Scan/SIM measurements)
Scan Mass Range : *m/z* 40 – 330
Scan Event Time : 0.1 sec
SIM Monitoring *m/z* : See Table 1.12.1
Sim Event Time : 0.3 sec

Table 1.12.1 Monitoring *m/z* for Target Compounds

	Monitoring *m/z*		Monitoring *m/z*
Methyl methanesulfonate	80, 95	Methyl *p*-toluenesulfonate	155, 186
Ethyl methanesulfonate	109, 97	Ethyl *p*-toluenesulfonate	155, 200
Isopropyl methanesulfonate	123, 79	Isopropyl *p*-toluenesulfonate	172, 155
n-Propyl methanesulfonate	109, 97	*n*-Propyl *p*-toluenesulfonate	155, 172
Methyl benzenesulfonate	172, 141	Butyl *p*-toluenesulfonate	173, 91
Ethyl benzenesulfonate	141, 186		
Butyl benzenesulfonate	141, 159		

1.12 Analysis of Sulfonic Acid Esters (2) - GC/MS

■Results



ID	Compound Name	Retention Time	ID	Compound Name	Retention Time
1	Methyl methanesulfonate	5.252	7	Methyl *p*-toluenesulfonate	10.911
2	Ethyl methanesulfonate	6.052	8	Ethyl *p*-toluenesulfonate	11.345
3	Isopropyl methanesulfonate	6.355	9	Isopropyl *p*-toluenesulfonate	11.462
4	*n*-Propyl methanesulfonate	6.944	10	Butyl benzenesulfonate	11.598
5	Methyl benzenesulfonate	9.865	11	*n*-Propyl *p*-toluenesulfonate	11.883
6	Ethyl benzenesulfonate	10.356	12	Butyl *p*-toluenesulfonate	12.491

Fig. 1.12.2 Total Ion Current Chromatogram of Sulfonic Acid Esters

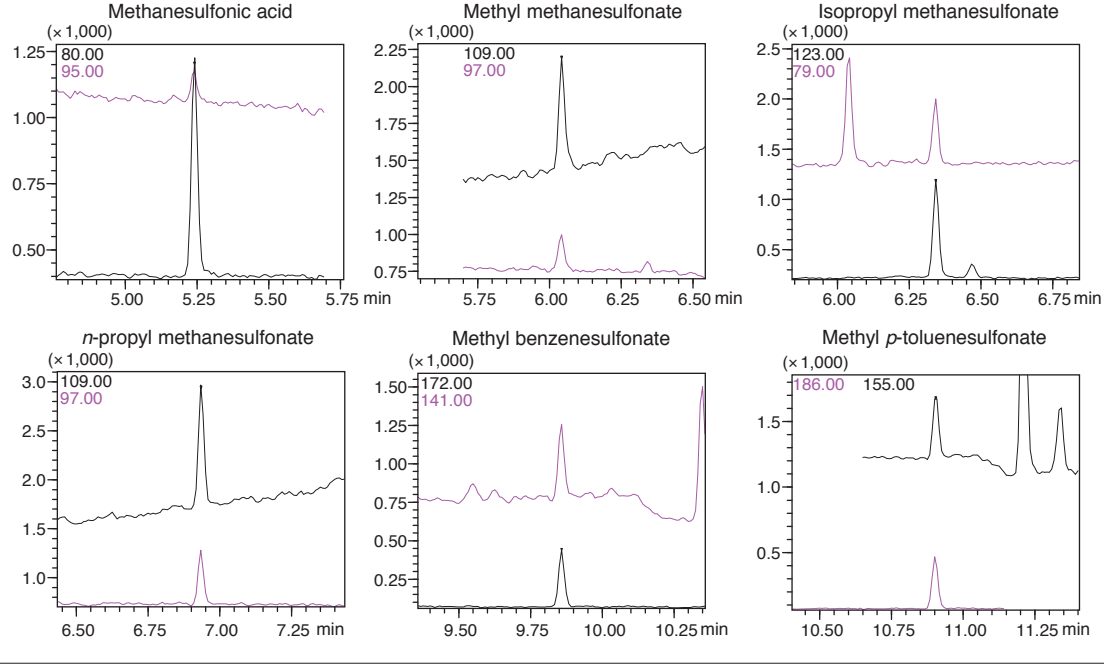


Fig. 1.12.3 Mass Chromatograms of Sulfonic Acid Esters

The standard sample concentration is 10 ng/mL. It is equivalent to 1 ng/mg in active pharmaceutical ingredients when diluted to 1/100th in pretreatment with a recovery ratio of 100 %.

1.13 Analysis of Haloalcohols and Glycidol (1) - GC/MS

■Explanation

Haloalcohols (Fig. 1.13.1) are used as synthetic materials in pharmaceuticals, and are considered potential genotoxic impurities (PGI). In addition, glycidol has been identified as a cancer-causing agent, and has been assigned to Group 2A (probably carcinogenic to humans) in terms of carcinogenic risk by the International Agency for Research on Cancer (IARC). Here we introduce analysis of haloalcohols and glycidol in an active pharmaceutical ingredient (API) using the GC-MS.

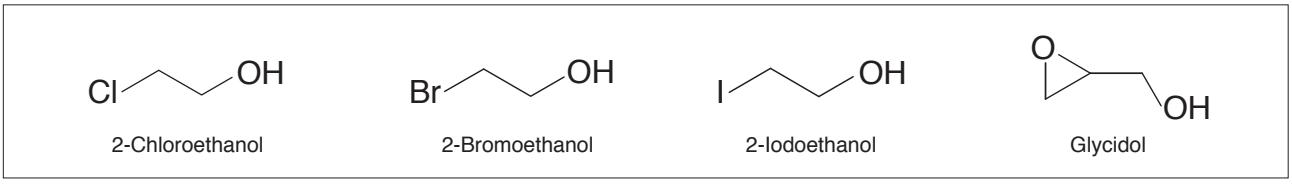


Fig. 1.13.1 Compound Structures of Typical Haloalcohols and Glycidol

■Experimental

Many APIs are compounds with a high boiling point, and can cause GC-MS and column contamination; therefore, it is critical to extract the target compounds from the API matrix prior to analysis by. Haloalcohols and glycidol are highly polar, making them difficult to extract with organic solvents. Accordingly, the target compounds were subjected to trimethylsilyl (TMS) derivatization before a solvent extraction was performed utilizing water and dichloromethane, thereby removing as much of the API as possible [1]. In addition, 1, 1, 2, 2-bromoethanol-D4 was utilized as the internal standard substance, and 50 ng of that was added to 200 µL of solution. Fig. 1.13.2 shows the detailed pretreatment procedure.

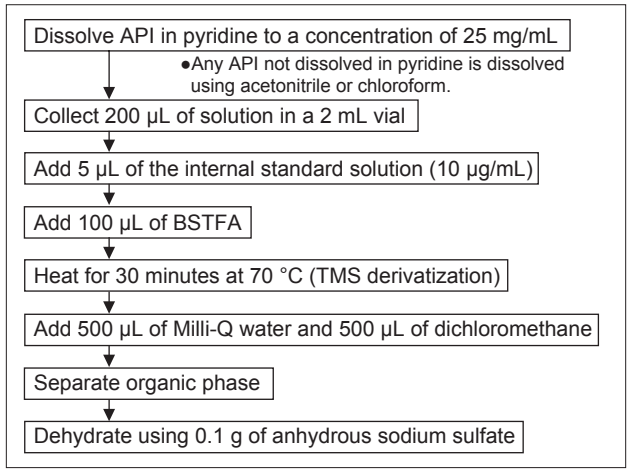


Fig. 1.13.2 Sample Preparation Procedure

■Analytical Conditions

FASST (Fast Automated Scan/SIM Type), which is capable of simultaneous Scan and SIM measurements, was used as the measurement mode.

Instrument	: GCMS-QP2010 Ultra
Column	: Rtx-200 (30 m × 0.25 mm I.D. df = 0.25 µm)
Glass Liner	: Deactivated Split insert with glass wool
[GC]	
Injector Temp.	: 280 °C
Column Temp.	: 50 °C (5 min) → 10 °C/min → 100 °C → 20 °C/min→ 320 °C (3 min)
Flow Control Mode	: Linear velocity (32.4 cm/sec)
Injection Method	: Split Injection
Split Ratio	: 1 : 30
Injection Volume	: 1.0 µL
[MS]	
Interface Temp.	: 280 °C
Ion Source Temp.	: 230 °C
Measurement Mode	: FASST (simultaneous Scan/SIM measurements)

Scan Mass Range	: *m/z* 30 – 450
Scan Event Time	: 0.2 sec
SIM Event Time	: 0.3 sec
SIM Monitoring *m/z*	:
2-Chloroethanol-TMS	93, 95
2-Bromoethanol-TMS	181, 183
2-Bromoethanol-D4-TMS	187
Glycidol-TMS	101, 59
2-Iodoethanol-TMS	185, 229

1.13 Analysis of Haloalcohols and Glycidol (2) - GC/MS

■Results

Fig. 1.13.3 shows the total ion current chromatogram of a 25 µg/mL standard sample (equivalent to 1000 ng/mg in the pharmaceuticals), and Fig. 1.13.4 shows the scan mass spectra.

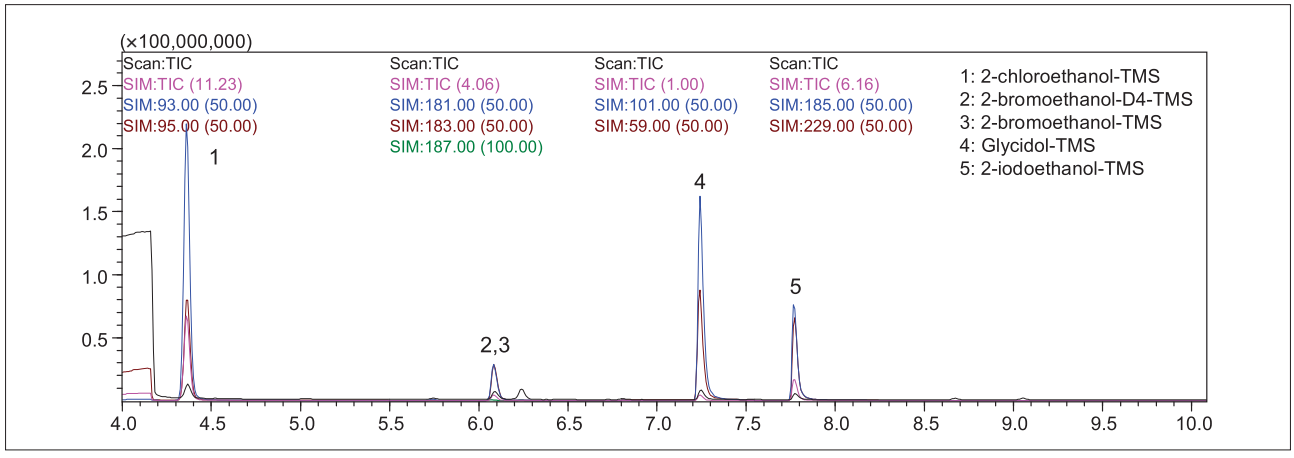


Fig. 1.13.3 Total Ion Current Chromatogram

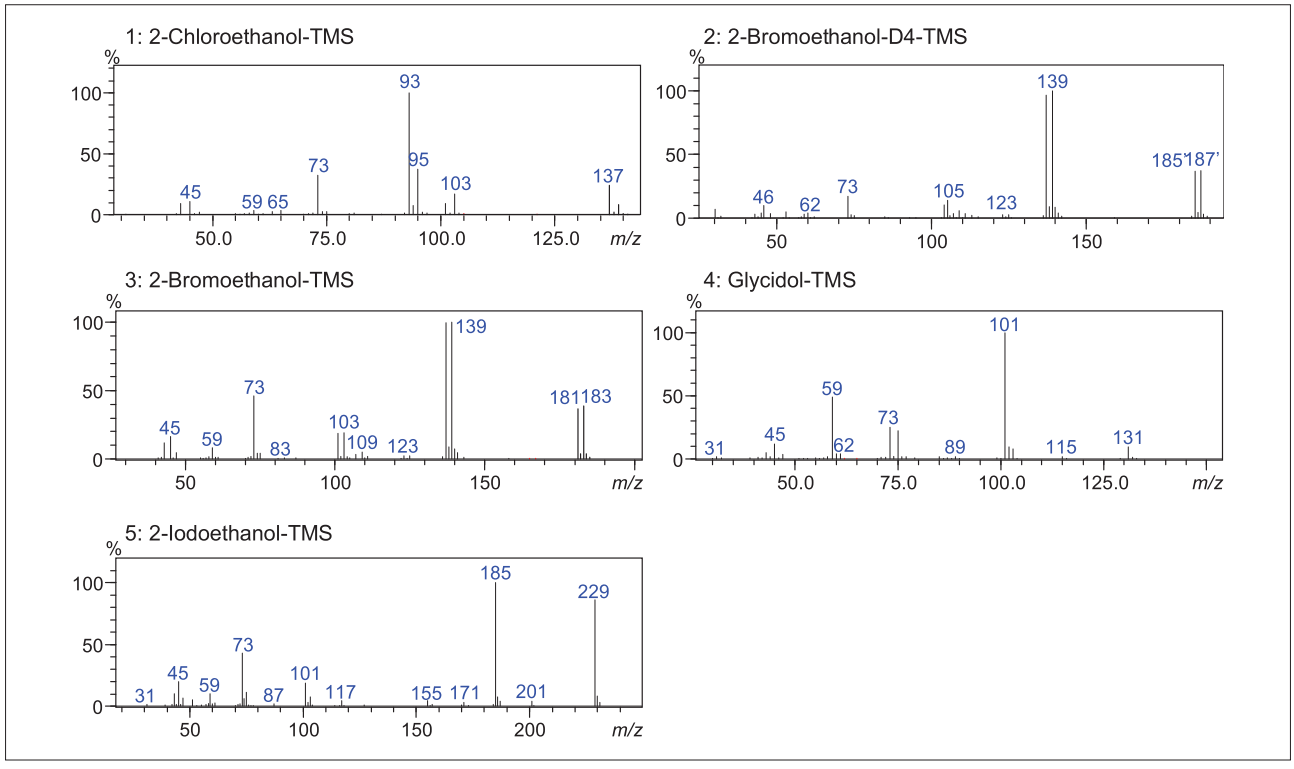


Fig. 1.13.4 Scan Mass Spectra of Haloalcohols and Glycidol

[Reference]
1) Frank David, Karine Jacq, Pat Sandra, Andrew Baker and Matthew S. Klee: Analysis of potential genotoxic impurities in pharmaceuticals by two-dimensional gas chromatography with Deans switching and independent column temperature control using a low-thermal-mass oven module, Anal Bioanal Chem, 396, 1291-1300 (2010)

1.14 Analysis of Alkyl Halides (1) - GC/MS

■Explanation

Alkyl halides are used as an alkylating agent for raw ingredients in the synthesis of pharmaceuticals or are generated as a byproduct of drug synthesis. They have been identified as potential carcinogens or genotoxins. Here we show an example of analyzing 18 alkyl halides using headspace-GC/MS.

■Experimental

Standard mixtures were prepared by diluting 18 types of alkyl halides in methanol to 0.2, 2, 10, 20, and 100 µg/mL concentrations. An internal standard solution was prepared by diluting fluorobenzene in methanol to a 20 µg/mL concentration. Test samples were prepared by placing 20 mg of the pharmaceutical ingredients in a 20 mL screw-cap vial (La-Pha-Pack P/N: 18 09 1307), diluting it with 10 mL of Milli-Q water, adding 10 µL of the internal standard solution, and then quickly sealing the vial by screwing on the magnetic screw-cap (La-Pha-Pack P/N: 18 09 1309). Standard aqueous samples were prepared by adding 10 µL of each standard alkali halide mixture and 10 µL of the internal standard solution to 10 mL Milli-Q water. The concentrations of the prepared standard aqueous samples were 0.2, 2, 10, 20, and 100 ng/mL (equivalent to 0.1, 1, 5, 10, and 50 ng/mg concentrations in the active pharmaceutical ingredients), respectively.

■Analytical Conditions

FASST (Fast Automated Scan/SIM Type), which is capable of simultaneous Scan and SIM measurements, was used as the measurement mode.

Instrument	: GCMS-QP2010 Ultra
Autosampler	: AOC-5000 Plus (HS)
Column	: Rtx-1 (60 m × 0.25 mm I.D. df=1.0 µm)
Glass Insert	: Deactivated Split insert with wool

[AOC-5000 Plus (HS)]

Incubation Temp.	: 80 °C
Incubation Time	: 30 min
Syringe Temp.	: 100 °C
Agitator Speed	: 250 rpm
Fill Speed	: 500 µL/sec
Pull Up Delay	: 500 msec
Inject to	: GC Inj 1
Injection Speed	: 500 µL/sec
Pre Inject Delay	: 500 msec
Flush Time	: 5 min
GC Run Time	: 25 min
Injection Volume	: 1 mL

[GC]

Injector Temp.	: 230 °C
Column Temp.	: 40 °C (2 min) → 20 °C/min → 250 °C (4 min)
Carrier Gas	: He
Flow Control Mode	: Linear velocity (25.5 cm/sec)
Injection Method	: Split Injection
Split Ratio	: 1 : 10

[MS]

Interface Temp.	: 230 °C
Ion Source Temp.	: 230 °C
Tuning Mode	: High sensitivity
Measurement Mode	: FASST (simultaneous Scan/SIM measurements)
Scan Mass Range	: *m/z* 30 - 270
Scan Event Time	: 0.05 sec
Scan Speed	: 10,000 *u*/sec
SIM Event Time	: 0.3 sec

SIM Monitoring *m/z*:

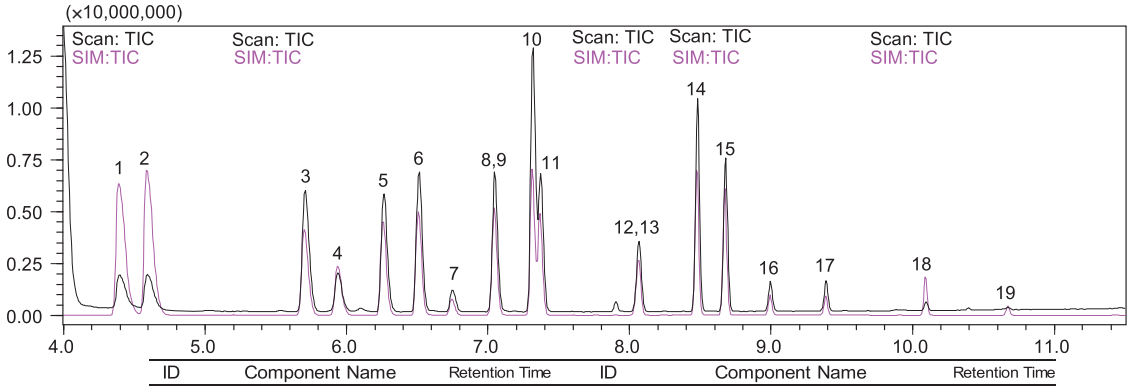
Chloromethane	50, 52
Vinyl chloride	62, 64
2-Chloropropane	43, 78
Iodomethane	142, 127
1-Chloropropane	42, 78
trans-1,2-Dichloroethylene	61, 96
2-Bromopropane	43, 122
cis-Dichloroethylene	61, 96
2-Chloroacrylonitrile	87, 52
1-Chloro-2-methylpropene	55, 90
1-Bromopropane	43, 122
2-Iodopropane	43, 170
Fluorobenzene	96, 70
1-Bromo-2-methylpropene	55, 134
1-Iodopropane	43, 170
trans-1,2-Dibromoethylene	186, 105
cis-1,2-Dibromoethylene	186, 105
trans-3-Bromo-2-methylacrylonitrile	66, 145
cis-3-Bromo-2-methylacrylonitrile	66, 145

1.14 Analysis of Alkyl Halides (2) - GC/MS

■Results

The total ion current chromatogram for the 100 ng/mL concentration standard aqueous solution (equivalent to 50 ng/mg concentration* in the active pharmaceutical ingredients) is shown in Fig. 1.14.1. The SIM chromatograms for six typical components in the 0.2 ng/mL concentration standard aqueous solution (equivalent to 0.1 ng/mg concentration* in the pharmaceutical) are shown in Fig. 1.14.2.

* 1, 2-Dibromoethylene and 3-Bromo-2-methylacrylonitrile concentrations include both *cis* and *trans* forms.



ID	Component Name	Retention Time	ID	Component Name	Retention Time
1	Chloromethane	4.391	11	1-Bromopropane	7.367
2	Vinyl chloride	4.590	12	2-Iodopropane	8.049
3	2-Chloropropane	5.700	13	Fluorobenzene (ISTD)	8.068
4	Iodomethane	5.938	14	1-Bromo-2-methylpropene	8.476
5	1-Chloropropane	6.258	15	1-Iodopropane	8.672
6	*trans*-1,2-Dichloroethylene	6.507	16	*trans*-1,2-Dibromoethylene	8.994
7	2-Bromopropane	6.744	17	*cis*-1,2-Dibromoethylene	9.386
8	*cis*-Dichloroethylene	7.041	18	*trans*-3-Bromo-2-methylacrylonitrile	10.088
9	2-Chloroacrylonitrile	7.051	19	*cis*-3-Bromo-2-methylacrylonitrile	10.671
10	1-Chloro-2-methylpropene	7.313			

Fig. 1.14.1 Total Ion Current Chromatogram

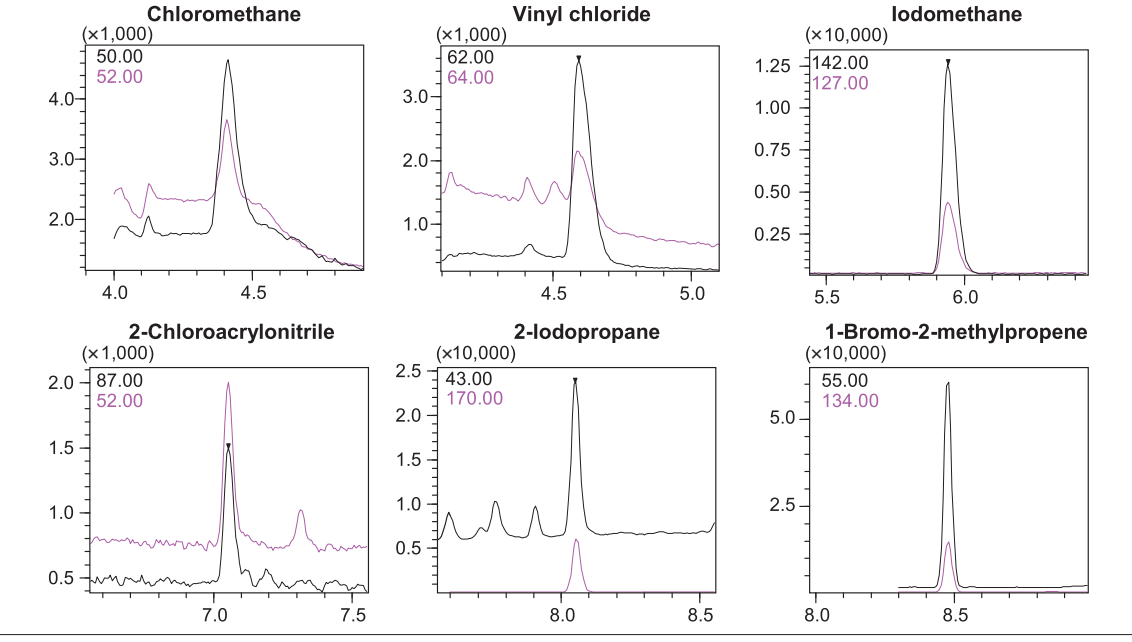


Fig. 1.14.2 Typical SIM Mass Chromatograms for 0.1 ng/mg Concentration in Active Pharmaceutical Ingredients

1.15 Ultra-High-Speed and Ultra-High-Resolution Analysis of Drug Analogs - LC

■Explanation

As analogs in drug products often have similar structures, high resolution is demanded for their analysis. A long, sub-2 µm column is effective in such cases but this requires a UHPLC system with high pressure tolerance, such as the Shimadzu Nexera (130 MPa pressure tolerance). Here we introduces the ultra-high-speed and ultra-high-resolution analysis of ketoprofen and its analogs.

■Analysis of Ketoprofen and Its Analogs

Nexera was used to analyze ketoprofen and its analogs under conventional conditions, ultra-high-speed conditions, and ultra-high-resolution conditions. Ultra-high-speed conditions employ a column with 1.8 µm particle size to increase the linear velocity and reduce the analysis time to one-tenth that for conventional analysis, while maintaining the resolution. Ultra-high-resolution conditions use a 1.8 µm particle size, 250 mm-long column to achieve complete resolution of components that are difficult to resolve under conventional conditions. The 130 MPa pressure tolerance of the Shimadzu Nexera permits selection of the 118 MPa system pressure load required under ultra-high-resolution conditions.

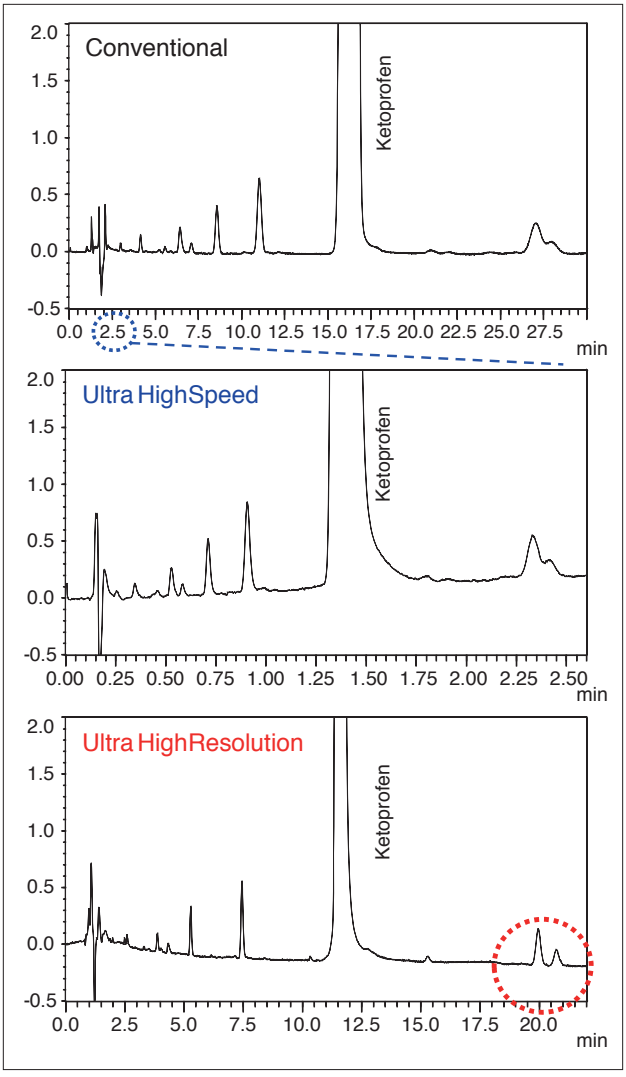


Fig. 1.15.1 Chromatograms of Ketoprofen and Its Analogs

■Analytical Conditions

(Conventional)

Column	: ODS (150 mmL. × 4.6 mm I.D.,4.6 µm)
Mobile Phase	: 0.1 % Formic Acid in Water/Acetonitrile = 65/35 (v/v)
Flowrate	: 1.0 mL/min
Column Temp.	: 40 ºC
Detection	: UV Absorbance Detector at 254 nm
Flow Cell	: Conventional Cell
Pressure	: 6.5 MPa

(Ultra High Speed)

Column	: ODS (50 mmL. × 2.1 mm I.D., 1.8 µm)
Mobile Phase	: 0.1 % Formic Acid in Water/Acetonitrile = 65/35 (v/v)
Flowrate	: 0.8 mL/min
Column Temp.	: 40 ºC
Detection	: UV Absorbance Detector at 254 nm
Flow Cell	: Semi-micro Cell
Pressure	: 50 MPa

(Ultra High Resolution)

Column	: ODS (250 mmL. × 2.1 mm I.D., 1.8 µm)
Mobile Phase	: 0.1 % Formic Acid in Water/Acetonitrile = 65/35 (v/v)
Flowrate	: 0.5 mL/min
Column Temp.	: 40 ºC
Detection	: UV Absorbance Detector at 254 nm
Flow Cell	: Semi-micro Cell
Pressure	: 118 MPa

1.16 Determination of Counter-ions and Impurity Ions by Ion Chromatography (1) - LC

■Explanation

Approximately 50 % of all drug molecules used in pharmaceutical products are reported to be ionic compounds[1]. Furthermore, ionic species are analyzed for various purposes through many stages of the pharmaceutical process, from development through quality control. Here we present examples of analysis of counter-ions and impurity ions at micro levels in drugs using ion chromatography.

■Analysis of Counterions

In drug development, the formation of various salts is examined as a factor influencing such physical properties as crystallinity and solubility of the principle drug ingredient (Active Pharmaceutical Ingredient: API). The selection and evaluation of an ion accompanying that principle active ingredient, referred to as a counter-ion is important in characterizing those properties. Fig. 1.16.1 shows the types and ratios of the most common anion and cation counterion listed in the 2006 USP (United States Pharmacopeia) [1].

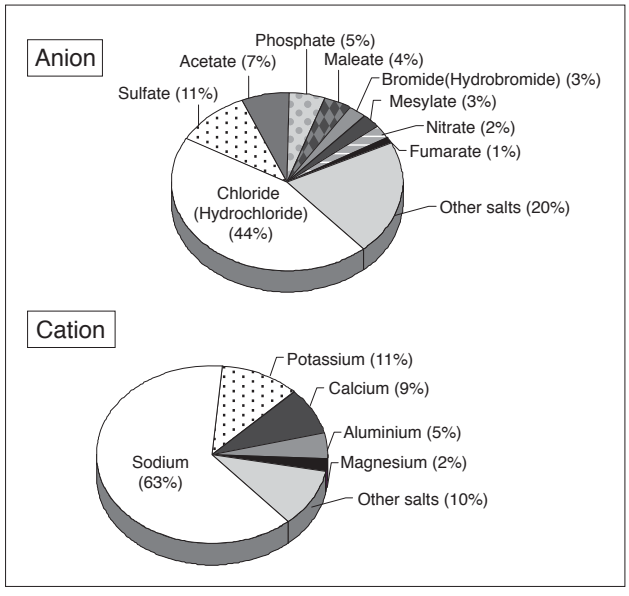


Fig. 1.16.1 Counter-ions in Drugs

Fig. 1.16.2 shows the results of analysis of a standard solution (250 mg/L) of the principle active ingredient hydroxocobalamin including acetate, and Fig. 1.16.3 shows a chromatogram obtained from analysis of a standard solution (100 mg/L) of the principle active ingredient sodium diclofenac including sodium salt. For detection, an electroconductivity detector was used without a suppressor.

■Analytical Conditions

(Anion)

Column	: Shim-pack IC-A3 (150 mmL. × 4.6 mm I.D.)
Guard Column	: Shim-pack IC-GA3 (10 mmL. × 4.6 mm I.D.)
Mobile Phase	: 8.0 mmol/L *p*-Hydroxybenzoic Acid/ 3.2 mmol/L Bis-Tris / 50 mmol/L Boric Acid
Flowrate	: 1.2 mL/min
Column Temp.	: 40 ºC
Detection	: Conductivity Detector CDD-10A*VP* (Non-suppressor)
Injection Volume	: 50 µL

(Cation)

Column	: Shim-pack IC-C4 (150 mmL. × 4.6 mm I.D.)
Guard Column	: Shim-pack IC-GC4 (10 mmL. × 4.6 mm I.D.)
Mobile Phase	: 2.5 mmol/L Oxalic Acid
Flowrate	: 1.0 mL/min
Column Temp.	: 40 ºC
Detection	: Conductivity Detector CDD-10A*VP* (Non-suppressor)
Injection Volume	: 50 µL

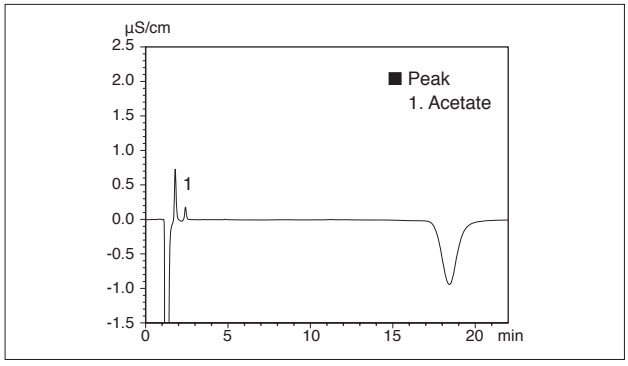


Fig. 1.16.2 Chromatogram of Hydroxocobalamin Acetate

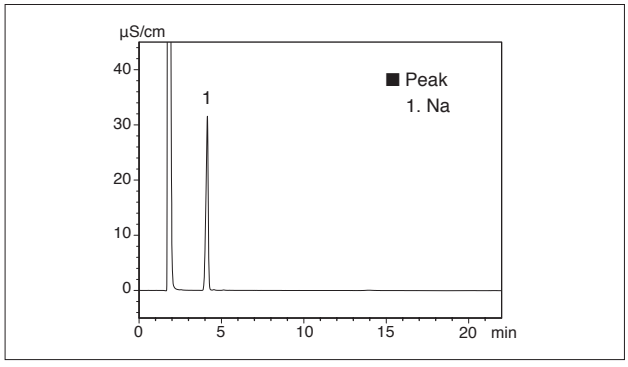


Fig. 1.16.3 Chromatogram of Sodium Diclofenac

1.16 Determination of Counter-ions and Impurity Ions by Ion Chromatography (2) - LC

■Analysis of Impurity Ions

Controlling impurities is critically important in ensuring drug quality. It is known that residual inorganic impurities such as catalysts and ions from the development phase adversely affect the solubility and stability in some drug products. Micro quantities of impurities in a drug can be analyzed with high sensitivity using ion chromatography. Fig. 1.16.4 shows the results of analysis of a sample consisting of a standard solution (1000 mg/L) of sodium diclofenac spiked with 7 anions, each at 0.02 % (0.2 mg/L). In addition, Fig. 1.16.5 shows the results of analysis of a sample consisting of a standard solution (1000 mg/L) of trazodone hydrochloride spiked with 6 cations, each at 0.02 % (0.2 mg/L).

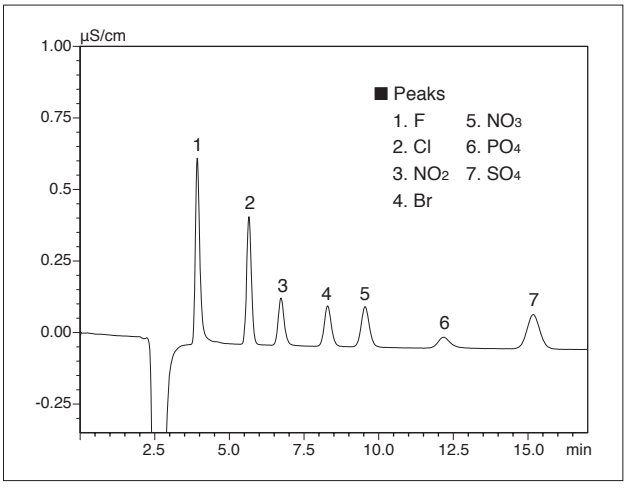


Fig. 1.16.4 Chromatogram of Sodium Diclofenac Solution (Anions spiked, 0.2 mg/L each)

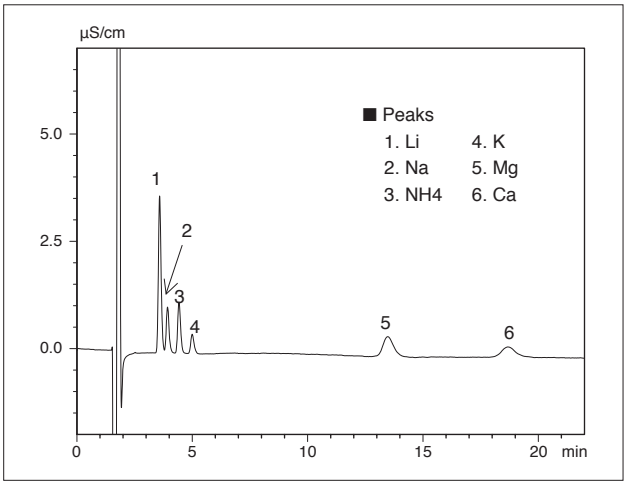


Fig. 1.16.5 Chromatogram of Trazodone Hydrochloride Solution (Cations spiked, 0.2 mg/L each)

■Analytical Conditions

(Anion)

Column	: Shim-pack IC-SA2 (250 mmL. × 4.0 mm I.D.)
Guard Column	: Shim-pack IC-SA2 (G) (10 mmL. × 4.6 mm I.D.)
Mobile Phase	: 12.0 mmol/L Sodium Bicarbonate 0.6 mmol/L Sodium Carbonate
Flowrate	: 1.0 mL/min
Column Temp.	: 30 ºC
Detection	: Conductivity Detector CDD-10A*SP* (Non-suppressor)
Injection Volume	: 50 µL

(Cation)

Column	: Shim-pack IC-C4 (150 mmL. × 4.6 mm I.D.)
Guard Column	: Shim-pack IC-GC4 (10 mmL. × 4.6 mm I.D.)
Mobile Phase	: 2.5 mmol/L Oxalic Acid
Flowrate	: 1.0 mL/min
Column Temp.	: 40 ºC
Detection	: Conductivity Detector CDD-10A*VP* (Non-suppressor)
Injection Volume	: 50 µL

[Reference]

1) Loken Kumer, Aeshna Amin, Arvind K.Bansal, Pharmaceutical Technology March 2, 2008

1.17 Ultra High Performance Liquid Chromatography/ Mass Spectrometry Using Open Solution Software (1) - LC/MS

■Explanation

With the demand for faster research and development and improved data quality, the spread of Ultra High Performance Liquid Chromatography (UHPLC) is rapidly advancing. The development of minute particle size packing material and high-pressure tolerance liquid chromatographs have made these higher speeds possible, but in order to further improve throughput, the importance of a high performance mass spectrometer coupled with well-developed, user- friendly software is also recognized. The LCMS-2020, with its high scanning speed (up to 15,000 u/sec) and high-speed polarity switching (15 msec polarity switching), demonstrates the performance that satisfies the demands of UHPLC. The powerful user interface of Open Solution makes data review and verification easy, supporting the chemist who wishes to make rapid yet accurate decisions related to his ongoing research.

■Analysis and Data Processing Using Open Solution Software

Open Solution is a superb software for routine high-speed analysis. For example, when conducting confirmation of synthetic compounds in pharmaceutical product research and development, compounds having different molecular weights and various physical properties are required to be measured using the same analytical conditions in a short period of time. Under these circumstances, the shorter the time spent on analytical separations and data verification, the better, so software that provides simplified sample login and LCMS operation, and quickly indicates pertinent information becomes a real asset. Fig. 1.17.1 shows an Open Solution sample login screen, and Fig. 1.17.2 shows an example of a Results View page. The compounds shown in Table 1.17.1 are the test compounds that were analyzed for this application news article. The compounds are easily confirmed using the mass spectral data displayed in the Results View window by comparing theoretical and actual *m/z* values.

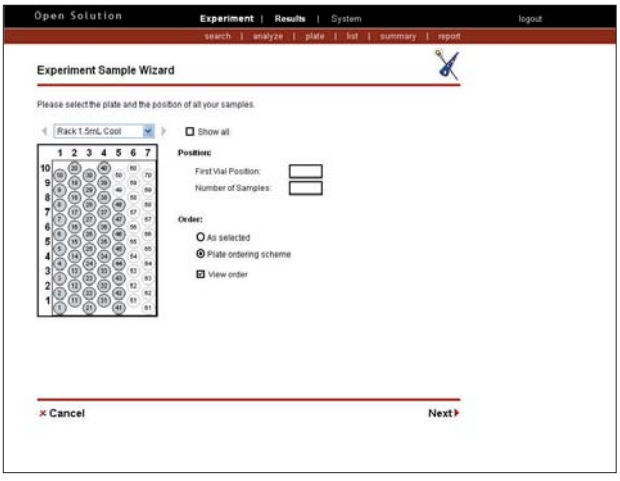


Fig. 1.17.1 Open Solution (Sample Login Screen)

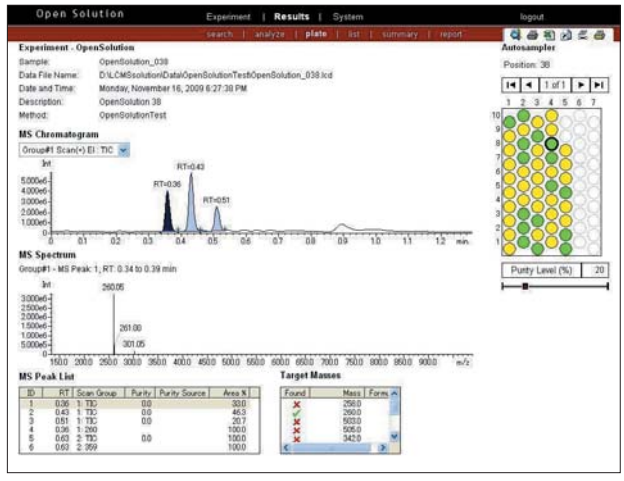


Fig. 1.17.2 Open Solution (Result View)

Table 1.17.1 Test Compounds (*: negative ion detection, others: positive ion detection)

No.	Compound	*m/z*	No.	Compound	*m/z*	No.	Compound	*m/z*
1	Atenolol	267	11	Doxepin	280	21	Isopropylantipyrine	231
2	Procaine	237	12	Dipyridamol	505	22	Alprazolam	309
3	Lidocaine	235	13	Desipramine	267	23	Triazolam	343
4	Atropine	290	14	Imipramine	281	24	Cilostazol	370
5	Yohimbine	355	15	Nortriptyline	264	25	Nifedipine	347
6	Chlorpheniramine	275	16	Amitriptyline	278	26	Diazepam	285
7	Propranolol	260	17	Dibucaine	344	27	Warfarin	309
8	Alprenolol	250	18	Verapamil	455	28	*Cefuroxime	423
9	Tetracaine	265	19	Reserpine	609	29	*Chloramphenicol	321
10	Diphenhydramine	256	20	Carbamazepine	237	30	*Nitrendipine	359

1.17 Ultra High Performance Liquid Chromatography/ Mass Spectrometry Using Open Solution Software (2) - LC/MS

■Ultra Fast Analysis of Drug Mixture Using LCMS-2020

We conducted UHPLC analysis of a solution containing a mixture of 30 drug compounds. A concentration of 5 µg/mL was used for substances analyzed using positive detection, and 50 µg/mL for substances analyzed using negative detection. Fig. 1.17.3 shows the mass chromatograms (displayed using Data Browser of LCMSsolution Ver. 5). For the Liquid chromatograph, the Prominence UFLCXR was used. All of the constituents were eluted within 0.7 min using a Shim-pack XR-ODS Ⅱ column (30 mmL. × 1 mm I.D., 2.2 µm, P/N 228-59907-91). Even after 700 successive analyses, stable measurement is achieved (Fig. 1.17.4).

■Analytical Conditions

Instruments	: Prominence UFLCXR + LCMS-2020
Column	: Shim-pack XR-ODS Ⅱ (30 mmL. × 1.5 mm I.D., 2.2 µm)
Mobile Phase A	: 0.1 % Formic Acid in Water
Mobile Phase B	: 0.1 % Formic Acid in Acetonitrile
	Gradient Elution Method
Time Program	: B 8 % (0 min) → 95 % (0.5 min) → 8 % (0.51 min) → STOP (1.3 min)
Flowrate	: 1.2 mL/min
Column Temp.	: 50 ºC
Injection Volume	: 1 µL
Rinsing Pump	: 3 sec (Methanol)
Mixer Volume	: 100 µL
Probe Voltage	: +4.5 kV (ESI-Positive Mode) -3.5 kV (ESI-Negative Mode)
Nebulizing Gas Flow	: 1.5 L/min
Drying Gas Flow	: 20.0 L/min
DL Temp.	: 250 ºC
BH Temp.	: 450 ºC
DL,Q-array Voltage	: Default Values
Event Time	: 0.1 sec
Scan Range	: *m/z* 150-1000

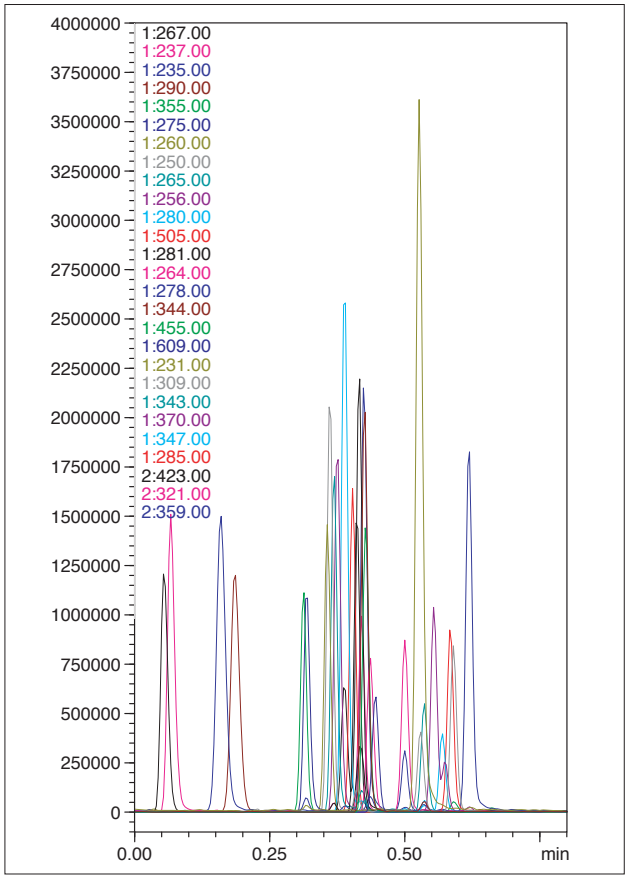


Fig. 1.17.3 Chromatograms of Drugs (Injection 1)

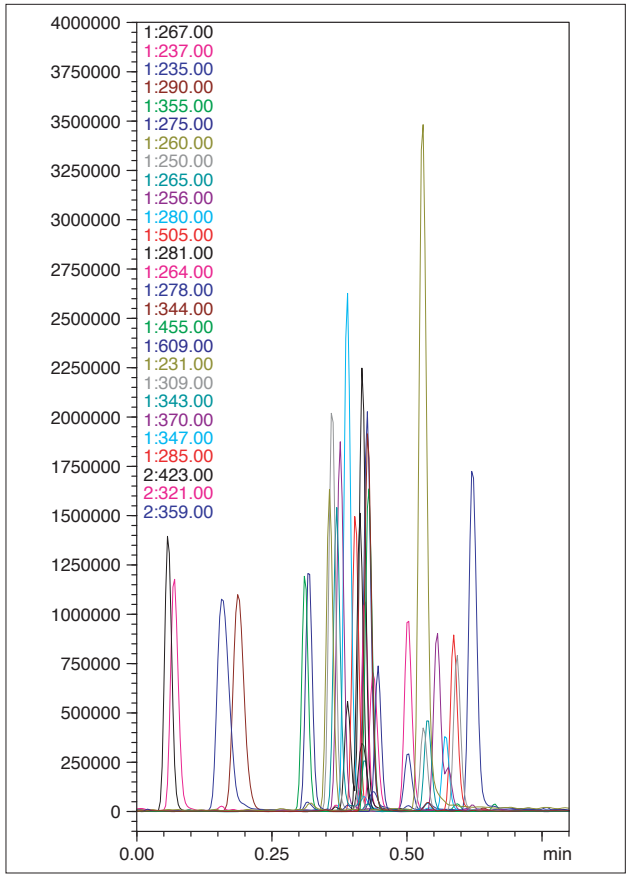


Fig. 1.17.4 Chromatograms of Drugs (Injection 700)

2. Cosmetics

2.1 Analysis of Fragrances of Cosmetics (1) - GC/MS

■Explanation

The 7th revision of the Cosmetics Directive was proposed in the European Union (EU) in March, 2003, and in that directive, the names of 26 compounds deemed to be allergens contained in cosmetics were published. If any of those compounds are contained in concentrations of 10 ppm and 100 ppm or greater in leave-on and rinse-off products, respectively, their content must be displayed. This also applies to cosmetics imported into the EU.
These substances are terpene alcohols, aldehydes and esters, etc. That list is shown in Table 2.1.1, and analysis for these substances is conducted using GC/MS. Introduced here is an example of analysis of a standard solution containing these substances.

This investigation was performed in cooperation with Takasago International Corporation.

We investigated to use Silicon and WAX columns, however, the results introduced here were obtained using a WAX column. The *m/z* used in the SIM analysis are summarized in Table 2.1.1.
Fig. 2.1.1 shows the TIC chromatogram. Overlapping occurs at 2 locations, however, quantitation can be performed without problem using the MC or SIM.
Fig. 2.1.2 shows the SIM chromatogram for each compound. The concentration of each compound is 0.5 ppm. From this data, it is clear that detection sensitivity is sufficient for analysis of the regulation concentration of 10 ppm.

■Analytical Conditions

Instrument : GCMS-QP2010
-GC-
Column : Stabilwax
(30 m × 0.25 mm I.D. df = 0.25 μm)
Column Temp. : 50 ºC-15 ºC/min-100 ºC-5 ºC/min-250 ºC (10 min)
Carrier Gas : He, 45.0 cm/sec:
(Constant Linear Velocity Mode)
Injector Temp. : 230 ºC
Injection Method : Split Injection
Split Ratio : 1 : 40
Injection Volume : 1 μL
-MS-
Interface Temp. : 230 ºC
Ion Source Temp. : 200 ºC
Ionization Method : EI
Scan Range : *m/z* 35-500
Scan Interval : 0.5 sec

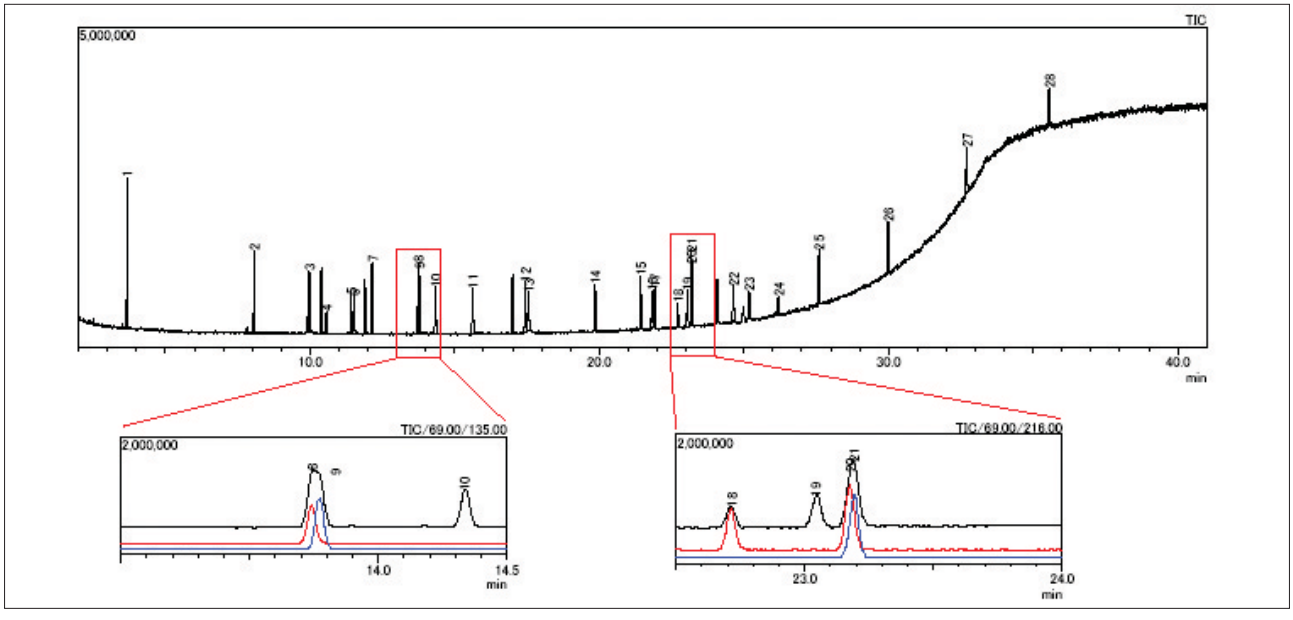


Fig. 2.1.1 Total Ion Current Chromatogram of SCAN Mode (10 ppm sample)

2.1 Analysis of Fragrances of Cosmetics (2) - GC/MS

Table 2.1.1 Compound Name and SIM

Peak No.	Compound Name	SIM *(m/z)*			Peak No.	Compound Name	SIM *(m/z)*		
1	Limonene	68.00	67.00	93.00	14	Eugenol	164.00	149.00	103.00
2	Linalool	93.00	71.00	121.00	15	Amyl cinnamal	202.00	129.00	201.00
3	Methyl heptin carbonate	95.00	79.00	123.00	16	Anisyl alcohol	138.00	109.00	137.00
4	Citral 1	69.00	94.00	109.00	17	Cinnamyl alcohol	92.00	115.00	134.00
5	1,4-dibromobenzene (IS)	236.00	234.00	238.00	18	Farnesol 1	69.00	81.00	93.00
6	Citral 2	69.00	94.00	109.00	19	Isoeugenol	164.00	149.00	131.00
7	Citronellol	69.00	81.00	95.00	20	Farnesol 2	69.00	81.00	93.00
8	Geraniol	69.00	41.00	123.00	21	Hexyl cinnam-aldehyde	216.00	129.00	215.00
9	3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-yl)-3-buten-2-one	135.00	206.00	150.00	22	Coumarin	146.00	118.00	89.00
					23	2-(4-tert-Butylbenzyl)propionaldehyde	136.00	149.00	192.00
10	Benzyl alcohol	108.00	79.00	107.00	24	Amylcin namyl alcohol	115.00	133.00	204.00
11	Hydroxy-citronellal	59.00	43.00	71.00	25	Benzyl benzoate	105.00	212.00	194.00
12	Cinnamal	131.00	132.00	103.00	26	Benzyl salicylate	91.00	65.00	228.00
13	Hydroxy-methylpenthylcyclohexen ecarboxaldeh	189.00	147.00	204.00	27	4,4'-Dibromobiphenyl (IS)	312.00	310.00	314.00
					28	Benzyl cinnamate	131.00	192.00	193.00

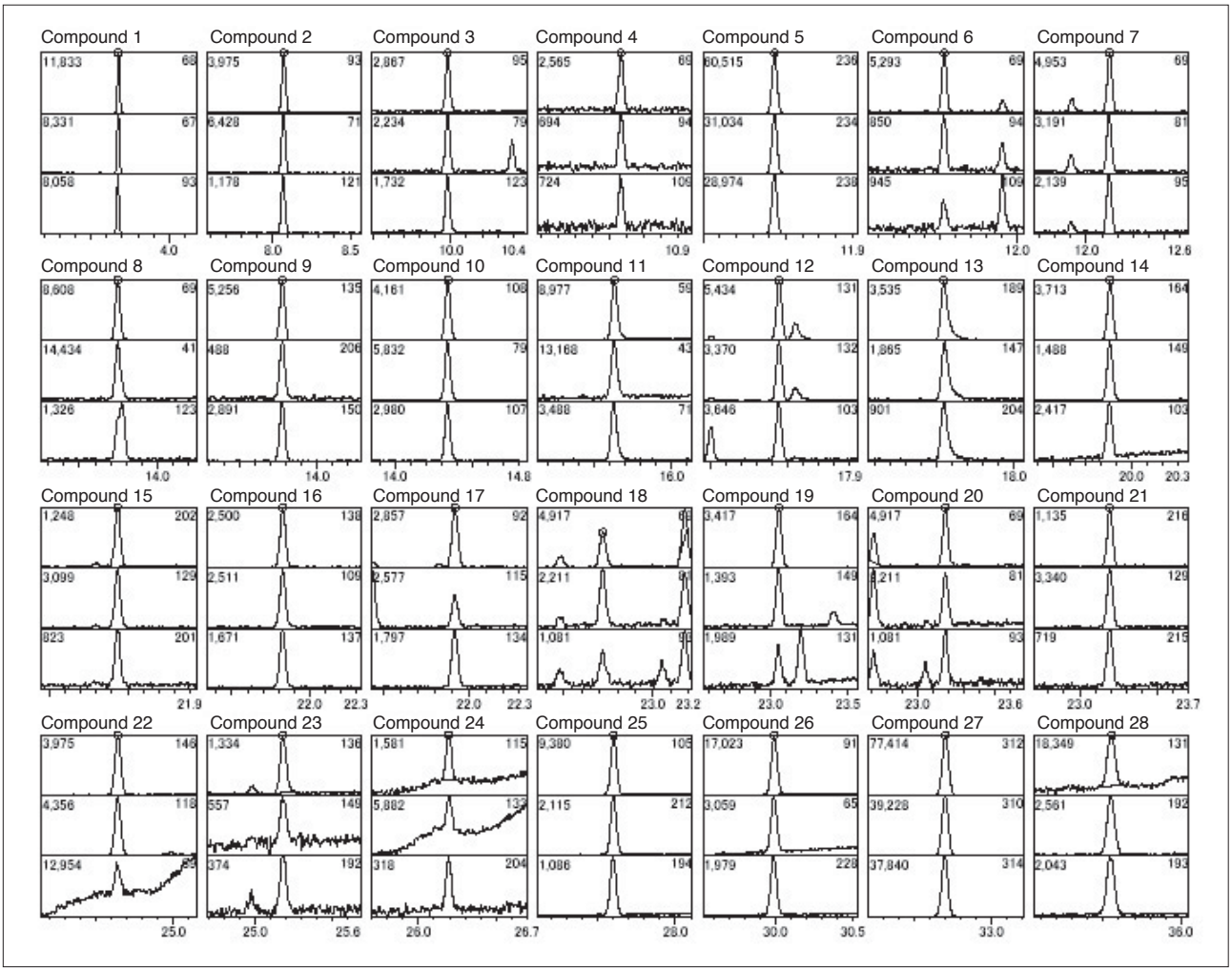


Fig. 2.1.2 Mass Chromatograms of SIM Mode (0.5 ppm samples)

2.2 Analysis of Cosmetics (1) - LC

●Analysis of Glycyrrhizic Acid and Piroctone in Shampoo

■Explanation

This data gives an analysis example for glycyrrhizic acid and piroctone in commercially available shampoo. It is recommended that the flow route and syringe, etc., be washed in advance with EDTA-2Na (ethylenediamine tetraacetic acid - 2-sodium) and the greatest care be taken to prevent as best as possible the effects of metal such as contamination that occurs with separation in order to suppress peak shape turbulence due to the metal coordination of piroctone.

■Pretreatment

Weigh sample 200-300 mg
↓
Add 50 mL of 10 mmol/L HCl aq. containing 2 mmol/L EDTA·2Na/Methanol=1/1
↓
Ultrasonication for 5 min
↓
Keep at 60 °C for 10 min
↓
Shake Vigorously
↓
Filtration
↓
Injection of 10 µL

■Analytical Conditions

Column : L-Column ODS
(150 mmL. × 4.6 mm I.D.)

Mobile Phase : Gradient Program A→B
A : 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 0.1 mmol/L EDTA·2Na /Acetonitrile = 4/1
B : 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 0.2 mmol/L EDTA·2Na /Acetonitrile = 1/3

Flowrate : 1.0 mL/min

Column Temp. : 40 °C

Detection : UV-VIS Absorbance Detector at 250 nm
Atten 7 (change to 8 at 6 min)

Time Program

#	TIME	FUNC	VALUE
0	3.00	B.CONC	40.0
1	6.00	WAVE A	300
2	10.00	B.CONC	100.0
3	15.00	B.CONC	100.0
4	15.01	B.CONC	40.0
5	20.90	WAVE A	250
6	21.00	ZERO A	
7	22.00	STOP	

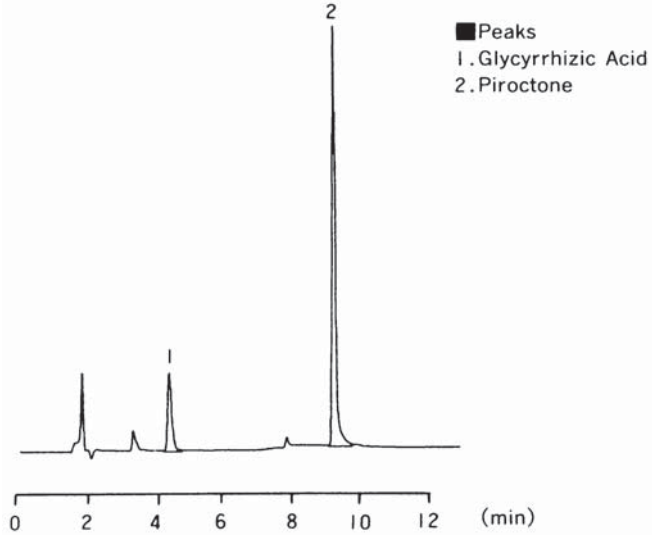


Fig. 2.2.1 Analysis of Commercially Available Shampoo

2.2 Analysis of Cosmetics (2) - LC

●Analysis of Glycyrrhizic Acid in Cosmetics

■Explanation

Glycyrrhizic acid ($C_{42}H_{62}O_{16}$; M.W. = 822.92) is a constituent of licorice, which is known as a crude drug, and is generally known to offer pharmacological benefits such as anti-allergy, anti-inflammatory, and detoxifying effects. Because no significant level of toxicity can be found, in addition to commercial drug products, such as gastrointestinal medicine, eye drops, and medicine for allergic rhinitis, it is also contained in various cosmetics, such as hair-growth agents, bathwater additives, shampoos, and lotions. It is a chemical substance that we often encounter in our daily lives.

■Analysis of Medicated Whitening Lotion

Fig. 2.2.3 shows an analysis example for medicated whitening lotion. Alcohol odor was detected for this commercial cosmetic and so, in pretreatment, the sample was diluted with the buffer solution used in the mobile phase, and the resulting solution was filtered with a water-system filter.

■Pretreatment of Medicated Whitening Lotion

Sample 2 mL
↓ ← Add 2mL of 10 mmol/L Sodium Phosphate Buffer (pH 2.6)
Mix
↓
Filter with a 0.45 µm membrane filter
↓
Inject 5 µL

■Analysis of Medicated Calamine Lotion

Fig. 2.2.4 shows an analysis example for commercial calamine lotion, which soothes burning of the skin caused, for example, by excessive exposure to sunlight. This type of cosmetic usually incorporates zinc-oxide powder containing a small amount of iron oxide, and the liquid phase often splits into two layers. Here, the liquid phase is extracted and used as the test liquid.

■Pretreatment of Medicated Calamine Lotion

Sample 2 mL
↓ ← Add 2 mL of Methanol
Mix
↓
Dilute 2 folds with 10 mmol/L Sodium Phosphate Buffer (pH 2.6)
↓
Filter with a 0.45 µm membrane filter
↓
Inject 20 µL

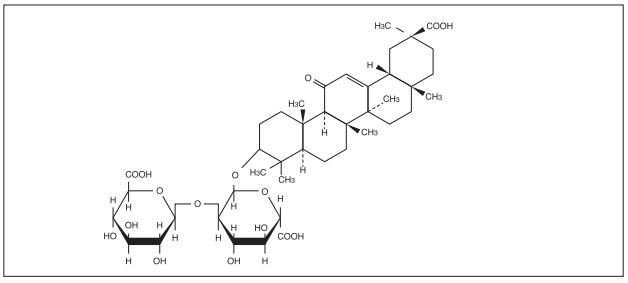


Fig. 2.2.2 Chemical Structure of Glycyrrhizic Acid

■Analytical Conditions

Column : Shim-pack FC-ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase : 10 mmol/L Sodium Phosphate Buffer (pH 2.6)
/Acetonitrile = 3/2 (v/v) (Fig. 2.2.3)
= 5/3 (v/v) (Fig. 2.2.4)
Florate : 0.8 mL/min.
Column Temp.: 40 ˚C
Detection : Photodiode Array UV-VIS Absorbance Detector SPD-M10A*VP* at 250 nm

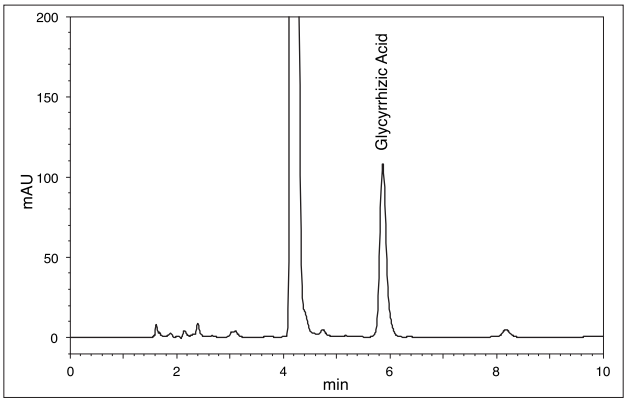


Fig. 2.2.3 Analysis of Medicated Whitening Lotion

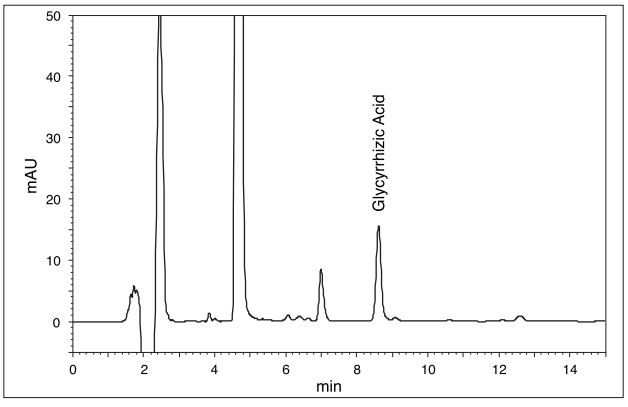


Fig. 2.2.4 Analysis of Medicated Calamine Lotion

2.2 Analysis of Cosmetics (3) - LC

●Analysis of Dimethicone (Dimethylpolysiloxane) in Hair Care Products

■Explanation

Dimethicone (dimethylpolysiloxane), a type of silicone oil, is widely used in hair care products such as conditioners and shampoos as an emollient that effectively utilizes the water repellent property of this substance. Here, we introduce examples of analysis of dimethicone in hair care products using the ELSD-LD Ⅱ evaporative light scattering detector.

Fig. 2.2.5 shows the structure of dimethicone.

Dimethicone is a strongly hydrophobic compound in which the dimethylsiloxane radical basic unit is polymerized, and the application of the compound varies with the degree of polymerization due to the associated distinct property variations. Because dimethicone has no chromophores, the evaporative light scattering detector is effective in HPLC analysis.

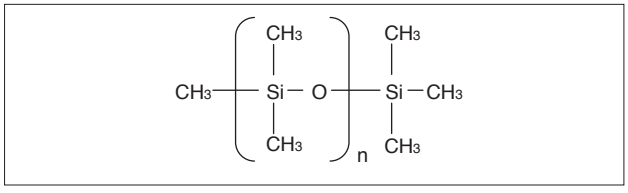


Fig. 2.2.5 Structure of Dimethicone

■Analysis of Dimethicone in Hair Care Products

Fig. 2.2.6 and Fig. 2.2.7 show chromatograms obtained from analysis of commercially available hair conditioner and shampoo products containing dimethicone. The samples were weighed out to 450 mg, 5 mL of tetrahydrofuran was added, and after agitation and ultrasonic extraction, the supernatant was filtered through a 0.22 µm membrane filter, and 2 µL was injected.

* The retention time varies slightly depending on the degree of polymerization of dimethicone. In order to conduct quantitation accurately, it is advisable to conduct analysis using standard samples that are prepared using additions of the actual dimethicone sample.

■Analytical Conditions

Column	: Presto FF-C18 (150 mmL. × 4.6 mm I.D., 2 µm)
Mobile Phase	: A : 0.5 % Formic Acid/Acetonitrile =1/1 (v/v) B : Tetrahydrofuran Gradient Elution Method
Time Program	: B 30 % (0-4 min) →100 % (5-9 min) →30 % (9.01-15 min)
Flowrate	: 0.5 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 2 µL
Detection	: Evaporative Light Scattering Detector ELSD-LT Ⅱ Temperature : 40 ºC Gain : 6 Nebulizer Gas : N_2 Gas Pressure : 350 kPa

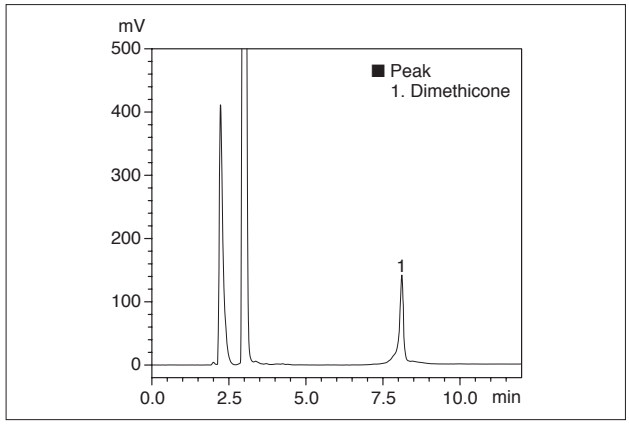


Fig. 2.2.6 Chromatogram of Hair Conditioner

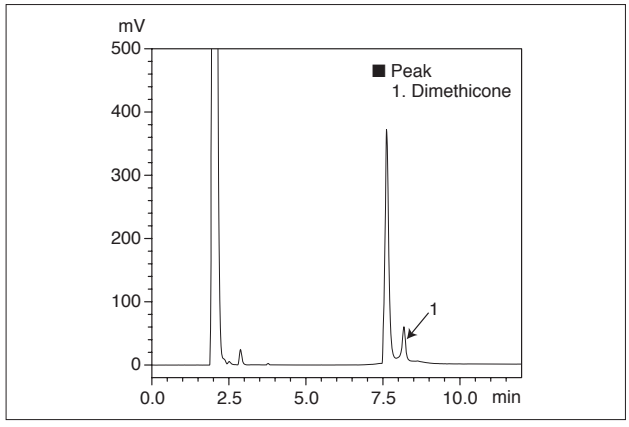


Fig. 2.2.7 Chromatogram of Hair Shampoo

2.3 Analysis of Cream (1) - LC

●High Speed, High Resolution Analysis of Preservatives in Cosmetics

■Explanation

Preservatives such as parabens and phenoxyethanol are used to maintain the safety and quality of cosmetics. Here, we introduces an example of ultra-high-speed analysis of reservatives found in cosmetics using the Nexra LC system in conjunction with the Shim-pack XR-ODS Ⅲ high-speed separation column. Fig. 2.3.1 shows the chromatograms of 12 substances (100 mg/L each) that are frequently used as preservatives in cosmetics. Fig. 2.3.2 shows the results of analysis of two commercially available cosmetic creams. Each sample was weighed out to 1.0 g, and after adding methanol, followed by ultrasonic extraction, the samples were brought to a volume of 100 mL each and passed through a membrane filter (pore size: 0.22 µm).

* Chromatograms are shown with the background subtracted.

■Analytical Conditions

Column	: Shim-pack XR-ODS Ⅲ (50 mmL. × 2.0 mm I.D., 1.6 µm)
Mobile Phase	: A : 5 mmol/L Sodium Citrate Buffer (pH 4.2) B : Acetonitrile Gradient Elution Method
Time Program	: B 25 % (0-0.6 min) → 40 % (0.7 min) → 45 % (2.0 min)
Flowrate	: 0.8 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 2 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 230, 255 nm
Flow Cell	: Semi-micro Cell

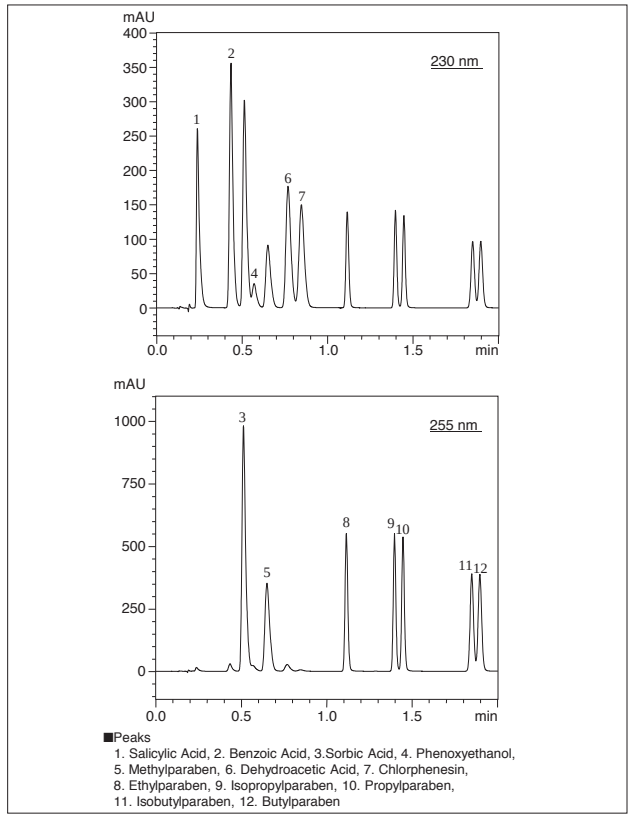


Fig. 2.3.1 Chromatograms of a Standard Mixture of 12 Preservatives (100 mg/L each)

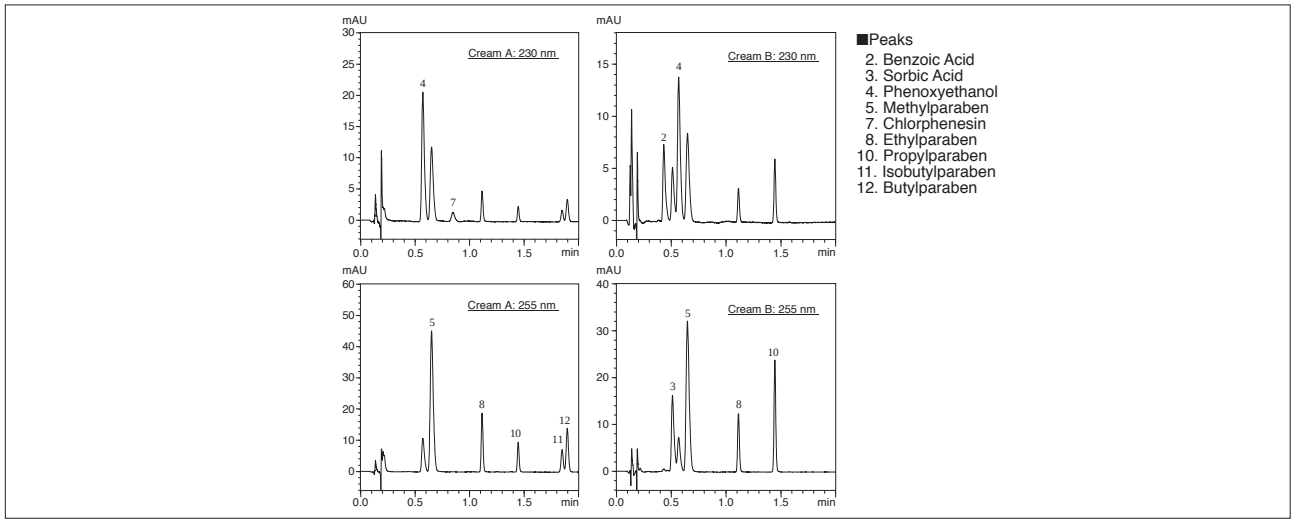


Fig. 2.3.2 Chromatograms of Cosmetics

[Reference]
Mori, Nakamura, Ohnuki, Terajima, Miyamoto, Ogino, and Saito: Annual Report of Tokyo Metropolitan Institute of Public Health, No. 58 (2007)

2.3 Analysis of Cream (2) - LC

●High Speed Analysis of Ultraviolet Absorbers in Cosmetics

■Explanation

Recently, cosmetics that protect the skin from ultraviolet radiation are being marketed worldwide. Ultraviolet absorbers and ultraviolet scatterers are used in these cosmetics, and analysis of ultraviolet absorbers is generally conducted by HPLC. Here we show an example of high-performance analysis of ultraviolet absorbers in cosmetics using the Prominence UFLCXR ultra fast, high-resolution LC system and a high-speed, high-resolution column.

■Analysis of Standard Solution

The analytes consisted of 11 components that were blended into the cosmetic product Fig. 2.3.3 shows the results of a standard mixture of 11 ultraviolet absorbers (10 mg/L each, prepared using methanol) using a 2 µL injection. The column used was a HALO® C18, 2.1 mm internal diameter (particle size 2.7 µm) (AMT Co.), and high-speed, high-resolution analysis was conducted with an elevated linear velocity and column temperature. A photodiode array detector was used for detection, and the peaks of benzoic acid, 2-[4-(diethylamino)-2-hydroxybenzoyl]-, hexyl ester and 4-*tert*-butyl-4'-methoxydibenzoylmethane were identified at 355 nm,with the other peaks being identified at 310 nm.

■Analytical Conditions

Column	: HALO® C18 (150 mmL. × 2.1 mm I.D., 2.7 µm)
Mobile Phase	: A : 0.085 % Phosphoric Acid in Water B : Acetonitrile Gradient Elution Method
Time Program	: B 30 % (0 min) → 70 % (3.5 min) → 75 % (7 min)
Flowrate	: 0.8 mL/min
Column Temp.	: 60 ºC
Injection Volume	: 2 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 310, 355 nm
Flow Cell	: Semi-micro Cell

■Analysis of Cosmetic

Fig. 2.3.4 shows an example of an analysis of a cosmetic cream. The sample amount used was 1.0 g, and after adding 10 mL of tetrahydrofuran, sonicated to extract the UV absorbing analytes. Then the sample was centrifuged and methanol was added to the supernatant to bring the volume to 250 mL. This was filtered through a 0.22 µm pore membrane filter, and 2 µL was injected.

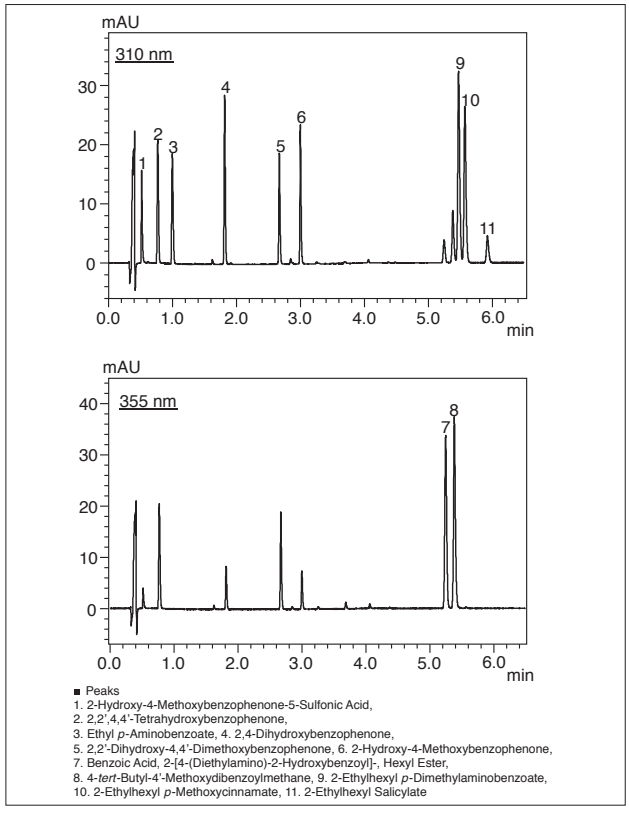


Fig. 2.3.3 Chromatograms of a Standard Mixture of 11 Ultraviolet Absorbers (10 mg/L each)

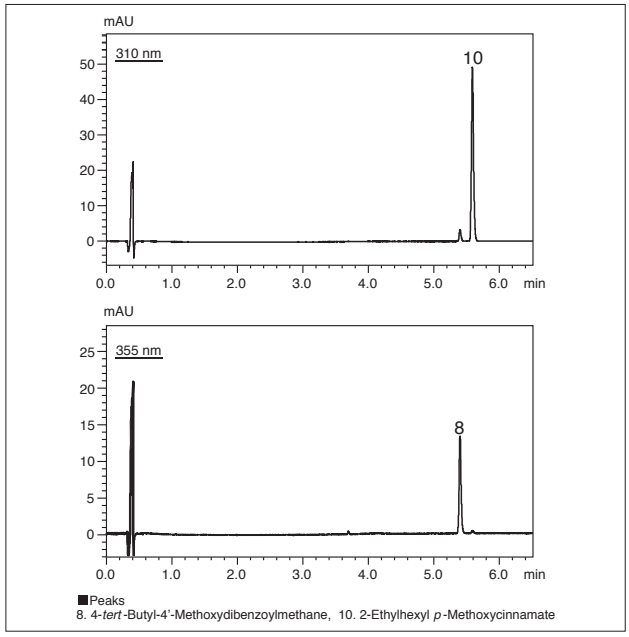


Fig. 2.3.4 Chromatograms of Cosmetic (Cream)

[Reference]
Y. Ikarashi *et al* : Bull. Natl. Inst. Health Sci., 126, 82-87 (2008)

2.3 Analysis of Cream (3) - LC

●Analysis of Allantoin in Cream

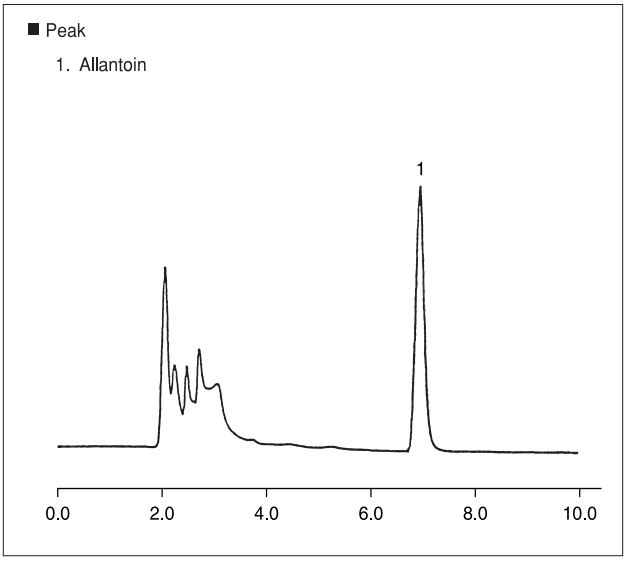


Fig. 2.3.5 Analysis of Allantoin in Cream

■Analytical Conditions

Column : Asahipak NH2P-50 4E
(250 mmL. × 4.6 mm I.D.)
Mobile Phase : A : 10 mmol/L Sodium Phosphate Buffer (pH 2.6)
B : Acetonitrile
A/B = 2/8 (v/v)
Flowrate : 1.0 mL/min
Column Temp. : 40 ˚C
Detection : UV Absorbance Detector at 200 nm

■Pretreatment

1. Add pure water to a sample
2. Ultrasonication for 5 min.
3. Heating at 60 ˚C for 5 min.
4. Filtration with membrane filter (0.45 µm) after shaking.
5. Inject 10 µL of filtrate after cooling

●Analysis of Zinc Pyrithione

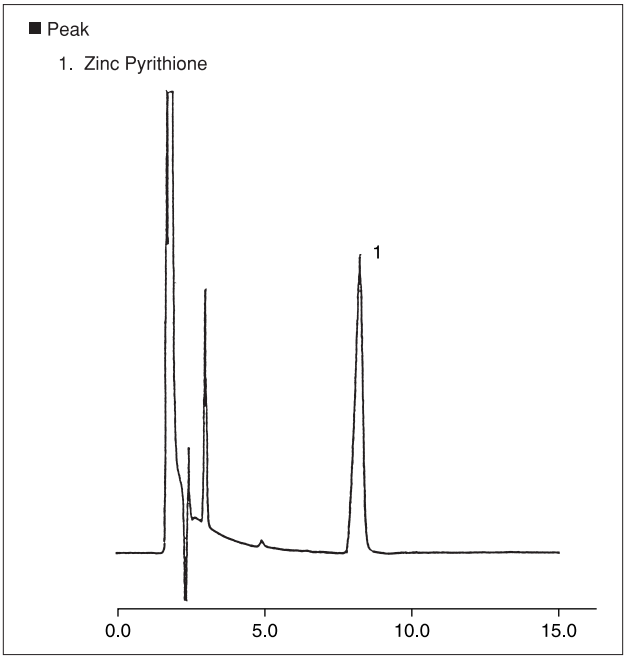


Fig. 2.3.6 Analysis of Zinc Pyrithione in Cream

■Analytical Conditions

Column : L-Column ODS
(150 mmL. × 4.6 mm I.D.)
Mobile Phase : A: 10 mmol/L Sodium Acetate Buffer (pH 4.0)
containing 3 mmol/L EDTA·2Na
B: Acetonitrile
A/B = 10/1 (v/v)
Flowrate : 0.8 mL/min
Column Temp. : 40 ˚C
Detection : UV Absorbance Detector at 250 nm

■Pretreatment

1. Add dilution solution to a sample
2. Dissolution by ultrasonication
3. Filtration with membrane filter (0.45 µm)
4. Inject 10 µL of filtrate

3. Quasi-Drugs

3.1 Analysis of Hair Tonic - LC

●Analysis of Resorcinol, D-Panthenol and Vitamin B

■Explanation

This data introduces analysis examples of resorcinol, D-panthenol and vitamin B_6 that are regarded as being effective for germ killing and the prevention of itchiness and hair loss. Fig. 3.1.1 shows a chromatogram of the standards while Fig. 3.1.2 and Fig. 3.1.3 show chromatograms of hair tonics A and B. All samples were diluted in methanol and injected for analysis.

■Analytical Conditions

Column : Shim-pack CLC-ODS (150 mmL. x 6.0 mm I.D.)
Mobile Phase : A: 10 mmol/L Phosphate Buffer (pH 2.6) containing 10 mmol/L Sodium Pentane Sulfonate
B: Methanol
A/B = 9/1 (v/v)
Flowrate : 1.0 mL/min
Column Temp. : 50 °C
Detection : UV-VIS Absorbance Detector at 210 nm

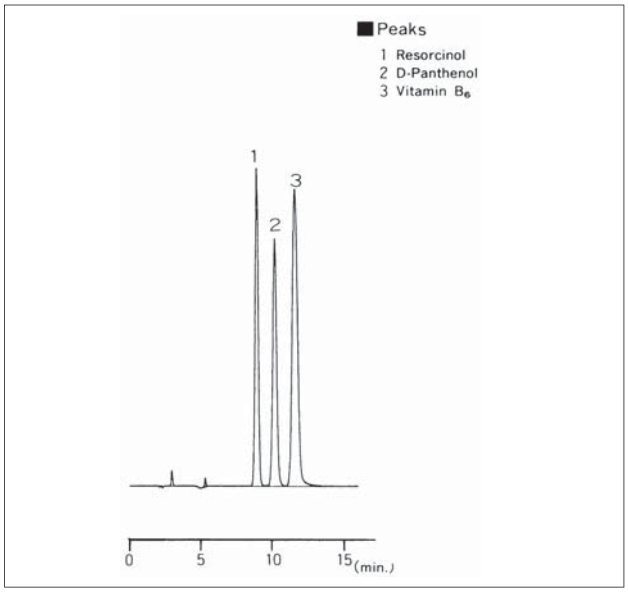


Fig. 3.1.1 Chromatogram of Resorcinol, D-panthenol and Vitamin B_6

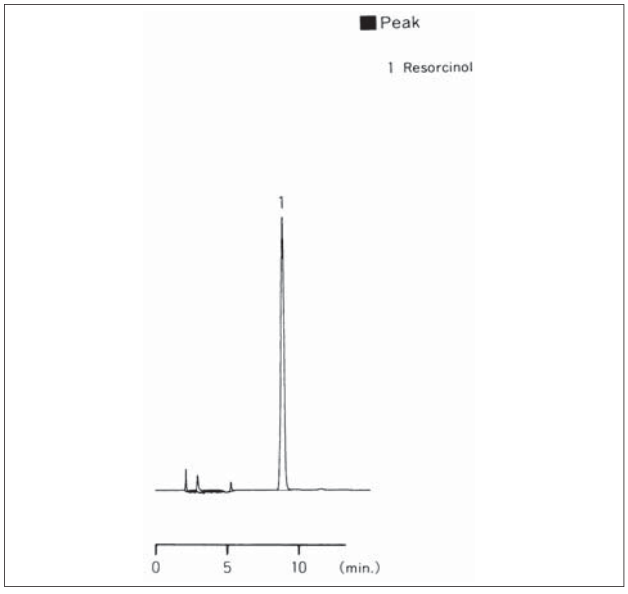


Fig. 3.1.2 Chromatogram of Hair Tonic A

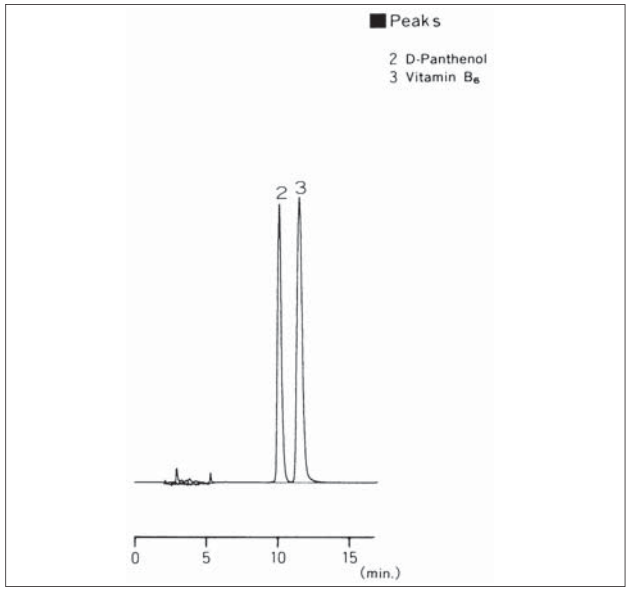


Fig. 3.1.3 Chromatogram of Hair Tonic B

3.2 Analysis of Hair Lotion - LC

■Explanation

This data introduces analysis examples of two types of hair lotion (A and B). The same care needs to be taken for lotion A as previously explained for piroctone because it contains hinokitiol, which causes metal coordination. Swertiamarin in lotion B is the effective element in senburi, a Japanese herbal plant.

■Pretreatment

Hair Lotion A was Diluted 20-fold with Methanol /2 mmol/L EDTA·2 Na=7/3

↓

Filtration

↓

Injection of 10 µL

■Pretreatment

Hair Lotion B was Diluted 5-fold with Methanol

↓

Filtration

↓

Injection of 10 µL

■Analytical Conditions

Column : L-Column ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase : Gradient Program A→B
A : 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 0.1 mmol/L EDTA·2Na /Acetonitrile = 6/1
B : 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 0.2 mmol/L EDTA·2Na /Acetonitrile = 1/3
Flowrate : 1.0 mL/min
Column Temp. : 40 °C
Detection : UV-VIS Absorbance Detector 275 nm
Time Program

#	TIME	FUNC	VALUE
0	2.00	B.CONC	0.0
1	6.00	WAVE A	400
2	10.00	B.CONC	100.0
3	11.50	WAVE A	260
4	16.00	B.CONC	100.0
5	16.01	B.CONC	0.0
6	20.80	WAVE A	275
7	21.00	ZERO A	
8	22.00	STOP	

■Analytical Conditions

Column : L-Column ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase : Gradient Program A 95 % → B 70 %
A : 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 100 mmol/L $NaClO_4$ /Acetonitrile = 10/1
B : 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 100 mmol/L $NaClO_4$ /Acetonitrile = 1/1
Flowrate : 1.0 mL/min
Column Temp. : 40 °C
Detection : UV-VIS Absorbance Detector 240 nm
Time Program

#	TIME	FUNC	VALUE
0	4.90	B.CONC	5.0
1	5.00	B.CONC	70.0
2	7.00	WAVE A	220
3	14.50	WAVE A	370
4	16.00	B.CONC	70.0
5	16.10	B.CONC	5.0
6	20.40	WAVE A	240
7	21.50	ZERO A	
8	22.00	STOP	

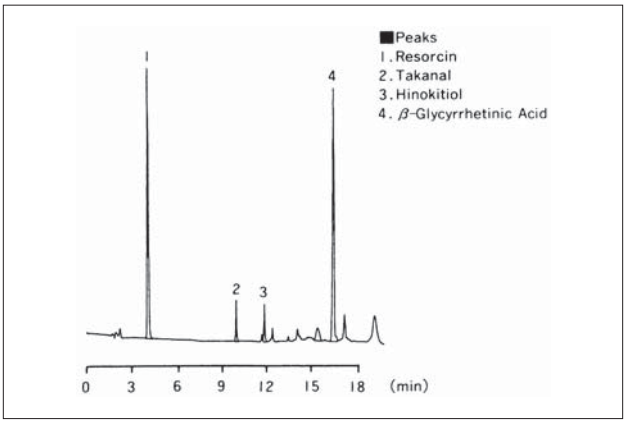


Fig. 3.2.1 Analysis of Hair Lotion A

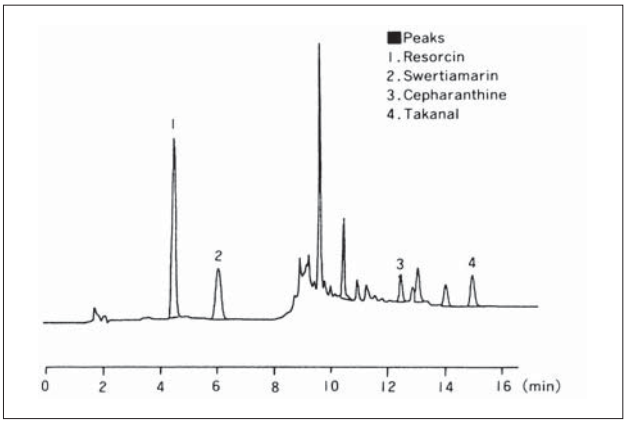


Fig. 3.2.2 Analysis of Hair Lotion B

3.3 Headspace Analysis of Volatile Components in Lip Cream and Toothpaste - GC

■Analytical Conditions

Column	: ULBON HR-20M (25 m × 0.32 mm I.D. df = 0.25 µm)
Column Temp.	: 200 °C
Injection Temp.	: 230 °C
Detector Temp.	: 230 °C (FID)
Carrier Gas	: He 1.2 mL/min
Injection Method	: Split Injection
Split Ratio	: 1 : 14
Sample Quantity	: 20 mg
Sample Thermostatting	: 150 °C, 60 min
Headspace Injection Volume	: 0.8 mL

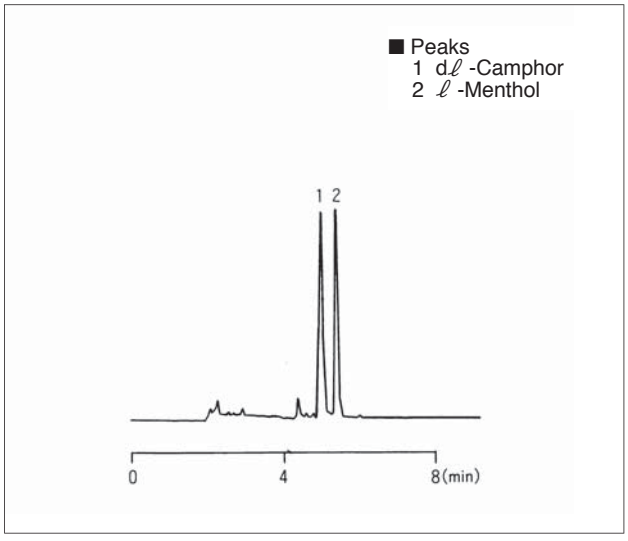


Fig. 3.3.1 Headspace Gas Chromatogram of Lip Cream

■Analytical Conditions

Column	: CBP1 (12 m × 0.53 mm I.D. df = 1.0 µm)
Column Temp.	: 60 °C-10 °C/min-200 °C
Injector Temp.	: 200 °C
Detector Temp.	: 200 °C (FID)
Carrier Gas	: He 15 mL/min
Injection Method	: Direct Injection
Sample Quantity	: 0.5 g
Sample Thermostatting	: 80 °C, 30 min
Headspace Injection Volume	: 0.4 mL

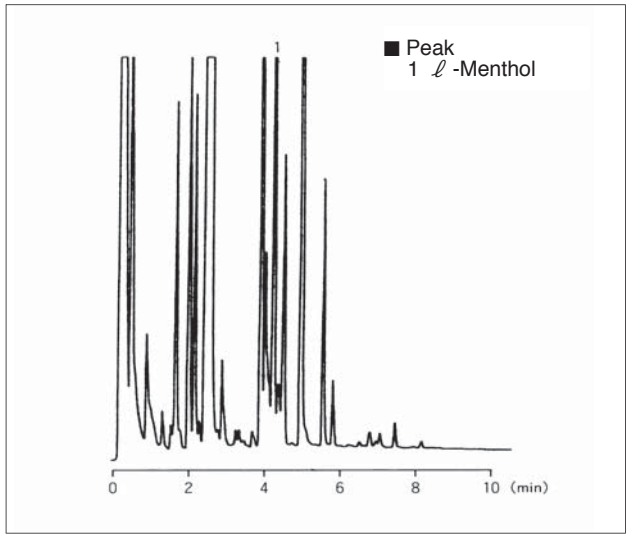


Fig. 3.3.2 Headspace Gas Chromatogram of Toothpaste A

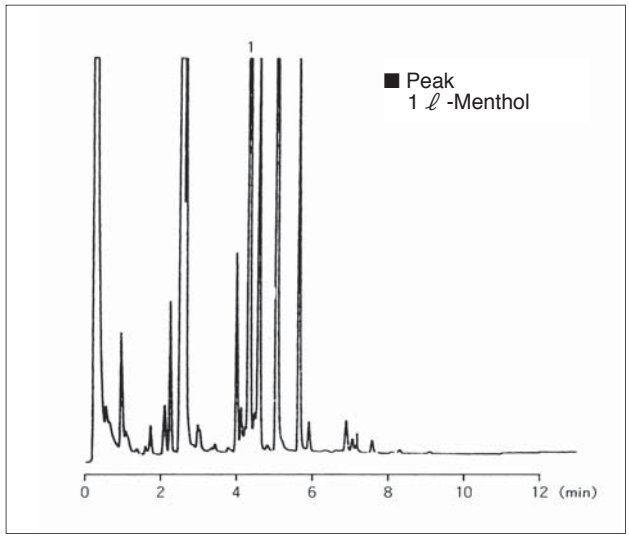


Fig. 3.3.3 Headspace Gas Chromatogram of Toothpaste B

3.4 Determination of Chlorhexidine, Benzethonium, and Benzalkonium in Disinfectants (1) - LC

■Explanation

In the last few years it has become a common practice to diligently disinfect one's hands due to the occurrence of health threats such as influenza epidemics. Ethanol is the principal ingredient in many of these hand sanitizers, but often there are other ingredients with sterilization properties that are included among the active ingredients. There are also bactericide / disinfectant products with sterilization action that are commercially available for topical use when minor injuries like scrapes or cuts are sustained. Here we introduce examples of HPLC analysis of 3 active ingredients (chlorhexidine, benzethonium, and benzalkonium) found in commercially available disinfectants.

■Analytical Conditions

Column	: Phenomenex Gemini-NX 5 µC18 (150 mmL. × 4.6 mm I.D.)
Mobile Phase	: 2 g/L SDS*, 6 mL/L Acetic Acid in Water/Acetonitrile/Tetrahydrofuran = 4/4/2 (v/v/v)
Flowrate	: 1.0 mL/min
Column Temp.	: 40 ºC
Detection	: UV Absorbance Detector SPD-20A at 258 nm (0-10 min), 272 nm (10-15 min)
Injection Volume	: 10 µL

* Sodium dodecyl sulfate

■Determination of Chlorhexidine and Benzethonium

Chlorhexidine is typically used in the form of a gluconate or hydrochloride salt, and benzethonium is widely used as a chloride salt (benzethonium chloride). Simultaneous analysis of benzethonium chloride and chlorhexidine gluconate was performed according to the analytical technique for chlorhexidine gluconate by reversed-phase ion pair chromatography published in the " Methods of Analysis in Health Science 2005". Fig. 3.4.1 shows a chromatogram of a standard solution of chlorhexidine diacetate and benzethonium chloride. Since these 2 constituents are both strongly basic, peak tailing sometimes occurs. Therefore, due to the importance of selecting a separation column with little adsorption of basic substances, the "Phenomenex Gemini-NX" was used for this analysis. In addition, since the maximum absorbance wavelength is different for these 2 substances, wavelength switching was conducted midway through the chromatogram. Fig. 3.4.2 shows examples of analysis of 3 types of commercial disinfectants (A - C). After preparing a 1/20 dilution of disinfectant A, and 1/10 dilutions of B and C using eluent, 10 µL each was injected.

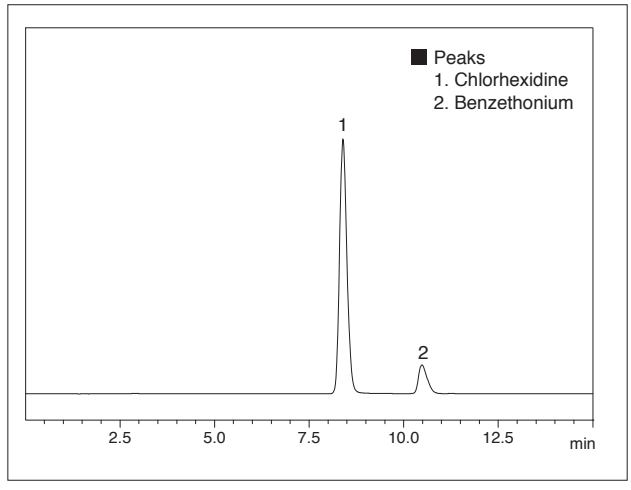


Fig. 3.4.1 Chromatogram of a Standard Solution of Chlorhexidine Diacetate (40 mg/L) and Benzethonium Chloride (100 mg/L)

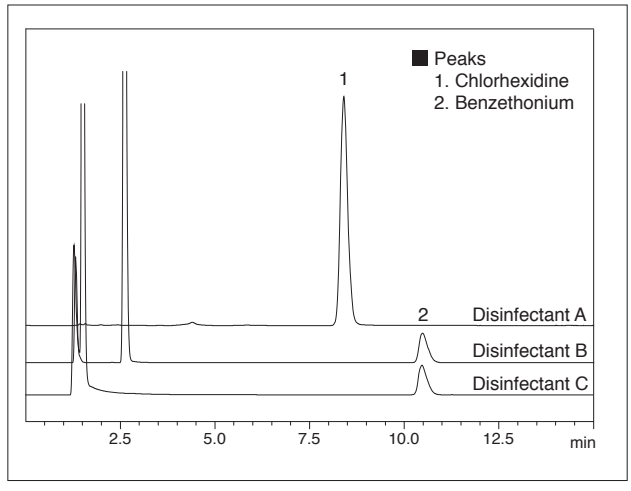


Fig. 3.4.2 Chromatograms of Commercial Disinfectants

3.4 Determination of Chlorhexidine, Benzethonium, and Benzalkonium in Disinfectants (2) - LC

■Determination of Benzalkonium

Benzalkonium is used as a chloride salt (benzalkonium chloride), and as shown in Fig. 3.4.3, its structural formula consists of a mixture of various even-numbered alkyl chain lengths from C8 to C18, (mainly C12 and C14). Here we conducted analysis using cation exchange chromatography and reversed-phase chromatography. Fig. 3.4.4 shows the chromatograms obtained using cation exchange mode. After preparing 1/100 dilutions of the commercial disinfectants (D and E) using purified water, 10 µL was injected for each analysis. Using a cation exchange column, the peaks were eluted in the order of longer to shorter alkyl chain lengths.

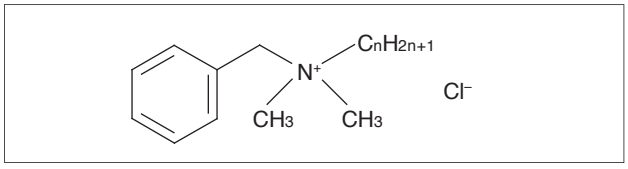


Fig. 3.4.3 Structure of Benzalkonium Chloride

■Analytical Conditions

Column	: Phenomenex Luna 5µSCX (250 mmL. × 4.6 mm I.D.)
Mobile Phase	: 20 mmol/L Sodium Perchlorate in Water/Acetonitrile = 6/4 (v/v)
Flowrate	: 1.0 mL/min
Column Temp.	: 45 ºC
Detection	: UV Absorbance Detector SPD-20A at 265 nm
Injection Volume	: 10 µL

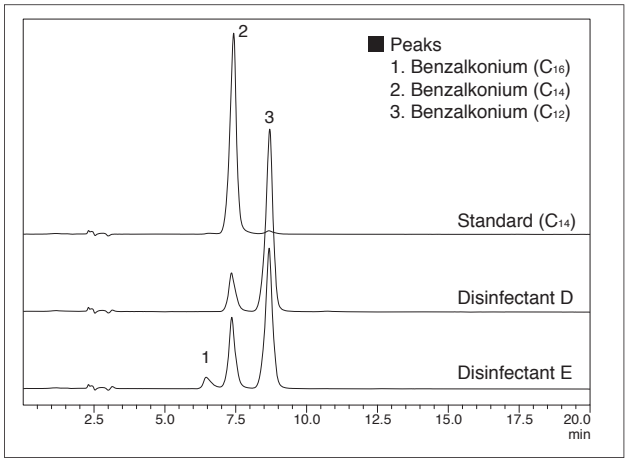


Fig. 3.4.4 Chromatograms of Benzalkonium Chloride Standard (C_{14}, 1000 mg/L) and Commercial Disinfectants using Cation Exchange Mode

Fig. 3.4.5 shows the chromatogram obtained using reversed-phase mode. The samples consisted of 1/1000 dilutions of commercial disinfectants (D and E) prepared using purified water, and 10 µL was injected for each analysis. In reversed-phase mode, the peaks were eluted in the order of shorter to longer alkyl chain lengths.

■Analytical Conditions

Column	: Phenomenex Luna 5µ C18 (150 mmL. × 4.6 mm I.D.)
Mobile Phase	: 20 mmol/L Sodium Perchlorate in Water/Acetonitrile = 2/8 (v/v)
Flowrate	: 1.0 mL/min
Column Temp.	: 45 ºC
Detection	: UV Absorbance Detector SPD-20A at 265 nm
Injection Volume	: 10 µL

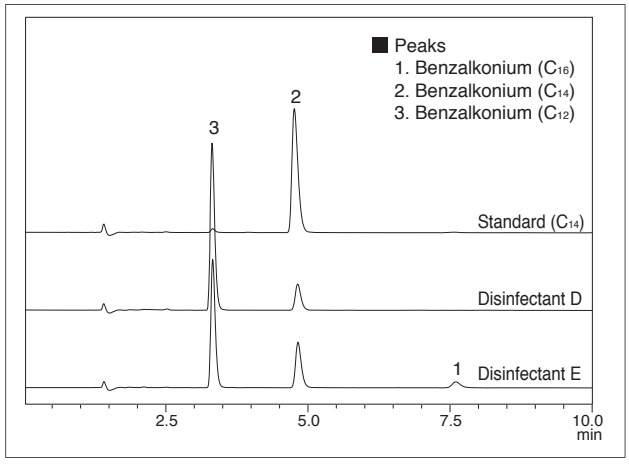


Fig. 3.4.5 Chromatograms of Benzalkonium Chloride Standard (C_{14}, 100 mg/L) and Commercial Disinfectants using Reversed-Phase Mode

[Reference]
Methods of Analysis in Health Science 2005, The Pharmaceutical Society of Japan (Kanehara & Co., Ltd.)

4. Antibiotics

4.1 Analysis of Actinomycin D - LC

■Explanation

This analysis example is for a standard of actinomycin D, which is used as an anticancer drug in chemotherapy for trophoblastic tumors and Wilms' tumors.

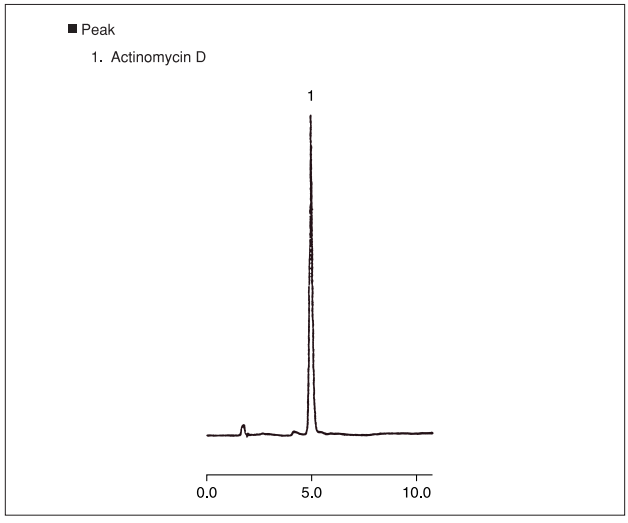


Fig. 4.1.1 Analysis of Actinomycin D

■Analytical Conditions

Column : STR ODS-M (150 mmL. × 4.6 mm I.D.)
Mobile Phase : A: 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 100 mmol/L $NaClO_4$
B: Methanol
A/B = 1/4 (v/v)
Flowrate : 1.0 mL/min
Column Temp. : 50 ˚C
Detection : UV Absorbance Detector at 240 nm

■Pretreatment

1. Dilute a sample with methanol
2. Filtration with membrane filter (0.45 µm)
3. Inject 5 µL of filtrate

4.2 Analysis of Mitomycin C - LC

■Explanation

Mitomycin C was discovered in Japan, and is currently used worldwide as an anticancer drug in the treatment of gastric cancer, colon cancer, uterine cancer, lung cancer, and breast cancer. An analysis example for a standard of mitomycin C is shown here.

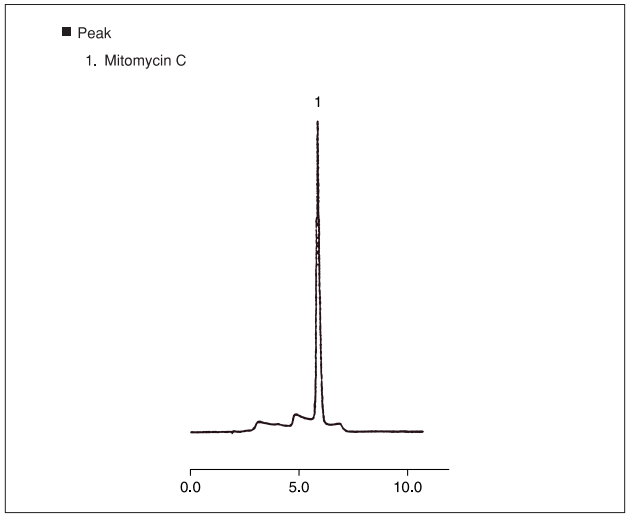


Fig. 4.2.1 Analysis of Mitomycin C Crystals

■Analytical Conditions

Column : STR ODS-M (150 mmL. × 4.6 mm I.D.)
Mobile Phase : A: 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 100 mmol/L $NaClO_4$
B: Methanol
A/B = 3/1 (v/v)
Flowrate : 1.0 mL/min
Column Temp. : 50 ˚C
Detection : UV Absorbance Detector at 360 nm

■Pretreatment

1. Dissolve a standard sample (crystal) in methanol (20 mg/mL)
2. Inject 5 µL of solution

4.3 Analysis of Amantadine - LC

■Explanation

Amantadine is well known as a drug used in the treatment of Parkinson's disease. It is also useful for the prevention and treatment of the type-A influenza virus. An analysis example for a standard of amantadine is shown here.

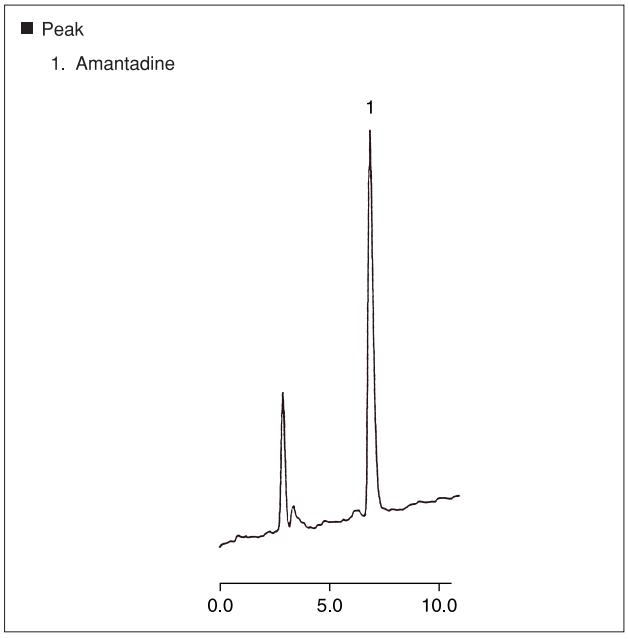


Fig. 4.3.1 Analysis of Amantadine

■Analytical Conditions

Column : STR ODS-Ⅱ (150 mmL. × 4.6 mm I.D.)
Mobile Phase : A: 20 mmol/L Sodium Citrate Buffer (pH 4.5) containing 100 mmol/L $NaClO_4$
B: Methanol
A/B = 3/2 (v/v)
Flowrate : 0.6 mL/min
Column Temp. : 40 ˚C
Detection Method : Post Column Derivatization Method
Reagent : A: 200 mmol/L Sodium Borate Buffer (pH 9.2) containing 1 mmol/L *β*-Mercaptopropionic Acid
B: 20 mmol/L *o*-Phthalaldehyde Methanol solution
A/B = 4/1 (v/v)
Flowrate : 0.3 mL/min
Reaction Coil : SUS (2 mL. × 0.5 mm I.D.)
Reaction Temp. : 80 ˚C
Detection : Fluorescence Detector (Ex: 330 nm Em: 460 nm)

■Pretreatment

1. Dissolve a standard sample in 100 mmol/L hydrochloric acid aq. solution
2. Inject 10 µL of solution

4.4 Analysis of Rifampicin - LC

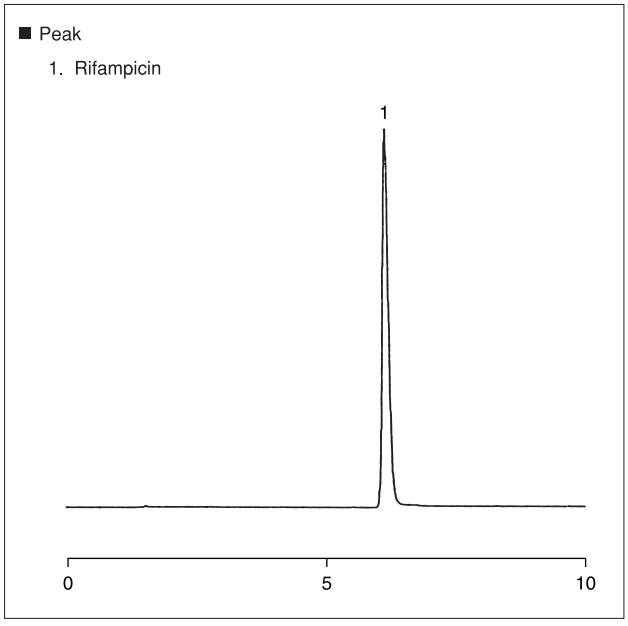


Fig. 4.4.1 Analysis of Rifampicin

■Explanation

This analysis example is for a standard of rifampicin, which is used as an antituberculosis drug and an antileprotic drug.

■Analytical Conditions

Column : STR ODS-M (150 mmL. × 4.6 mm I.D.)
Mobile Phase : A: 10 mmol/L Sodium Phosphate Buffer (pH 2.6) containing 100 mmol/L $NaClO_4$
B: Methanol
A/B = 1/3 (v/v)
Flowrate : 1.0 mL/min
Column Temp. : 40 ˚C
Detection : UV Absorbance Detector at 230 nm

4.5 High Speed Analysis of Cephem Antibiotics - LC

■Explanation

Cephem antibiotics are a sub-group of β-lactam antibiotics which, because of their wide antibacterial spectrum and powerful antibacterial effects, are commonly used as drugs, both injectable and orally administered. Here we introduce an example of high speed, batch analysis of cephem antibiotics using the ultra-fast LC system "Prominence UFLC" and the high performance "Shim-pack XR-ODS" column for high speed and high-resolution separation.

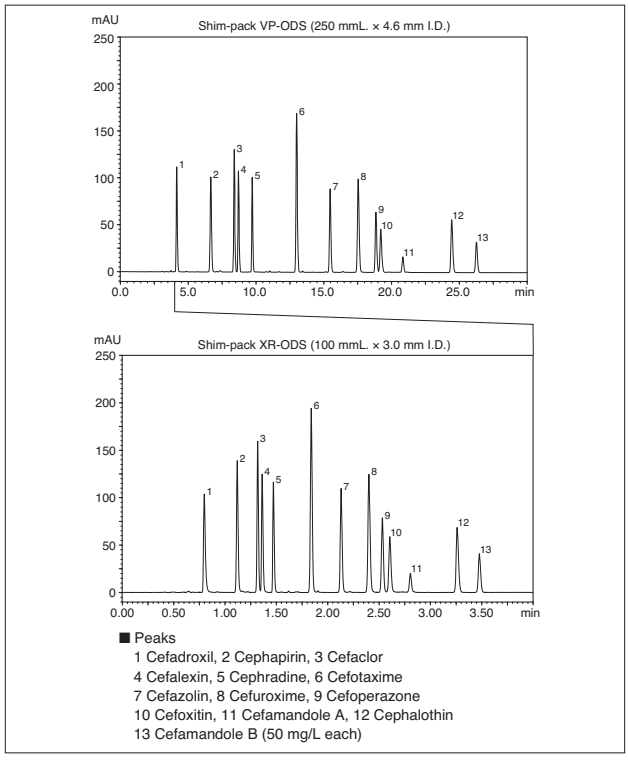


Fig. 4.5.1 Chromatograms of 12 Compounds of Cephem Antibiotic Standard Solution
(Upper: Shim-pack VP-ODS, Lower: Shim-pack XR-ODS)

The structures of these cephem antibiotics are shown in Fig. 4.5.2. With the Shim-pack XR-ODS, a high linear velocity of mobile phase, 2.4 times that with the Shim-pack VP-ODS column, shortens the time required for one analysis cycle to about 1/7 the time normally required, without sacrificing the high separation.

* Since two peaks were confirmed as Cefamandole, they are designated as Cefamandole A and B, respectively.

■Analytical Conditions

Column : Shim-pack VP-ODS
(100 mmL. × 3.0 mm I.D., 2.2 µm)
Shim-pack VP-ODS
(250 mmL. × 4.6 mm I.D., 4.6 µm)

Mobile Phase : A : 0.1 % Formic Acid-Water
B : Acetonitrile

Time Program : [XR-ODS]
B 15 % (0 min) → 55 % (3.5 min)
→ 15 % (3.51 - 6.5 min)
[VP-ODS]
B 15 % (0 min) → 55 % (30 min)
→ 15 % (30.01 - 45 min): 0.2 mL/min

Flowrate : 1.0 mL/min (XR-ODS)
1.0 mL/min (VP-ODS)

Column Temp. : 40 ˚C

Injection Volume : 4 µL (XR-ODS)
10 µL (VP-ODS)

Detection : Photodiode Array
UV-VIS Absorbanc Detector at 260 nm

Flow Cell : Semi-micro Cell (XR-ODS)
Conventional Cell (VP-ODS)

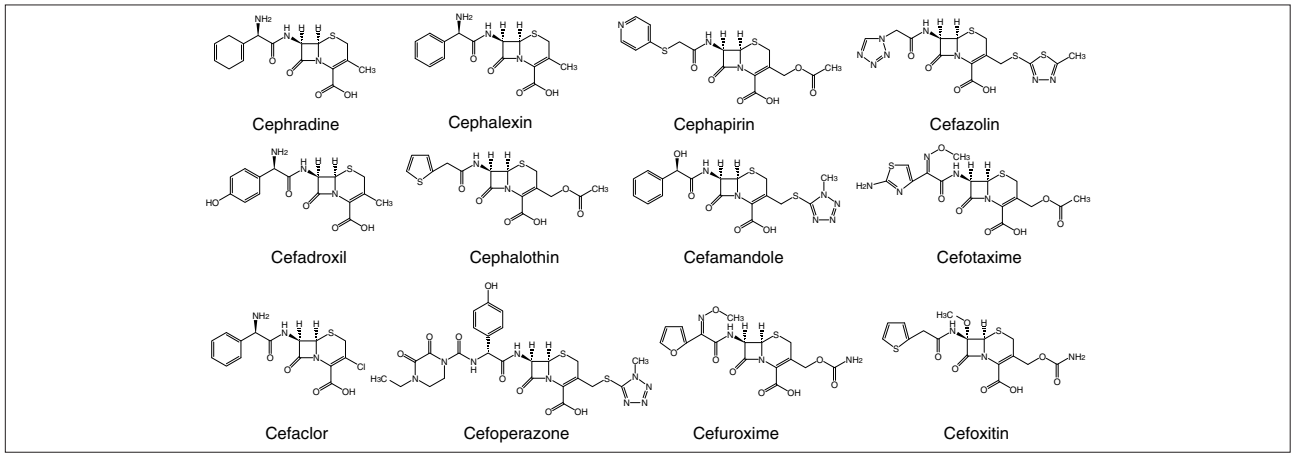


Fig. 4.5.2 Structural Formulas of 12 Cephem Antibiotic Compounds

4.6 Analysis of Penicillin Antibiotic - LC/MS

■Explanation

Analysis examples of representative synthetic-penicillin compounds are shown here. Ionization was performed using ESI. In the case of amoxicillin, ampicillin, and ticarcillin, positive ions to which protons had attached were detected, and in the case of flucloxacillin, negative ions from which protons had eliminated were detected. Solvent attached ions were also detected.

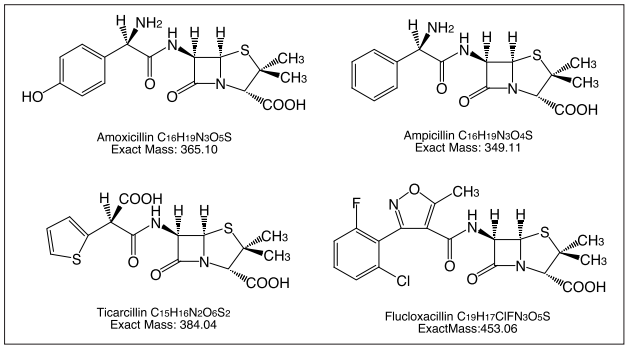


Fig. 4.6.1 Structure of Synthetic Penicillins

■Analytical Conditions

Column	: Shim-pack VP-ODS (150 mmL. × 2.0 mm I.D.)
Mobile Phase A	: 10 mmol/L Ammonium Acetate Buffer adjusted to pH 4.0 with Acetic Acid
Mobile Phase B	: Acetonitrile
Time Program	: B 5% (0-1min) → 80% (12-15 min) → 50 % (15.01-25 min)
Flowrate	: 0.2 mL/min
Injection Volume	: 50 µL
Column Temp.	: 40 ˚C
Probe Voltage	: (1) +4.5 kV (0- 10 min, ESI positive) → (2) -3.5 kV (10-20 min, ESI negative)
CDL Temp.	: 200 ˚C
BH Temp.	: 200 ˚C
Nebulizing Gas Flow	: 4.5 L/min
CDL Voltage	: (1) -20 V, (2) 20 V
Q-array DC	: Scan Mode
Q-array RF	: Scan Mode
Scan Range	: *m/z* 200-600

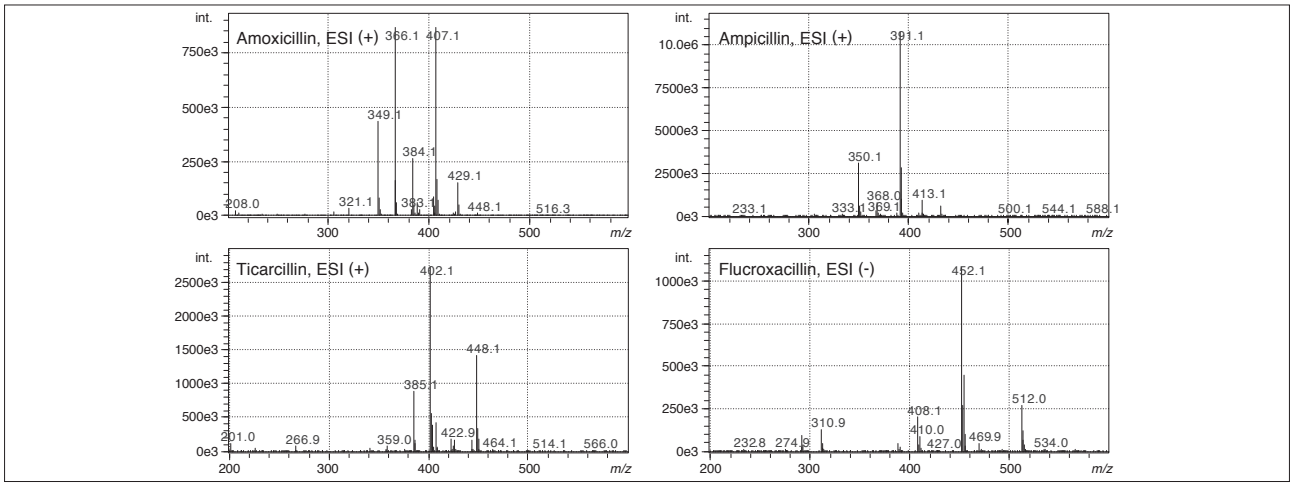


Fig. 4.6.2 ESI Mass Spectra for Synthetic Penicillins

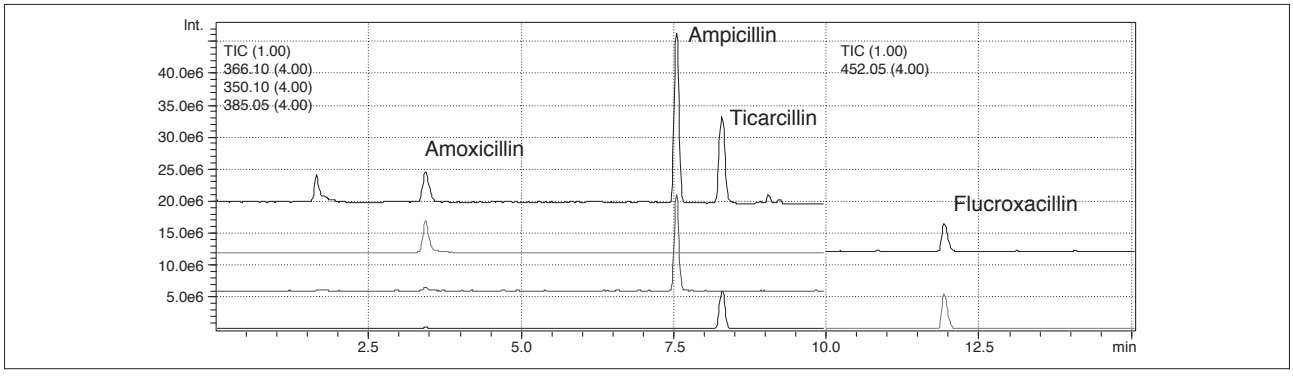


Fig. 4.6.3 SIM Chromatograms for Synthetic Penicillins

5. Crude Drugs

5.1 HPLC Analysis of a Crude Drug (1) - LC

■Explanation

Analysis of herbal medicines by HPLC generally requires separation of the impurities and active constituents, so the time to required to complete an analysis becomes relatively long. Here we introduce examples of high speed analysis of herbal medicines using the "Prominence UFLC" ultra-fast LC system with the "Shim-pack XR-ODS" high-speed, high-resolution column.

■Analysis of Sennosides in Senna

Sennosides, present in senna, display purgative action after decomposing in the intestine. For this reason, senna is used as a laxative. Fig. 5.1.1 shows an analysis example of sennosides A and B in senna. The sample preparation procedure[1] is shown in Fig. 5.1.2.

■Analytical Conditions

Column : Shim-pack XR-ODS
(75 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase : A : 100 mmol/L Sodium Acetate Buffer
(pH = 4.7)
B : Acetonitrile
Gradient Elution Method
Time Program: B 7 % (0 min) → 13 % (6 min) →
100 % (6.1-7.5 min) → 7 % (7.6-10 min)
Flowrate : 1.0 mL/min
Column Temp. : 40 ºC
Injection Volume : 2 µL
Detection : UV Absorbance Detector SPD-20A at 340 nm
Flow Cell : Semi-micro Cell

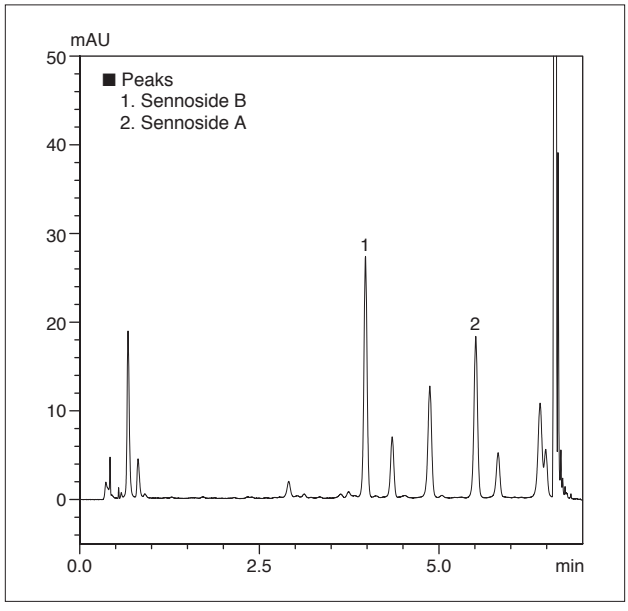


Fig. 5.1.1 Chromatogram of Senna

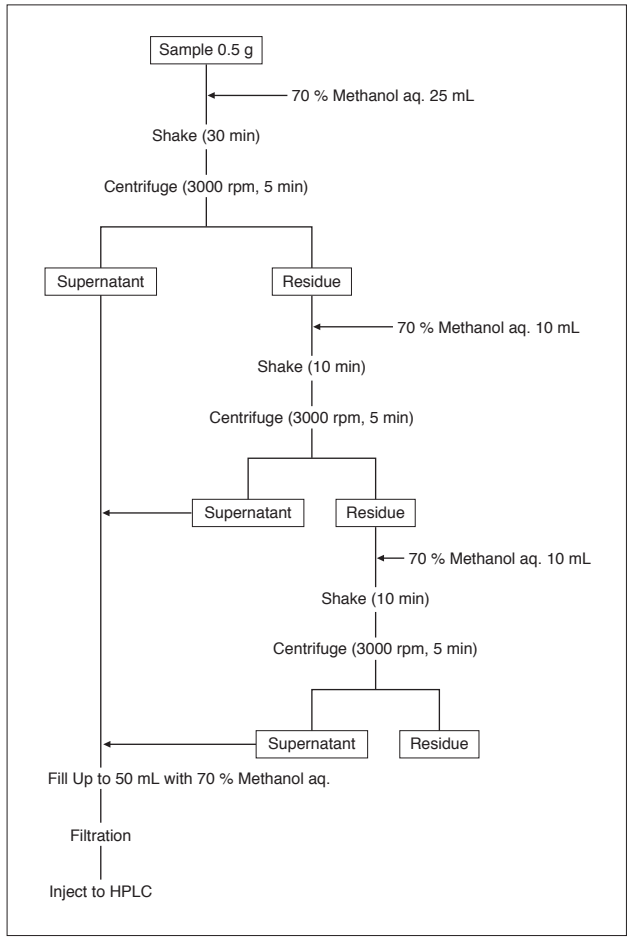


Fig. 5.1.2 Sample Preparation

[Reference]
1) Japan Pharmacopeia, 15th Revision Japan
(edited by Society of Japanese Pharmacopoeia)

5.1 HPLC Analysis of a Crude Drug (2) - LC

■Analysis of Curcumin in Turmeric

Curcumin, which is present in turmeric, is used not only as an artificial yellow coloring agent, but it is also effective for enhancing liver function and promoting bile secretion. Fig. 5.1.3 shows an analysis example of curcumin in turmeric. The sample preparation procedure[1] is shown in Fig. 5.1.4.

■Analytical Conditions

Column : Shim-pack XR-ODS (75 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase : A : 2 % Acetic Acid aq. Solution
B : Acetonitrile
A/B = 55/45 (v/v)
Flowrate : 1.0 mL/min
Column Temp. : 40 ºC
Injection Volume : 4 µL
Detection : UV-VIS Absorbance Detector SPD-20AV at 425 nm
Flow Cell : Semi-micro Cell

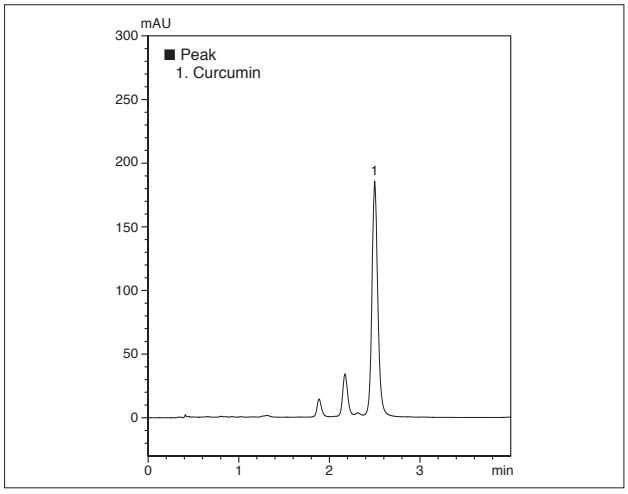


Fig. 5.1.3 Chromatogram of Turmeric

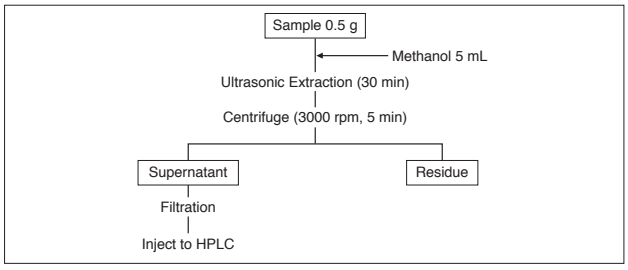


Fig. 5.1.4 Sample Preparation

■Analysis of Baicalin in Scutellaria Root

Baicalin, which is present in scutellaria root, is effective as a substance with anti-allergic action. Fig. 5.1.5 shows an analysis example of baicalin in scutellaria root. The sample preparation procedure is shown in Fig. 5.1.6.

■Analytical Conditions

Column : Shim-pack XR-ODS (75 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase : A : 10 mmol/L (Sodium) Phosphate Buffer (pH = 2.6)
B : Acetonitrile
A/B = 70/30 (v/v)
Flowrate : 1.0 mL/min
Column Temp. : 40 ºC
Injection Volume : 2 µL
Detection : UV Absorbance Detector SPD-20A at 280 nm
Flow Cell : Semi-micro Cell

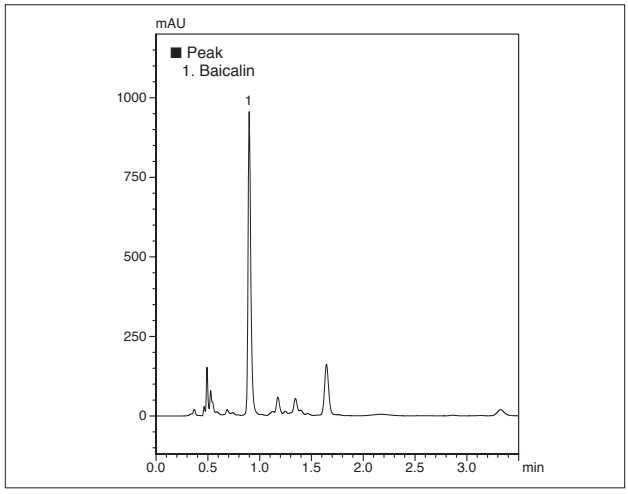


Fig. 5.1.5 Chromatogram of Scutellaria Root

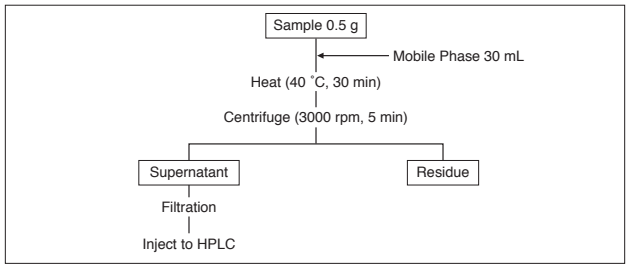


Fig. 5.1.6 Sample Preparation

[Reference]

1) Fukushima, Yazaki, Kase: Health Research Report of Chiba Prefectural Institute of Public Health, No. 20, 37-40 (1996)

5.1 HPLC Analysis of a Crude Drug (3) - LC

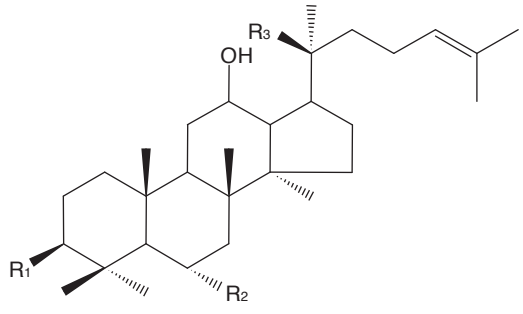
■Explanation

Ginseng is a widely used herbal medicine with a number of reported health benefits including stress reduction, building resistance to disease, and promoting concentration and memory function. Compounds called ginsenosides are believed to be the active constituents behind ginseng's efficacy.

Analysis of ginsenosides by HPLC has traditionally been a relatively time-consuming process due to the time required for separation of these structurally similar analytes as well as their separation from complex contaminants. Here we introduce an example of the analysis of ginsenosides in ginseng using the ultra fast LC system "Prominence UFLC" with the Phenomenex Synergi 2.5 µm Polar-RP high-speed, high-resolution column.

■Analysis of Standard Solution

The structural formulas of the 5 ginsenosides that are the subject of determination in this analysis are shown in Fig. 5.1.7. Here, separation of the ginsenosides Rg1 and Re in particular was conducted efficiently using the high-speed, high-resolution Phenomenex Synergi 2.5 µm Polar-RP (particle diameter 2.5 µm) column. Fig. 5.1.8 shows the results of analysis of a solution (60 % methanol aqueous solution) of the 5 ginsenosides, each present at 50 mg/L in the 2 µL sample.



Compound	R_1	R_2	R_3
Ginsenoside Rg1	OH	O-Glc	O-Glc
Ginsenoside Re	OH	O-Glc(2-1)Rha	O-Glc
Ginsenoside Rb1	O-Glc(2-1)Glc	H	O-Glc(6-1)Glc
Ginsenoside Rc	O-Glc(2-1)Glc	H	O-Glc(6-1)Araf
Ginsenoside Rd	O-Glc(2-1)Glc	H	O-Glc

Glc : β-D-glucose
Rha : α-L-rhamnose
Araf : α-L-arabinose (furanose)

Fig. 5.1.7 Structures of Ginsenosides

■Analytical Conditions

Column : Synergi 2.5 µm Polar-RP 100 Å (50 mmL. × 2.0 mm I.D., 2.5 µm)
Mobile Phase : A : Water
B : Acetonitrile
Gradient Elution Method
Time Program : B 15 % (0 min) → 20 % (3 min) → 25 % (4 min) → 30 % (8 min)
Flowrate : 0.6 mL/min
Column Temp. : 35 ºC
Injection Volume : 2 µL
Detection : UV Absorbance Detector SPD-20A at 203 nm
Flow Cell : Semi-micro Cell

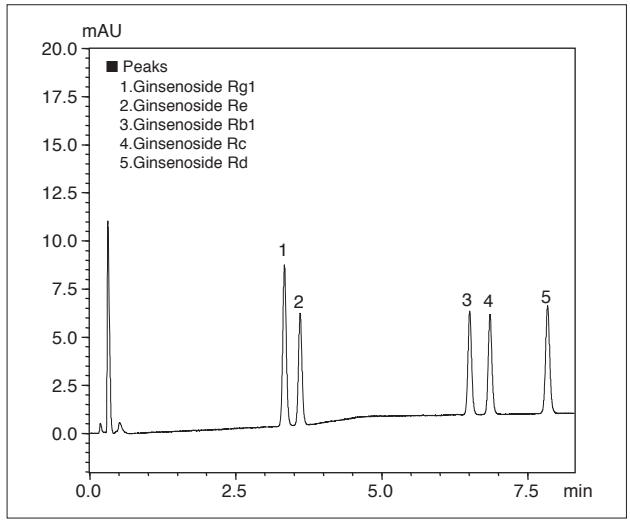


Fig. 5.1.8 Chromatogram of a Standard Mixture of 5 Ginsenosides (50 mg/L each)

5.1 HPLC Analysis of a Crude Drug (4) - LC

■Analysis of Powdered Ginseng

Fig. 5.1.9 shows the preparation procedure for herbal medicines as described in the Japanese Pharmacopeia. Fig. 5.1.10 shows the results of analysis of commercial ginseng powder, using a 2 µL injection of the sample prepared using the process shown in Fig. 5.1.9. Fig. 5.1.11 shows the procedure in which solid phase extraction (SPE) is incorporated in the sample preparation procedure of Fig. 5.1.9, using a reversed phase sorbent cartridge (Phenomenex "strata-X"), and the results of that analysis are shown in Fig. 5.1.12. Compared to the results of Fig. 5.1.10, it is clear that the high-polarity contaminants are effectively removed during the SPE step.

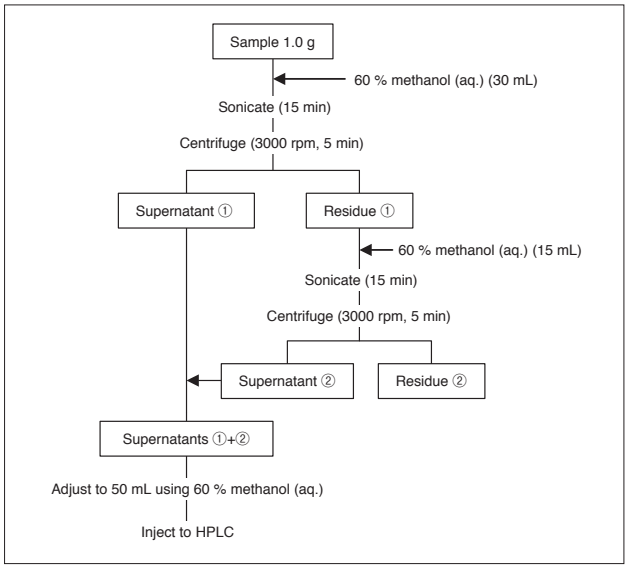


Fig. 5.1.9 Sample Preparation 1

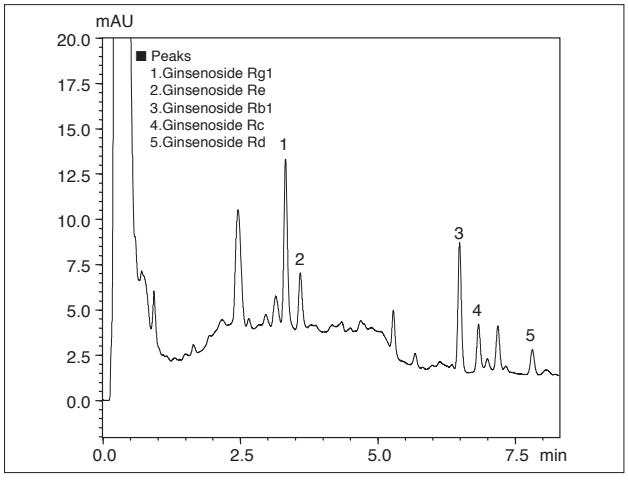


Fig. 5.1.10 Chromatogram of Powdered Ginseng (using Sample Preparation 1)

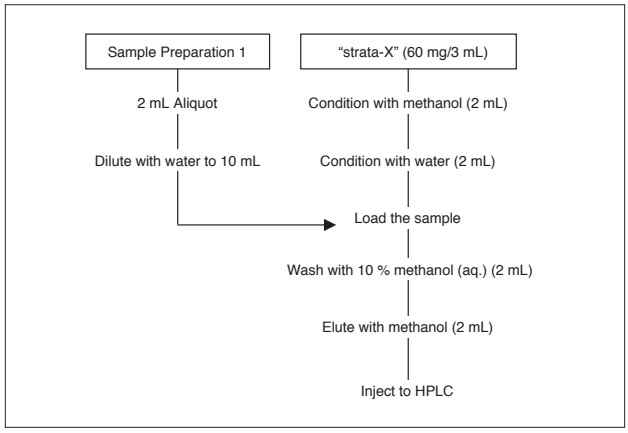


Fig. 5.1.11 Sample Preparation 2

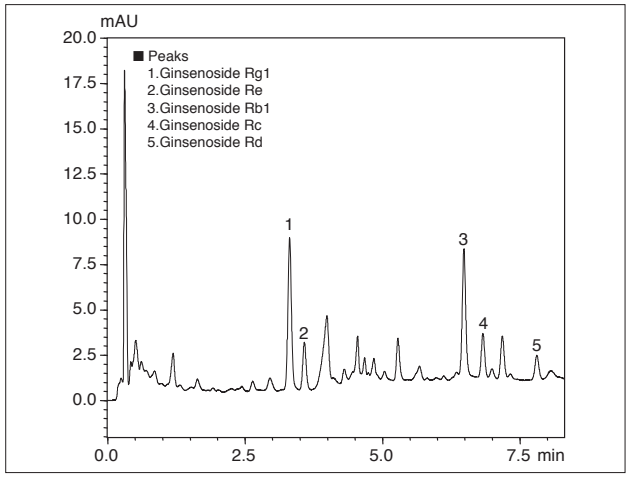


Fig. 5.1.12 Chromatogram of Powdered Ginseng (using Sample Preparation 2)

[Reference]

The 15th Revision of the Japanese Pharmacopeia (Society of Japanese Pharmacopeia)

5.1 HPLC Analysis of a Crude Drug (5) - LC

■Explanation

Peony root is an herbal medicine that is often used in Chinese herbal preparations as paeoniae radix for its anti-inflammatory, analgesic, antibacterial, hemostatic, and anticonvulsant activity. It is often mixed with other herbal medicines in traditional Chinese herbal remedies. Here we focus on paeoniflorin as one of the principle active substances in peony root, and introduce an example of analysis of paeoniflorin using the ultra high-speed LC system "Prominence UFLC" with the SPD-M20A photodiode array detector.

■Analysis of Standard Solution

Fig. 5.1.13 shows the structural formulas of paeoniflorin and albiflorin, also a constituent of peony root. The Japanese Pharmacopeia specifies that albiflorin be included with paeoniflorin in the resolution solution to verify that separation of the principal substance is adequate. Here, the retention time of paeoniflorin was observed at about 2 minutes. Fig. 5.1.14 shows the chromatogram of result. In addition, Fig. 5.1.15 shows the overlaid UV spectra of the 2 constituents, obtained using the photodiode array detector. Both show similar spectral patterns with absorbance maxima in the vicinity of 232 nm and 274 nm.

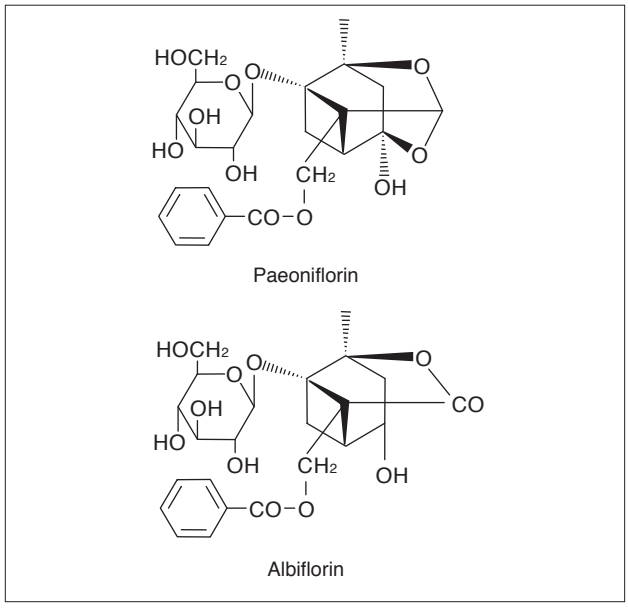


Fig. 5.1.13 Structures of Paeoniflorin and Albiflorin

■Analytical Conditions

Column	: Shim-pack XR-ODS (75 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase	: Water/Acetonitrile/Phosphoric Acid = 850/150/1 (v/v/v)
Flowrate	: 0.9 mL/min
Column Temp.	: 35 ºC
Injection Volume	: 4 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 232 nm
Flow Cell	: Semi-micro Cell

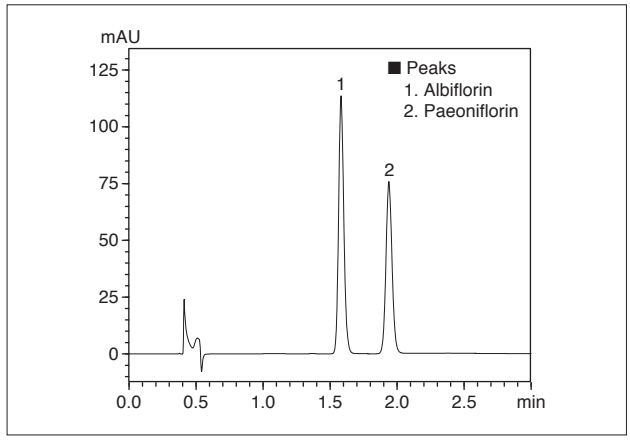


Fig. 5.1.14 Chromatogram of a Standard Mixture of Paeoniflorin and Albiflorin (100 mg/L each, 4 µL injected)

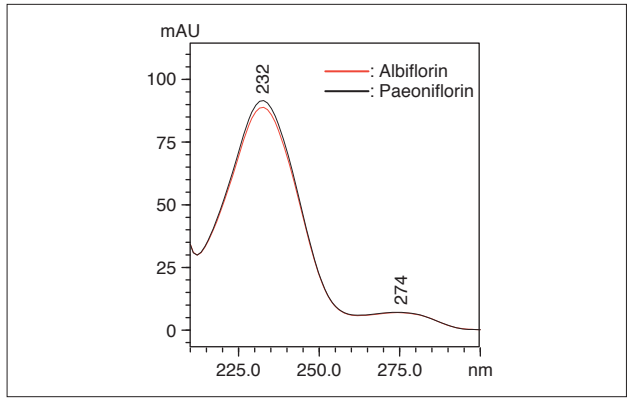


Fig. 5.1.15 UV Spectra of Paeoniflorin and Albiflorin

5.1 HPLC Analysis of a Crude Drug (6) - LC

■Analysis of Powdered Peony Root

The powdered peony root was prepared as indicated in Fig. 5.1.16, and then analyzed. Fig. 5.1.17 shows the chromatogram of result. Here, the retention time axis only shows as far as 3 minutes, but since there are some late-eluting substances, the actual analysis was conducted using a 10-minute cycle.

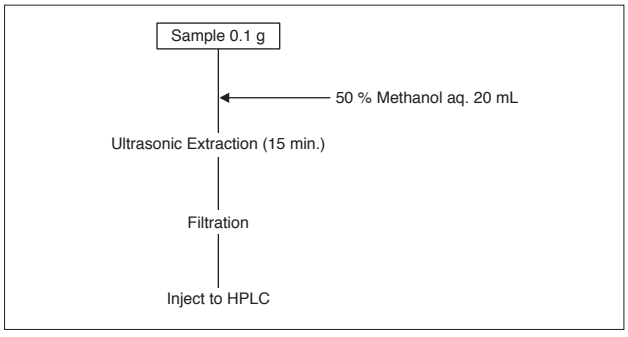


Fig. 5.1.16 Sample Preparation

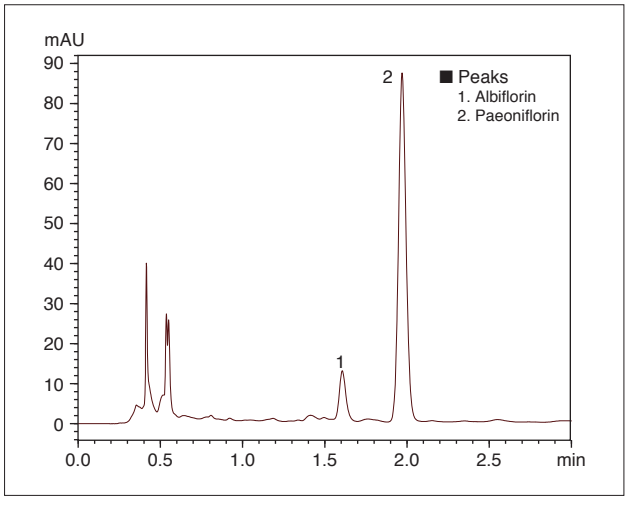


Fig. 5.1.17 Chromatogram of Powdered Peony Root

The paeoniflorin spectral pattern at the peak apex nearly perfectly matches that of the standard, with a greater than 0.99999 degree of similarity. In addition, when overlaying data at multiple wavelengths, and extracting spectra at different times from the paeoniflorin peak for a 3-point purity determination, nearly the same pattern was obtained, as shown in Fig. 5.1.18. Fig. 5.1.19 shows a contour plot of the powdered peony root analysis results.

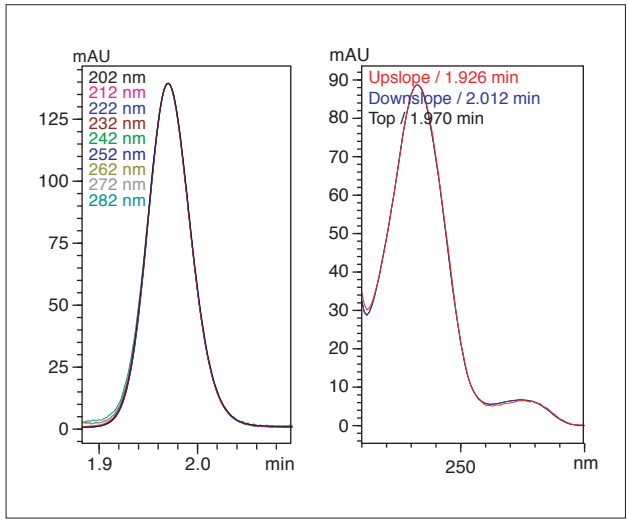


Fig. 5.1.18 Peak Profile and 3 point-Spectra of Paeoniflorin

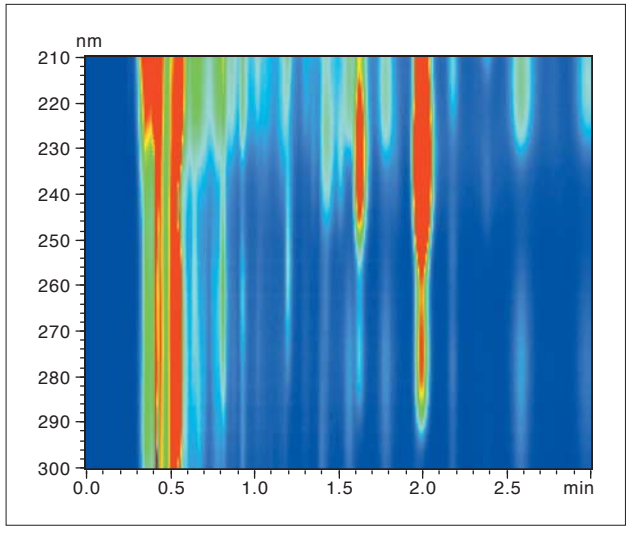


Fig. 5.1.19 Contour Plot

[Reference]
The 15th Revision of the Japanese Pharmacopeia
(Society of Japanese Pharmacopeia)

5.1 HPLC Analysis of a Crude Drug (7) - LC

■High-Speed Analysis of Berberine and Palmatine

This is an example of high-speed analysis of berberine, one of the alkaloids contained in cork tree bark. The ultra fast separation for berberine and palmatine was performed using a UFLCXR system. The two components were detected using a UV detector with a semi-micro flow cell. 20 mL of extraction solution* was added to 0.1 g of cork tree bark powder to extract the palmatine and berberine. The supernatant was filtered using a 0.45 µm membrane filter.

* Extraction solution: Methanol / 10 mM HCl (1/1, v/v)

■Analytical Conditions

Instrument	: Prominence UFLCXR System
Column	: Shim-pack XR-ODS (75 mmL. × 3.0 mm I.D.)
Mobile Phase	: Water/Acetonitrile = 1/1 (v/v) containing SDS (1.7 g/L) and Potassium Dihydrogenphosphate (3.4 g/L)
Flowrate	: 1.2 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 4 µL
Detection	: UV Absorbance Detector at 345 nm
Flow Cell	: Semi-micro Cell

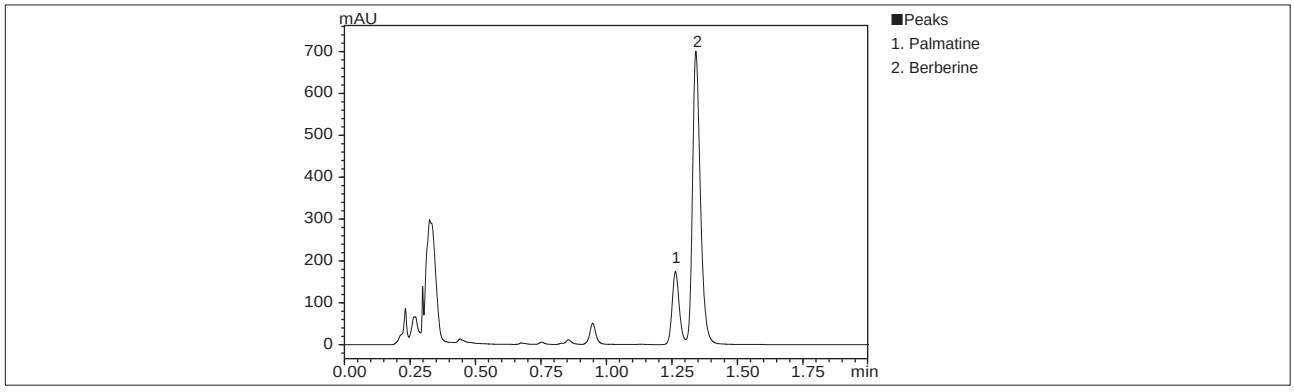


Fig. 5.1.20 Analysis of Berberine and Palmatine in Cork Tree Bark Powder

■High-Speed Analysis of Swertiamarin

Here we conducted high-speed analysis of Swertiamarin, a bitter ingredient of the natural medicine Japanese green gentian (Swertia japonica). 10 mL of water was added to 0.1 g of sample, and after extraction, the supernatant fluid was filtered through a 45 µm filter.

■Analytical Conditions

Instrument	: Prominence UFLC System
Column	: Shim-pack XR-ODS (75 mmL. × 3.0 mm I.D.)
Mobile Phase	: Water/Acetonitrile = 91/9 (v/v)
Flowrate	: 1.2 mL/min
Column Temp.	: 50 ºC
Injection Volume	: 1 µL
Detection	: UV Absorbance Detector at 238 nm
Flow Cell	: Semi-micro Cell

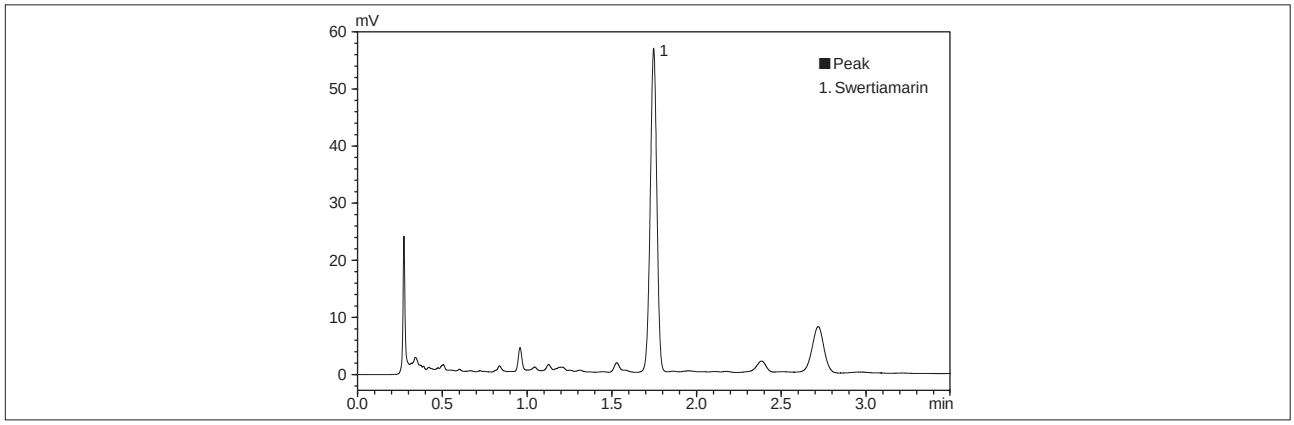


Fig. 5.1.21 Analysis of Swertianmarin in Japanese Green Gentian

5.1 HPLC Analysis of a Crude Drug (8) - LC

■High-Speed Analysis of Geniposide

Here we conducted high-speed analysis of geniposide, one of the iridoid glycosides contained in the natural medicine gardenia (gardenia fruit). To produce a mixture of 10 g/L, finely powdered tablet was mixed with 50 % methanol solution, and then filtered through a 0.45 µm membrane filter.

■Analytical Conditions

Instrument	: Prominence UFLC System
Column	: Shim-pack XR-ODS (50 mmL. × 3.0 mm I.D.)
Mobile Phase	: Water/Acetonitrile/Phosphoric Acid = 900/100/1 (v/v/v)
Flowrate	: 1.2 mL/min
Column Temp.	: 35 ºC
Injection Volume	: 4 µL
Detection	: UV Absorbance Detector at 240 nm
Flow Cell	: Semi-micro Cell

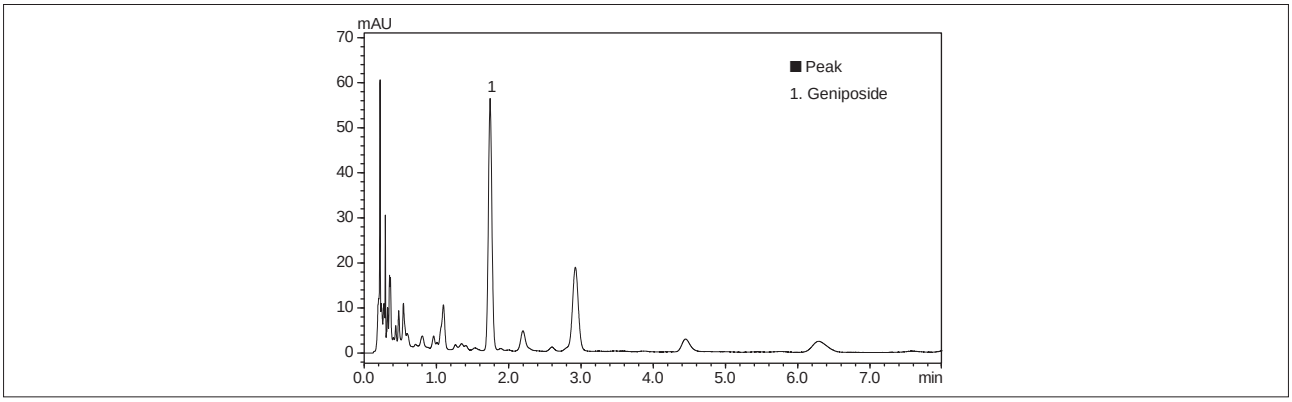


Fig. 5.1.22 Analysis of Geniposide Found in Traditional Chinese Medicine

■High Speed Analysis of Gentiopicroside

An internal stomachic mixed with gentian extract was analyzed by the Prominence UFLCXR system.

Some research has indicated that a bitter glycoside, gentiopicroside shows a stimulatory effect on taste buds to promote a reflex increase in the secretion of gastric juice. Sample was filtered through a 0.20 µm membrane.

* After 4 minutes elution, the column was washed to elute strongly retained matrices. The total cycle time including washing and equilibrating was 8 minutes.

■Analytical Conditions

Instrument	: Prominence UFLCXR system
Column	: Shim-pack XR-ODS Ⅱ (100 mmL. × 3.0 mm I.D.)
Mobile Phase	: 0.05 % Phosphoric Acid/Methanol = 4/1 (v/v)
Flowrate	: 1.1 mL/min
Column Temp.	: 50 ºC
Injection Volume	: 4 µL
Detection	: UV Absorbance Detector at 270 nm
Flow Cell	: Semi-micro Cell

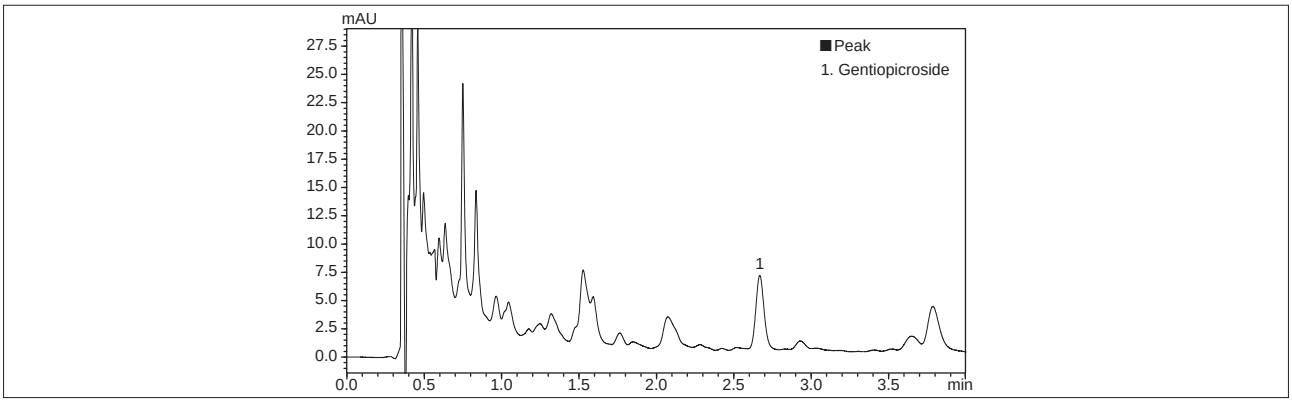


Fig. 5.1.23 Analysis of an Internal Stomachic Mixed with Gentian Extract

5.2 High Speed Analysis of Berberine and Glycyrrhizin - LC/MS

■High-Speed Analysis of Berberine

Here we used an LC/MS to conduct an analysis of berberine, an alkaloid that exists in the bark of the cork tree. Selective detection capability of the mass spectrometer is efficient in the separation of berberine and palmatine, enabling the analysis to be conducted in a short period of time. After adding 1 mL of methanol to 1 mg of finely powdered cork bark and centrifuge, the resulting supernatant fluid was filtered through a 0.45 µm membrane filter.

■Analytical Conditions

Instruments : Prominence UFLC System+LCMS-2010EV
Column : Shim-pack XR-ODS
(75 mmL. × 2.0 mm I.D.)
Mobile Phase : A : 0.1 % Formic Acid/Tetrahydrofuran
= 95/5 (v/v)
B : Acetonitrile
Gradient Elution Method
B 15 % (0 min) → 25 % (2 min)
Flowrate : 0.5 mL/min
Column Temp. : 40 ºC
Injection Volume : 0.5 µL
Detection : MS: ESI-Positive, Scan

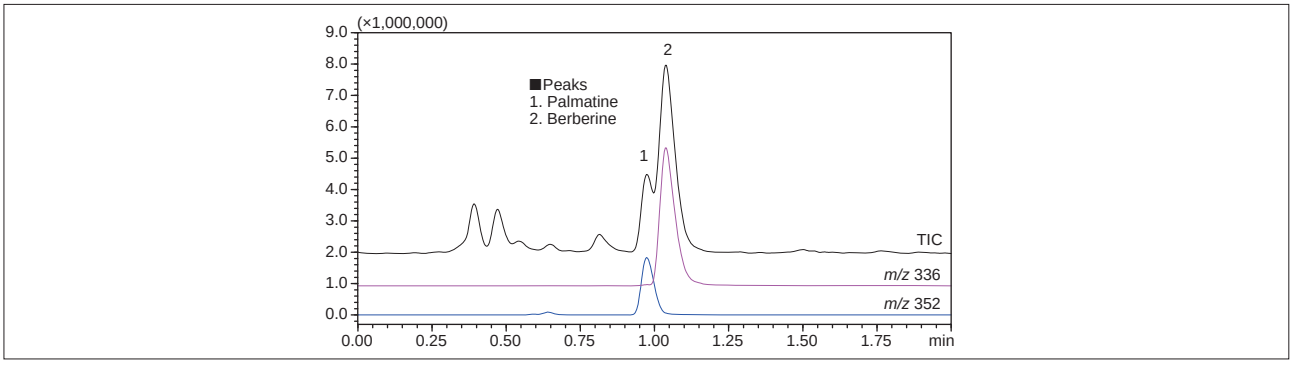


Fig. 5.2.1 Analysis of Berberine in Cork Tree Bark

■High-Speed Analysis of Glycyrrhizin

Here we used an LC/MS to conduct analysis of glycyrrhizin, one of the triterpene glycosides that exists in licorice. Negative ions were detected.
10 mL water was added to 100 mg licorice powder and left for 20 minutes at 60 ºC. After centrifuging, the resulting supernatant was filtered through a 0.45 µm membrane filter.

■Analytical Conditions

Instruments : Prominence UFLC System+LCMS-2010EV
Column : Shim-pack XR-ODS
(75 mmL. × 2.0 mm I.D.)
Mobile Phase : A : 50 mmol/L Ammonium Acetate (pH 4.7)
B : Acetonitrile
Gradient Elution Method
B 25 % (0min) → 35 % (4 min)
Flowrate : 0.6 mL/min
Column Temp. : 40 ºC
Injection Volume : 2 µL
Detection : MS: ESI-Negative, Scan

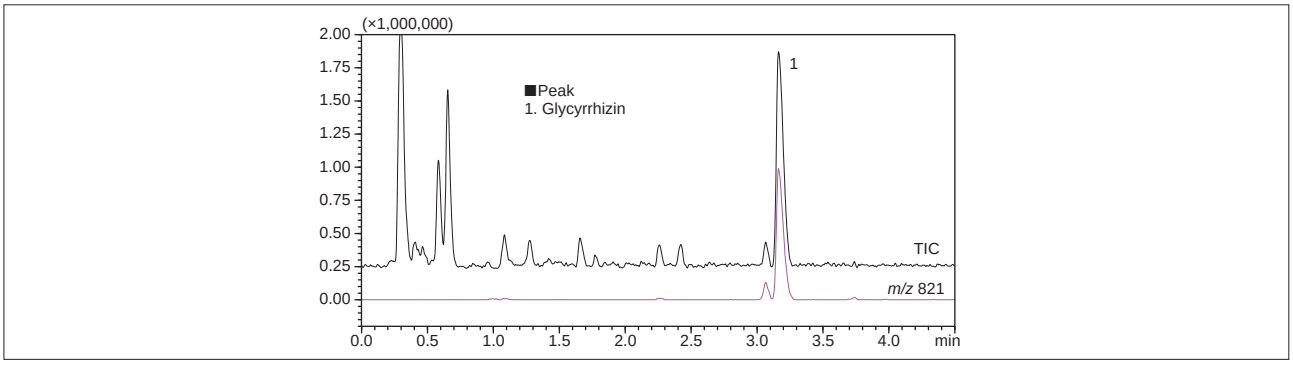


Fig. 5.2.2 Analysis of Glycyrrhizin in Licorice

5.3 Analysis of Herbal Medicines by ICP (1) - ICP

■Explanation

The use of herbal medicines as an alternative to traditional western medicine has been increasing, and consequently this trend has been accompanied by heightened interest and concern regarding the quality and safety of herbal medicines. Herbal medicines—medicinal products that naturally contain a variety of flora and fauna, bacteria, minerals, etc—are currently utilized as medications (over-the-counter drugs), foods, functional foods, and dietary supplements without purification of their active ingredients. Herbal medicines that are used as pharmaceuticals are subject to standards associated with their behavior, chemical properties, and safety as specified in official regulations, including the Japanese Pharmacopoeia. Safety is assessed using limit testing, including heavy metals testing and arsenic limit testing. However, since the biological effects of heavy metals vary depending on the metal, testing of metal content is required for each metal. Here, we present the results of our analysis of herbal medicines using the Shimadzu ICPE-9000 multi-type ICP emission spectrometer.

■Samples

The samples consisted of herbal medicines that are readily available in Japan.

■Sample Preparation

As acidification and heating will cause volatilization of low-boiling-point elements such as arsenic (As) and mercury (Hg), a pretreatment method that permits efficient dissolution of the sample with minimal loss of these elements is required. Here, we employed microwave assisted acid Closed Vessel digestion method for pretreatment. For the digestion, 7.5 mL of concentrated nitric acid and 0.5 mL of concentrated hydrochloric acid were added to 0.5 g of dried sample, which was then transferred to a microwave digestion system (ETHOS One microwave sample preparation system, from Milestone General K.K.).

Following the digestion, ultrapure water was added to the processed solution to bring the volume to 25 mL, and this was used as the analytical sample. Separately, a sample was prepared consisting of standard solution spiked with the analyte elements for use in spike and recovery testing.

■Analytical Instrument

Herbal medicines are said to number in the hundreds, and elemental analysis of these medicines must be conducted accurately and efficiently. The ICPE-9000 is a high-throughput, high-performance instrument that features ease of use, low running cost, high sensitivity, and low contamination.

■Analytical Coditions

Instrument	: ICPE-9000
RF Power	: 1.2 kw
Plasma Gas	: 10 L/min
Auxiliary Gas	: 0.6 L/min
Carrier Gas	: 0.7 L/min
Sample Introduction	: Coaxial Nebulizer
Misting Chamber	: Cyclone Chamber
Plasma Torch	: Mini Torch
View Method	: Axial Obsevation

■Analysis

Quantitation was conducted by the calibration curve method using the ICPE-9000. Elemental analysis was conducted for arsenic, cadmium, chromium, copper, mercury, lead, and tin; all elements that are considered to have relatively high oral toxicity.

■Results

Table 5.3.1 shows the semi-quantitation results (wt-%) for the principal components determined in qualitative analysis. The ICPE-9000 acquires and saves the qualitative data for all elements at the time of quantitative analysis. The semi-quantitative concentrations are calculated automatically from the values stored in the software-integrated database.

Herbal medicines often contain large amounts of calcium, potassium, and magnesium as coexisting substances. It is not uncommon for errors to occur in analytical values due to ionization interference and other factors when samples contain large amounts of coexisting components. In this regard, and compared to the typical torch, the high-temperature plasma achieved with the mini-torch adopted in the ICPE-9000 permits high sensitivity while suppressing the adverse effects of ionization interference. Table 5.3.2 shows the quantitation results, recovery rates, and detection limits. Excellent recovery results were achieved for all elements, and measurement was conducted without any interference from the principal components. Moreover, measurement was clearly conducted with sufficient sensitivity, and all the detection limits were below the specified Japanese Pharmacopoeia limit values, as well as the Chinese import/export standard values. The measurement cycle is very fast, taking only about two and a half minutes per sample, including the time required for sample introduction and rinse. Furthermore, use of the autosampler permits automated measurement of multiple samples, permitting even greater efficiency.

5.3 Analysis of Herbal Medicines by ICP (2) - ICP

Table 5.3.1 Semi-Quantitative Results for Herbal Medicines by Qualitative Analysis

	Ca	K	Mg	S	Al	P	Si	Fe	Mn	Ba	Sr	Na
Horny goat weed	2.6	1.0	0.4	0.3	0.2	0.2	0.14	0.13	0.05	0.02	0.01	0.01
Fang feng (Saposhnikoviae Radix)	1.2	0.5	0.4	0.2	0.2	0.2	0.08	0.10	0.004	0.01	0.02	0.08

Table 5.3.2 Quantitative Results for Herbal Medicines (μg/g)

	Element Name	As	Cd	Cr	Cu	Hg	Pb	Sn
	*Detection Limit	0.2	0.005	0.02	0.04	0.1	0.1	0.05
	Sample Name							
1.	Cardamom	<	0.07	<	5.5	<	0.2	<
2.	Cinnamon	0.3	<	0.5	7.0	<	0.5	<
3.	Horny goat weed	0.5	0.14	3.0	4.7	<	1.4	0.1
	Recovery Rate in Spike/Recovery Test (%)	101.1	98.5	98.8	95.2	97.9	95.2	100.3
4.	Carrot	<	0.03	0.04	5.2	<	<	<
5.	Rehmanniae radix	<	<	0.3	3.9	<	0.2	<
6.	Paeoniae radix	<	<	0.2	4.3	<	<	0.1
7.	Fang feng (Saposhnikoviae Radix)	<	<	0.4	7.0	<	0.2	<
	Recovery Rate in Spike/Recovery Test (%)	100.9	101.7	98.7	100.1	97.4	97.6	99.6
8.	Turmeric (Curcumae Radix)	<	0.05	0.1	2.2	<	4.5	<
	Japanese Pharmacopoeia Limit Value (varies with sample)	2 – 5	10 – 15 (heavy metal test: lead conversion)					
	WHO Recommended Level		0.3				10	
	People's Republic of China Herb Import/Export Standard Values	2	0.3		20	0.2	5	

* Detection limit : Detection limit (3σ) determined from standard deviation using N = 5 repeat measurements of calibration curve blank
* < : Below the limit of detection

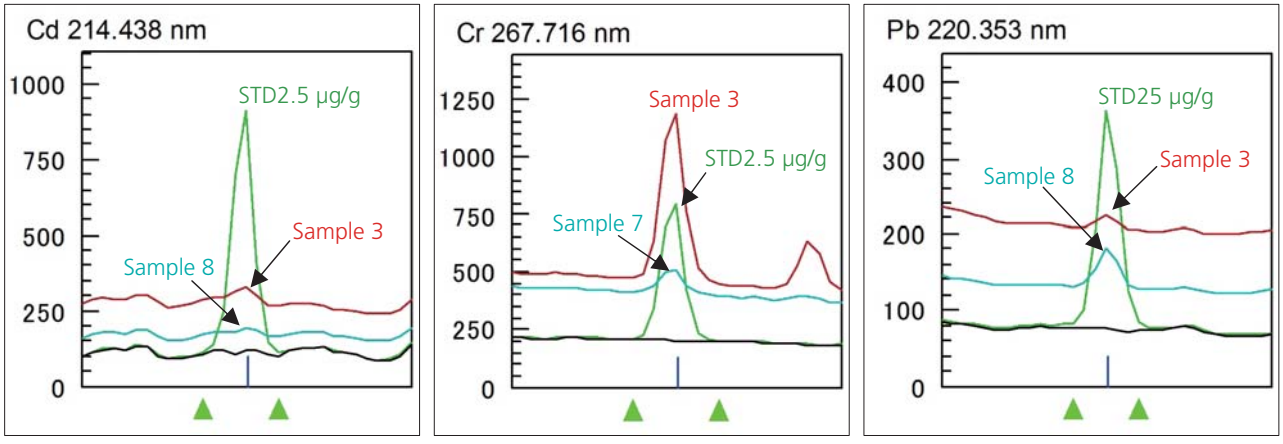


Fig. 5.3.1 Spectral Profiles of Cd, Cr, and Pb in Herbal Substances

* The concentrations in the figures refer to the concentrations in the samples (solid).

[Reference]

1) Sixteenth Edition of Japanese Pharmacopoeia (edited by Society of Japanese Pharmacopoeia)
2) WHO Guidelines for Assessing Quality of Herbal Medicines with Reference to Contaminants and Residues (issued March 2009 by Japan Self-Medication Industry)
3) Green Trade Standards of Importing & Exporting Medicinal Plants & Preparations (issued April 2001 and enacted July 1, 2001 by Ministry of Foreign Trade and Economic Cooperation, the People's Republic of China)

6. Clinical and Forensic Medicine

6.1 Analysis of Anti-Epilepsy Drug - GC

■Explanation

This data introduces direct analysis of an anti-epilepsy drug without derivatization.

■Pretreatment

1 g of anti-epilepy drug is dissolved in 10 mL of methanol, and 1 µL of this solution is injected.

■Analytical Conditions

Column	: DB-1 (30 m × 0.25 mm I.D. df = 0.25 µm)
Column Temp.	: 230 °C
Injector Temp.	: 300 °C
Detector Temp.	: 300 °C (FID)
Carrier Gas	: He 0.5 mL/min
Injection Method	: Split Injection
Split Ratio	: 1 : 100

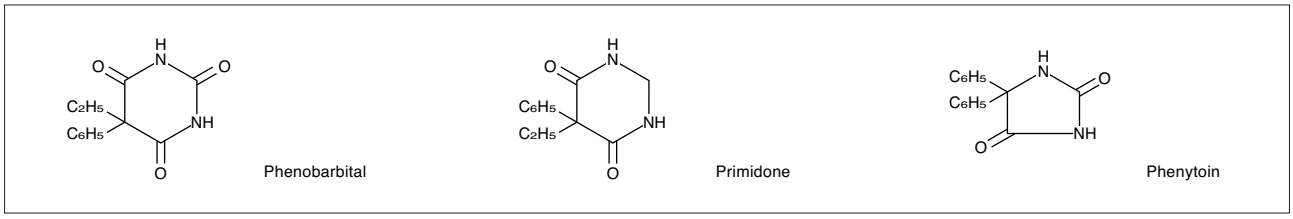


Fig. 6.1.1 Structural Formula of Elements in Anti-Epilepsy Drug

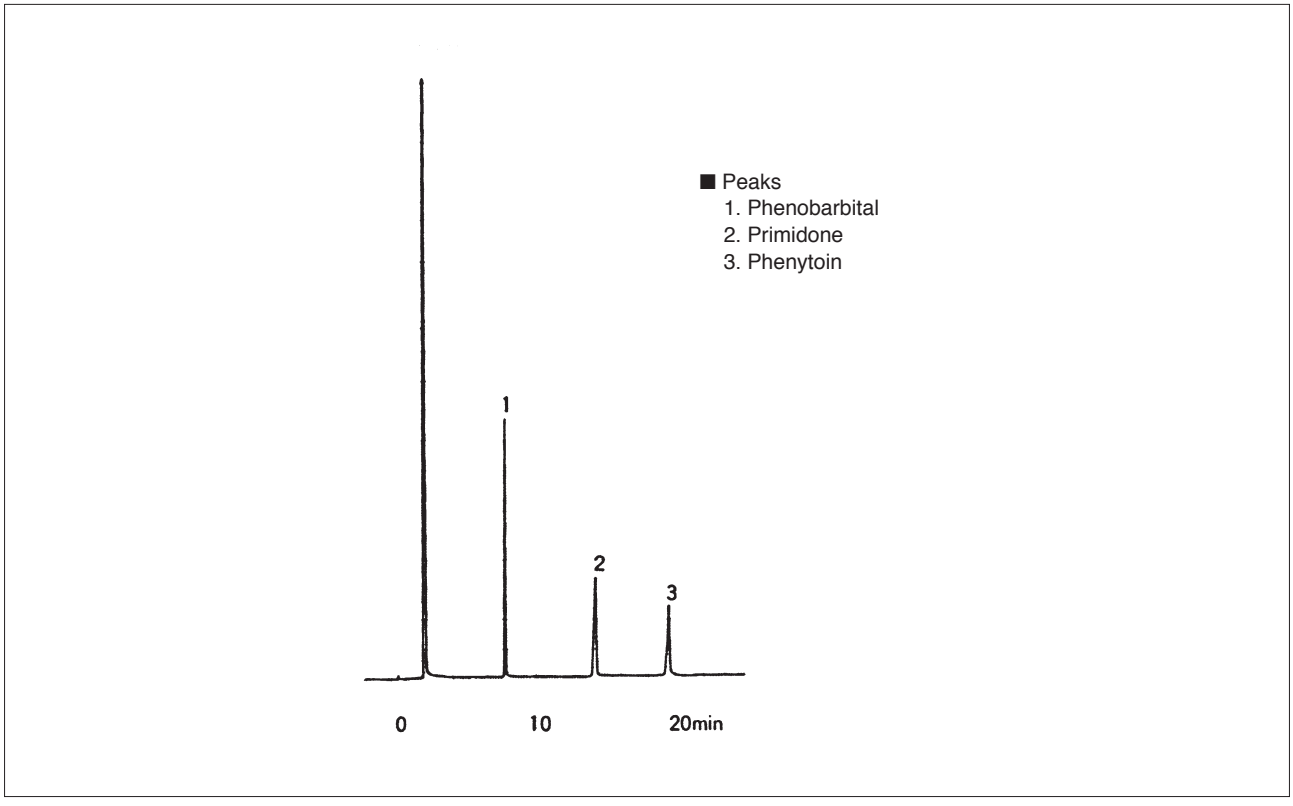


Fig. 6.1.2 Chromatogram of Anti-Epilepsy Drug

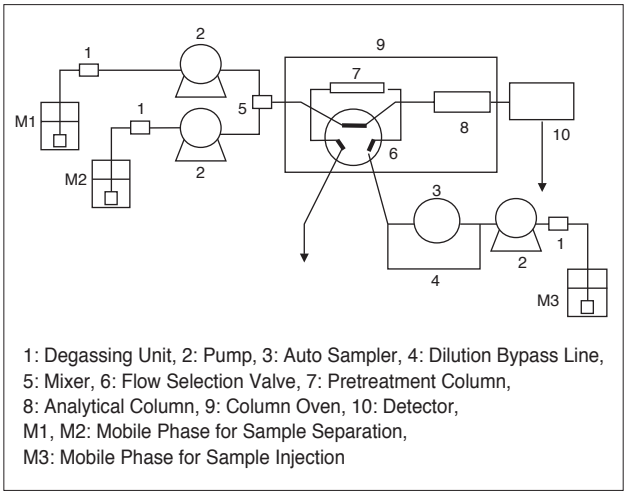
6.2 Analysis of Drugs in Blood Plasma Using "Co-Sense for BA" Bio-Sample Analysis System (1) - LC

■Explanation

When analyzing drugs contained in bio-samples such as blood plasma and serum using HPLC, unwanted substances such as proteins must be removed at the pretreatment stage. In general, protein removal is performed using centrifugal separation after the addition of an organic solvent or acid. Because this operation is rather complicated and is performed by hand, there is a risk of adversely affecting analysis accuracy. A technique that enables the automation of this operation is therefore desirable. The "Co-Sense for BA" bio-sample analysis system incorporates column-switching technology that uses a newly developed pretreatment column and an original online dilution bypass. In addition to automating pretreatment operations such as protein removal, it is a system that is designed to increase analysis accuracy.

■Operating Principle of "Co-Sense for BA"

Through the incorporation of a newly developed pretreatment column, "Shim-pack MAYI-ODS", and a dilution bypass, the "Co-Sense for BA" system enables the automation of the protein-removal operation performed, for example, before analyzing drugs in blood plasma. Fig. 6.2.1 shows the flow lines in "Co-Sense for BA". "Shim-pack MAYI-ODS" is an internal-surface reversed-phase column that is coated with a hydrophilic polymer. The structure is shown in Fig. 6.2.2. Large molecules, such as proteins, are blocked by the hydrophilic polymer, and cannot enter the pores. On the other hand, small molecules, such as drugs, can enter the pores and are consequently retained in the stationary phase. This makes it possible to selectively remove substances composed of large molecules, such as proteins, from the system.



1: Degassing Unit, 2: Pump, 3: Auto Sampler, 4: Dilution Bypass Line,
5: Mixer, 6: Flow Selection Valve, 7: Pretreatment Column,
8: Analytical Column, 9: Column Oven, 10: Detector,
M1, M2: Mobile Phase for Sample Separation,
M3: Mobile Phase for Sample Injection

Fig. 6.2.1 Flow-Line Diagram

■Analytical Procedure

The bio-sample (e.g. previously filtered blood plasma) is injected from an autosampler (3*) and, through the sample-introduction liquid (M3), it is conveyed to the Shim-pack MAYI-ODS pretreatment column (7). In order to increase the rate of recovery of the drug from proteins, a dilution bypass line (4) is incorporated in the autosampler section. Next, by turning the high-pressure flow-selection valve 60°, the drug trapped in Shim-pack MAYI-ODS is conveyed by the mobile phases for separation (M1 and M2) to the analysis column, where it is separated.

* These numbers correspond to those indicated in Fig. 6.2.1.

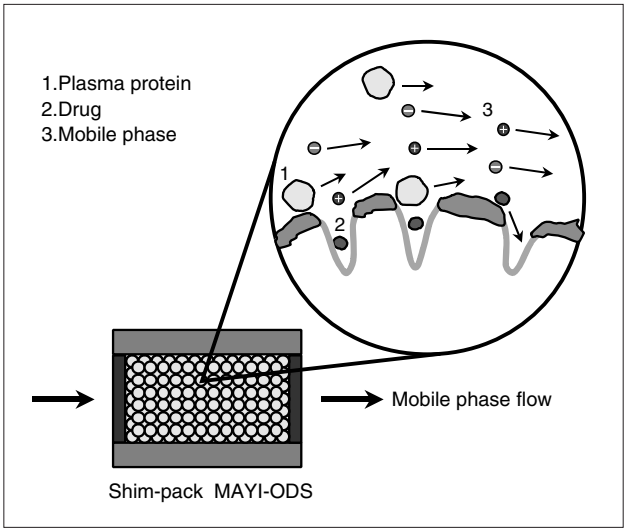


Fig. 6.2.2 Principle of Protein Removal

6.2 Analysis of Drugs in Blood Plasma Using "Co-Sense for BA" Bio-Sample Analysis System (2) - LC

■Analysis of Warfarin in Blood Plasma

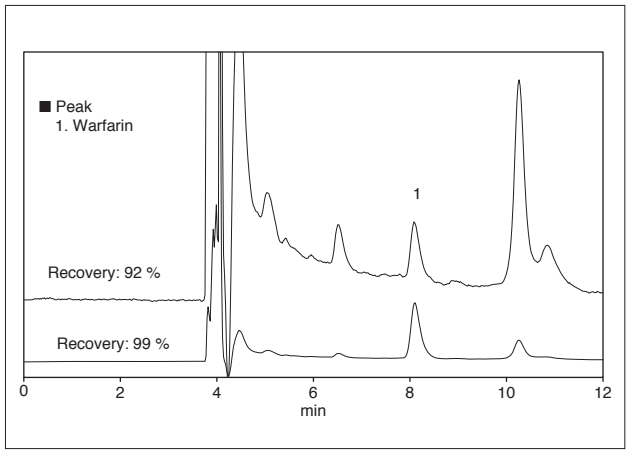


Fig. 6.2.3 Analysis of Warfarin in Blood Plasma
(Top: 0.1 mg/L added; Bottom: 1 mg/L added;
50 µL injected for both)

■Analytical Conditions

For Sample Injection

Column : Shim-pack MAYI-ODS
(10 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 100 mmol/L Acetate (Na) Buffer
(pH = 4.7)
B : Acetonitrile
A/B = 95/5 (v/v)

Flowrate : 2.0 mL/min

Dilution Factor : 8

For Separation

Column : Shim-pack FC-ODS
(75 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 20 mmol/L Phosphate (Na) Buffer
(pH = 2.5)
B : Methanol
A/B = 40/60 (v/v)

Flowrate : 1.0 mL/min

Column Temp. : 40 ˚C

Detection : Photodiode Array UV-VIS Absorbance Detector SPD- M10A*VP* at 315 nm

■Analysis of Naproxen in Blood Plasma

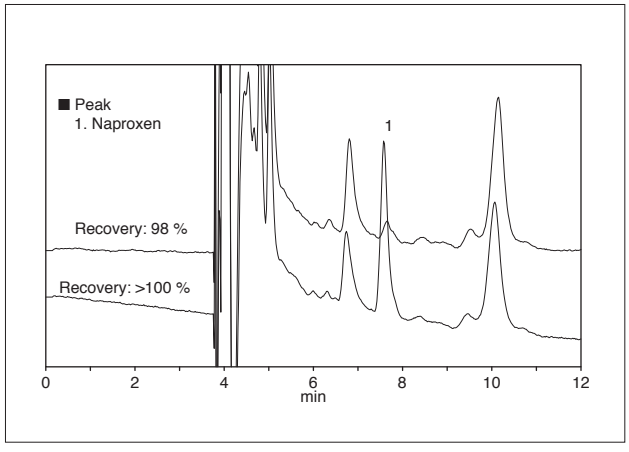


Fig. 6.2.4 Analysis of Naproxen in Blood Plasma
(Top: 0.1 mg/L added; Bottom: 1 mg/L added;
50 µL injected for both)

■Analytical Conditions

For Sample Injection

Column : Shim-pack MAYI-ODS
(10 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 0.1 % Phosphoric Acid
B : Acetonitrile
A/B = 95/5 (v/v)

Flowrate : 2.0 mL/min

Dilution Factor : 8

For Separation

Column : Shim-pack FC-ODS
(75 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 20 mmol/L Phosphate (Na) Buffer
(pH = 2.5)
100 mmol/L Sodium Perchlorate
B : Methanol
A/B = 40/60 (v/v)

Flowrate : 1.0 mL/min

Column Temp. : 40 ˚C

Detection : Photodiode Array UV-VIS Absorbance Detector SPD- M10A*VP* at 330 nm

6.2 Analysis of Drugs in Blood Plasma Using "Co-Sense for BA" Bio-Sample Analysis System (3) - LC

■Analysis of Six Acidic Drugs in Blood Plasma

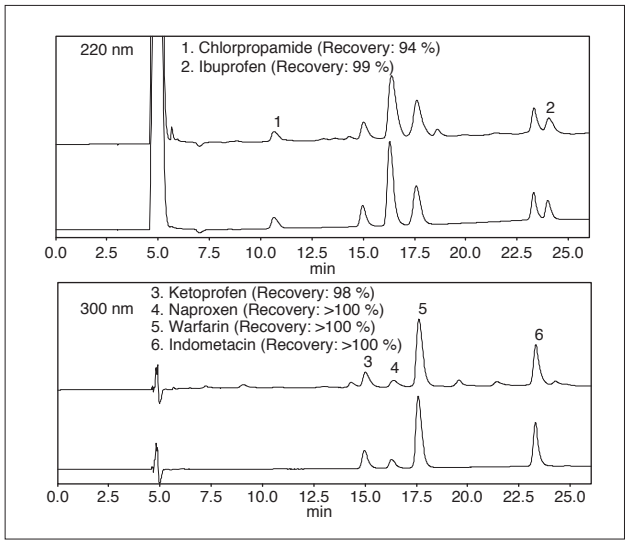


Fig. 6.2.5 Analysis of Six Acidic Drugs in Blood Plasma
(Top: 2 mg/L added for each; Bottom: 2 mg/L of standard preparation for each; 100 µL injected)

■Analytical Conditions

For Sample Injection

Column : Shim-pack MAYI-ODS (10 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 100 mmol/L Acetate (Na) Buffer (pH = 4.7)
B : Acetonitrile
A/B = 90/10 (v/v)

Flowrate : 2.0 mL/min

Dilution Factor : 8

For Separation

Column : Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 20 mmol/L Phosphate (Na) Buffer (pH = 2.5)
B : Methanol
Linear Gradient B 50 % → 70 % (4 → 19 min.)

Flowrate : 1.0 mL/min

Column Temp. : 40 ˚C

Detection : Photodiode Array UV-VIS Absorbance Detector SPD- M10A*VP* at 220 nm and 300 nm

■Analysis of Eight Drugs in Blood Plasma

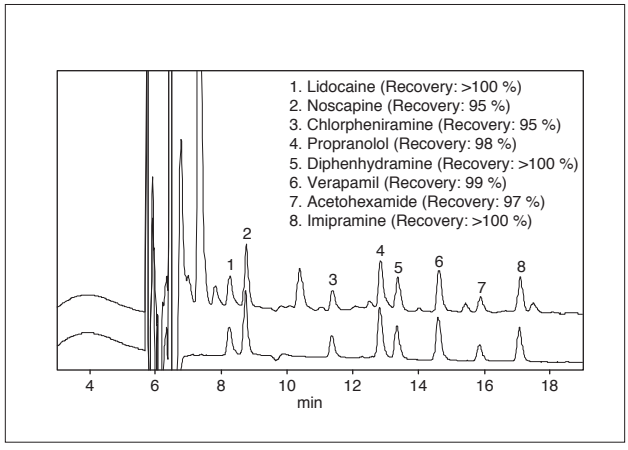


Fig. 6.2.6 Analysis of Eight Drugs in Blood Plasma
(Top: 0.5 mg/L added for each; Bottom: 0.5 mg/L of standard preparation for each; 50 µL injected for each)

■Analytical Conditions

For Sample Injection

Column : Shim-pack MAYI-ODS (10 mmL. × 4.6 mm I.D.)

Mobile Phase : 100 mmol/L Acetate (Na) Buffer (pH = 4.7)

Flowrate : 2.0 mL/min

Dilution Factor : 8

For Separation

Column : Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 20 mmol/L Phosphate (Na) Buffer (pH = 2.5)
100 mmol/L Sodium Perchlorate
B : Methanol
Linear Gradient B 50 % → 70 % (4 → 15 min.)

Flowrate : 1.0 mL/min

Column Temp. : 40 ˚C

Detection : Photodiode Array UV-VIS Absorbance Detector SPD- M10A*VP* at 205 nm

6.2 Analysis of Drugs in Blood Plasma Using "Co-Sense for BA" Bio-Sample Analysis System (4) - LC

■Analysis of Phenylbutazone in Blood Plasma

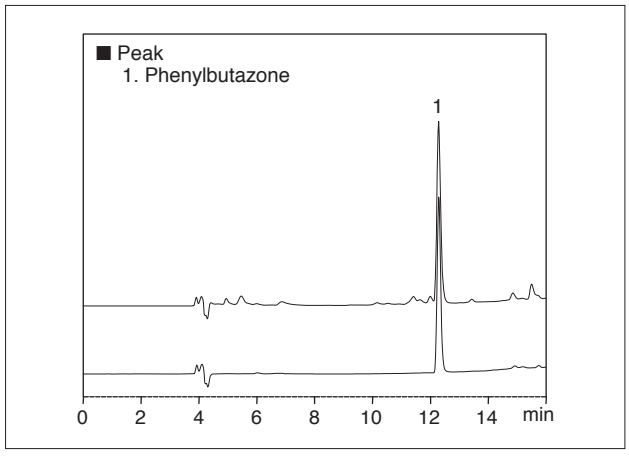


Fig. 6.2.7 Analysis of Phenylbutazone in Blood Plasma
(Top: Added; Bottom: Standard preparation;
4 µg/mL, 50 µL injected)

■Analytical Conditions

For Sample Injection

Column : Shim-pack MAYI-ODS
(10 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 100 mmol/L Acetate (Na) Buffer
(pH = 4.7)
B : Acetonitrile
A/B = 95/5 (v/v)

Flowrate : 2.0 mL/min

Dilution Factor : 8

For Separation

Column : Shim-pack FC-ODS
(75 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 20 mmol/L Phosphate (Na) Buffer
(pH = 6.9)
100 mmol/L Sodium Perchlorate
B : Methanol
Linear Gradient B 30 % → 80 % (4→12 min.)

Flowrate : 1.0 mL/min

Column Temp. : 40 ˚C

Detection : Photodiode Array UV-VIS Absorbance
Detector SPD- M10A*VP* at 265 nm

■Analysis of Ibuprofen in Blood Plasma

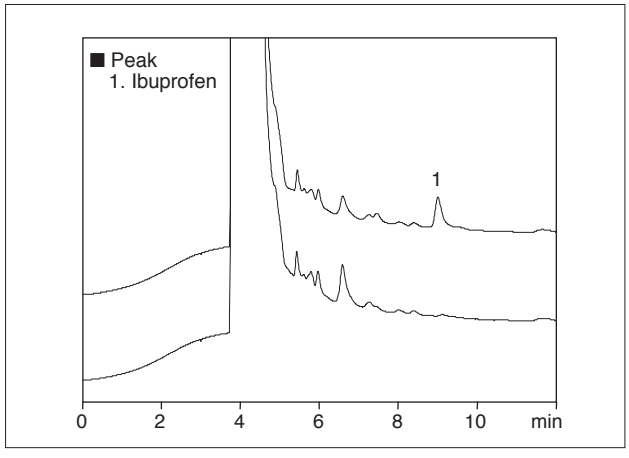


Fig. 6.2.8 Analysis of Ibuprofen in Blood Plasma
(Top: Added; Bottom: Not added;
1 µg/mL, 50 µL injected)

■Analytical Conditions

For Sample Injection

Column : Shim-pack MAYI-ODS
(10 mmL. × 4. 6mm I.D.)

Mobile Phase : A : 100 mmol/L Acetate (Na) Buffer
(pH = 4.7)
B : Acetonitrile
A/B = 90/10 (v/v)

Flowrate : 2.0 mL/min

Dilution Factor : 8

For Separation

Column : Shim-pack FC-ODS
(75 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 20 mmol/L Phosphate (Na) Buffer
(pH = 6.9)
B : Methanol
A/B = 45/55 (v/v)

Flowrate : 1.0 mL/min

Column Temp. : 40 ˚C

Detection : Photodiode Array UV-VIS Absorbance
Detector SPD- M10A*VP* at 210 nm

6.2 Analysis of Drugs in Blood Plasma Using "Co-Sense for BA" Bio-Sample Analysis System (5) - LC

■Analysis of Indometacin in Blood Plasma

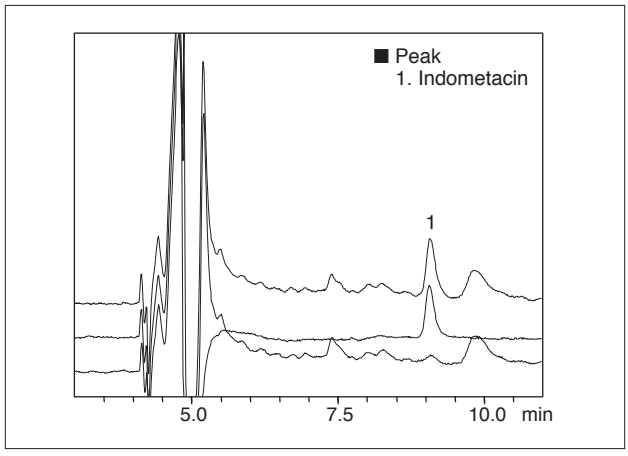


Fig. 6.2.9 Analysis of Indometacin in Blood Plasma
(Top: Added; Middle: Standard; Bottom: Not added;
100 ng/mL, 50 µL injected)

■Analytical Conditions

For Sample Injection

Column : Shim-pack MAYI-ODS
(10 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 100 mmol/L Ammonium Acetate
B : Acetonitrile
A/B = 90/10 (v/v)

Flowrate : 4.0 mL/min

Dilution Factor: 8

For Separation

Column : Shim-pack VP-ODS
(150 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 20 mmol/L Phosphate (Na) Buffer
(pH = 6.9)
B : Acetonitrile
Linear Gradient B 30 % → 35 % (4 → 6 min.)

Flowrate : 1.2 mL/min

Column Temp. : 40 ˚C

Detection : Photodiode Array UV-VIS Absorbance
Detector SPD- M10A*VP* at 270 nm

■Analysis of Lidocaine in Blood Plasma

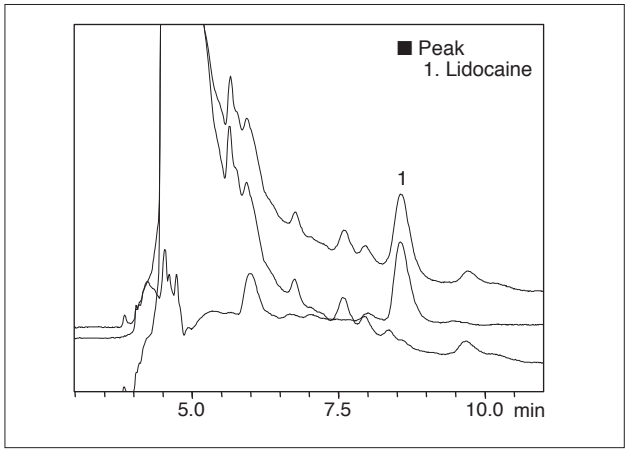


Fig. 6.2.10 Analysis of Lidocaine in Blood Plasma
(Top: Added; Middle: Standard; Bottom: Not added;
1 µg/mL, 50 µL injected)

■Analytical Conditions

For Sample Injection

Column : Shim-pack MAYI-ODS
(10 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 50 mmol/L Phosphate (Na) Buffer
(pH = 6.9)
B : Acetonitrile
A/B = 90/10 (v/v)

Flowrate : 4.0 mL/min

Dilution Factor : 8

For Separation

Column : Shim-pack VP-ODS
(150 mmL. × 4.6 mm I.D.)

Mobile Phase : A : 20 mmol/L Phosphate (Na) Buffer
(pH = 6.9)
B : Acetonitrile
A/B = 45/55 (v/v)

Flowrate : 1.2 mL/min

Column Temp. : 40 ˚C

Detection : Photodiode Array UV-VIS Absorbance
Detector SPD- M10A*VP* at 220 nm

6.2 Analysis of Drugs in Blood Plasma Using "Co-Sense for BA" Bio-Sample Analysis System (6) - LC

■Analysis of Triazolam in Blood Plasma

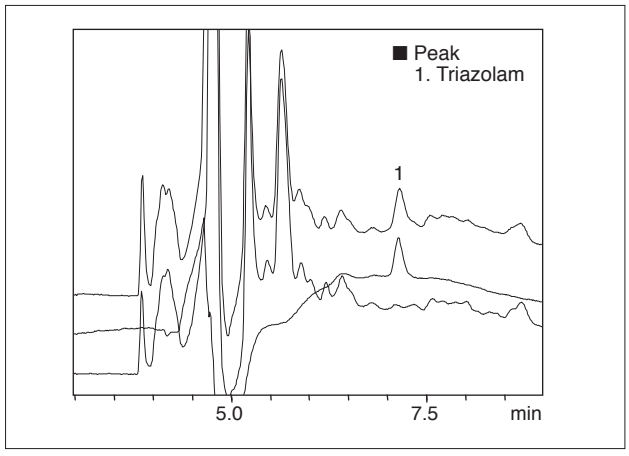


Fig. 6.2.11 Analysis of Triazolam in Blood Plasma
(Top: Added; Middle: Standard; Bottom: Not added; 20 ng/mL, 200 µL injected)

■Analytical Conditions

For Sample Injection

Column	: Shim-pack MAYI-ODS (10 mmL. × 4.6 mm I.D.)
Mobile Phase	: A : 20 mmol/L Ammonium Acetate B : Acetonitrile A/B = 90/10 (v/v)
Flowrate	: 4.0 mL/min
Dilution Factor	: 8

For Separation

Column	: Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase	: A : 10 mmol/L Ammonium Acetate B : Acetonitrile Linear Gradient B 45 % → 50 % (4 → 6 min.)
Flowrate	: 1.2 mL/min
Column Temp.	: 40 ˚C
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD- M10A*VP* at 250 nm

■Analysis of Atenolol in Blood Plasma

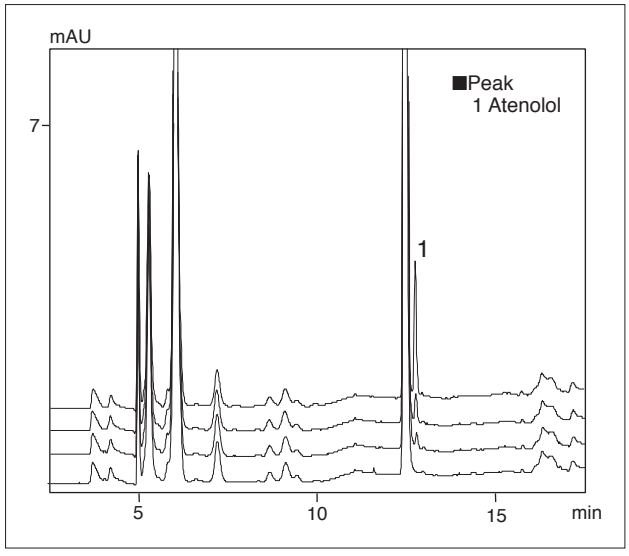


Fig. 6.2.12 Analysis of Atenolol in Blood Plasma
(from upper to lower, spiked with 5, 1, 0.5 µg/mL, lowest, unspiked) (10 µL injected)

■Analytical Conditions

For Sample Injection

Column	: Shim-pack MAYI-SCX (10 mmL. × 4.6 mm I.D.)
Mobile Phase	: 0.1 % Acetic Acid
Flowrate	: 3.0 mL/min
Extraction Time	: 2 min
Inj. Volume	: 10 µL
Dilution Factor	: 8

For Separation

Column	: Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase	: A : 10 mmol/L Ammonium Acetate Buffer (pH 4.7) B : Acetonitrile Linear Gradient B 2 % → 35 % (5 → 6 min.)
Flowrate	: 1.0 mL/min
Column Temp.	: 40 ˚C
Detection	: UV Absorbance Detector at 274 nm

6.2 Analysis of Drugs in Blood Plasma Using "Co-Sense for BA" Bio-Sample Analysis System (7) - LC

■Analysis of Procainamide in Blood Plasma

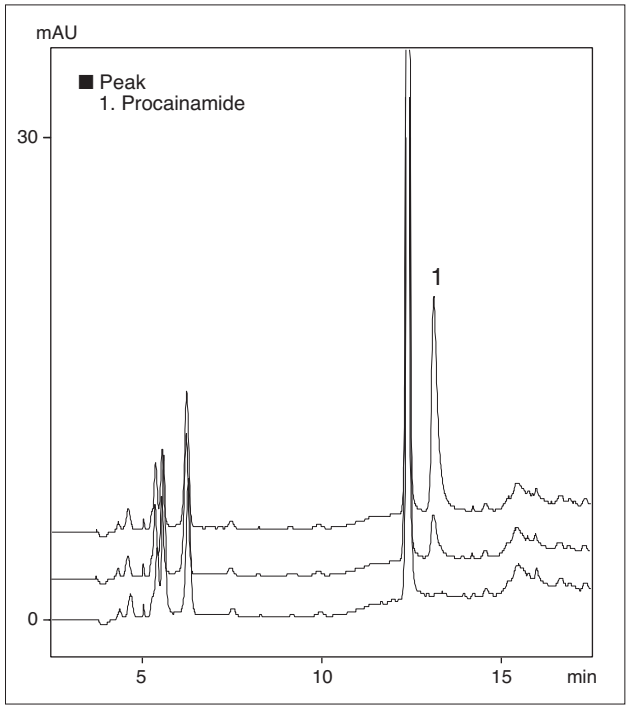


Fig. 6.2.13 Analysis of Procainamide in Blood Plasma (from upper to lower, spiked with 2.5, 0.5 µg/mL, lowest, unspiked) (20 µL injected)

■Analytical Conditions

For Sample Injection

Column	: Shim-pack MAYI-ODS (10 mmL. × 4.6 mm I.D.)
Mobile Phase	: A : 20 mmol/L Ammonium Acetate B : Acetonitrile A/B = 90/10 (v/v)
Flowrate	: 4.0 mL/min
Dilution Factor	: 8

For Separation

Column	: Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase	: A : 10 mmol/L Ammonium Acetate B : Acetonitrile Linear Gradient B 45 % → 50 % (4 → 6 min.)
Flowrate	: 1.2 mL/min
Column Temp.	: 40 ˚C
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD- M10A*VP* at 250 nm

6.3 Analysis of Drugs in Blood Plasma Using "Co-Sense for BA" Bio-Sample Analysis System - LC/MS

■Explanation

The "Co-Sense for BA" bio-sample analysis system makes it possible for filtered blood-plasma and blood-serum samples to be injected directly into the system. Its most important feature is that it enables the automation of pretreatment operations, such as protein removal. In the example shown here, drugs in blood plasma are analyzed with "Co-Sense for BA - LC/MS", which uses the LCMS-2010A single quadrupole high-performance liquid chromatograph mass spectrometer. The LCMS-2010A is particularly effective for samples with complex matrices, such as bio-samples, and offers reductions in analysis times.

■System Flow Lines

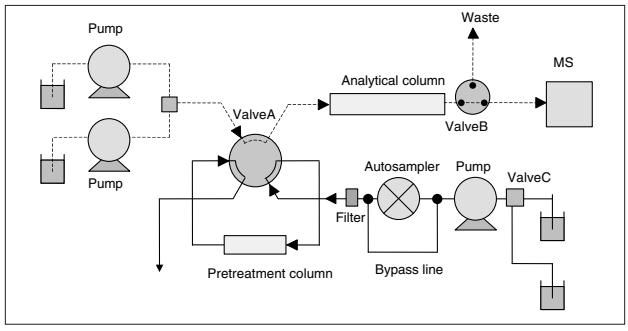


Fig. 6.3.1 System Flow Lines

■Analysis of Basic Drugs

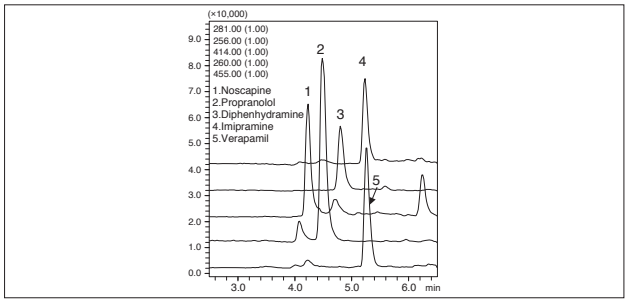


Fig. 6.3.2 SIM Chromatograms for Five Basic Drugs in Blood Plasma

■Analytical Conditions

For Sample Injection
- Column : Shim-pack MAYI-ODS (10 mmL. × 4.6 mm I.D.)
- Mobile Phase : Water/Acetonitrile = 95/5 (v/v) containing 0.1% Formic Acid
- Flowrate : 3.0 mL/min
- Dilution Factor : 8

For Separation
- Column : Chromolith SpeedRod (50 mmL. × 4.6 mm I.D.)
- Mobile Phase : A : Water/Acetonitrile = 95/5 (v/v) containing 0.1 % Formic Acid
  B : Acetonitrile containing 0.1 % Formic Acid
  Linear Gradient B 30 % → 90 % (2 → 5 min)
- Flowrate : 0.5 mL/min
- Column Temp. : 40 ˚C
- Probe Voltage : 4.5 kV (ESI-positive mode)
- Nebulizing Gas : 1.5 L/min
- Drying Gas : 0.1 MPa

■Analysis of Ketoprofen and Warfarin

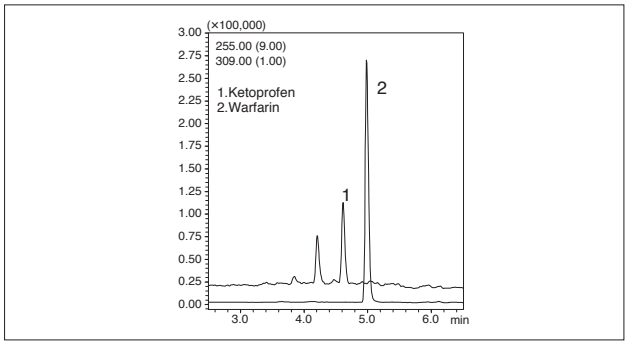


Fig. 6.3.3 SIM Chromatograms for Ketoprofen and Warfarin in Blood Plasma

■Analytical Conditions

For Sample Injection
- Column : Shim-pack MAYI-ODS (10 mmL. × 4. 6mm I.D.)
- Mobile Phase : A: 10 mmol/L Ammonium Formate Buffer (pH = 3.7)
  B: Acetonitrile
  A/B = 90/10 (v/v)
- Flowrate : 3.0 mL/min
- Dilution Factor : 8

For Separation
- Column : Shim-pack FC-ODS (75 mmL. × 4.6 mm I.D.)
- Mobile Phase : A: 10 mmol/L Ammonium Formate Buffer (pH = 3.7)
  B: Acetonitrile
  Linear Gradient B 50 % → 90 % (2 → 5 min)
- Flowrate : 0.8 mL/min
- Column Temp. : 40 ˚C
- Probe Voltage : 4.5 kV (ESI-positive mode)
- Nebulizing Gas : 1.5 L/min
- Drying Gas : 0.2 MPa

■Analysis of Ibuprofen

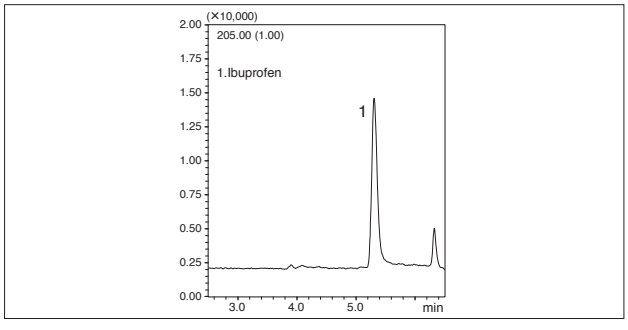


Fig. 6.3.4 SIM Chromatogram for Ibuprofen in Blood Plasma

■Analytical Conditions

For Sample Injection
- Column : Shim-pack MAYI-ODS (10 mmL. × 4.6 mm I.D.)
- Mobile Phase : 10 mmol/L Ammonium Acetate /Acetonitrile = 95/5 (v/v)
- Flowrate : 3.0 mL/min
- Dilution Factor : 8

For Separation
- Column : Shim-pack FC-ODS (75 mmL. × 4.6 mm I.D.)
- Mobile Phase : A: 10 mmol/L Ammonium Acetate
  B: Acetonitrile
  Linear Gradient B 40 % → 90 % (2 → 4 min)
- Flowrate : 0.6 mL/min
- Column Temp. : 40 ˚C
- Probe Voltage : -3.5 kV (ESI-positive mode)
- Nebulizing Gas : 1.5 L/min
- Drying Gas : 0.2 MPa

6.4 High Speed, High Resolution Analysisof Mexiletine, Pilsicainide, and Zonisamide in Serum (1) - LC

■Explanation

HPLC separation and quantitation are often used for measurement of drug concentrations in the blood. When analyzing the concentration of a drug in the blood using HPLC, the separation of contaminants and analytes requires a long analysis time. For that reason, there is demand for faster HPLC analysis to improve the productivity of drug pharmacokinetic studies and clinical testing, particularly when handling many samples. Here we introduce analyses of mexiletine, pilsicainide, and zonisamide in serum using the ultra-high speed Prominence UFLC system and high-speed Shim-pack XR-ODS separation column.

■Analysis of Mexiletine

Mexiletine (Fig. 6.4.1) is a type of anti-arrhythmic medicine effective for treating irregular heartbeat. After subjecting the serum sample to organic solvent extraction, the extract solution was filtered through a 0.22 µm membrane filter, and 20 µL was injected. Fig. 6.4.2 shows the chromatogram.

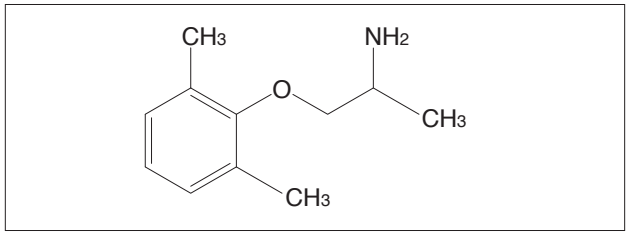


Fig. 6.4.1 Structure of Mexiletine

■Analytical Conditions

Column	: Shim-pack XR-ODS (75 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase	: 25 mmol/L KH_2PO_4 aq. Solution /Acetonitrile = 83/17 (v/v) containing 1.0 mmol/L $NaClO_4$
Flowrate	: 0.8 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 20 µL
Detection	: UV-VIS Absorbance Detector SPD-20AV at 205 nm
Flow Cell	: Semi-micro Cell

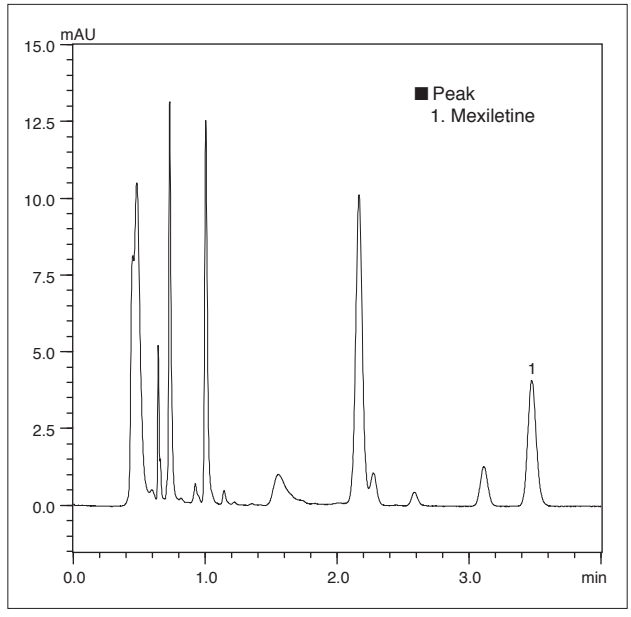


Fig. 6.4.2 Chromatogram of Mexiletine in Serum Sample

6.4 High Speed, High Resolution Analysisof Mexiletine, Pilsicainide, and Zonisamide in Serum (2) - LC

■Analysis of Pilsicainide

Pilsicainide (Fig. 6.4.3) is used for treatment of tachyarrhythmia (rapid heart-rate with arrhythmia) After subjecting the serum sample to organic solvent extraction, the extract solution was filtered through a 0.22 µm membrane filter, and 20 µL was injected. Fig. 6.4.4 shows the chromatogram.

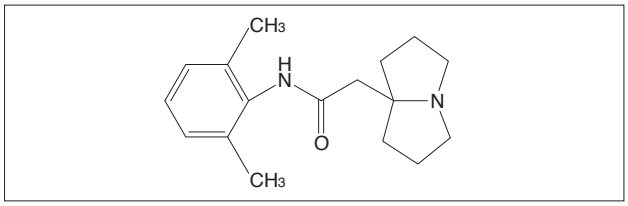


Fig. 6.4.3 Structure of Pilsicainide

■Analytical Conditions

Column	: Shim-pack XR-ODS (75 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase	: 25 mmol/L KH_2PO_4 aq. Solution /Acetonitrile /Metanol = 85/10/5 (v/v/v)
Flowrate	: 0.8 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 20 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 210 nm
Flow Cell	: Semi-micro Cell

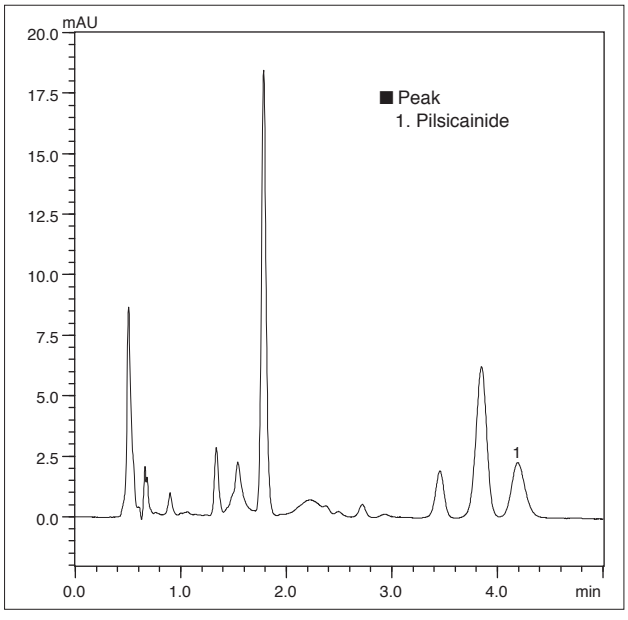


Fig. 6.4.4 Chromatogram of Pilsicainide in Serum Sample

■Analysis of Zonisamide

Zonisamide (Fig. 6.4.5) is a type of anticonvulsant medicine. After subjecting the serum sample to organic solvent extraction, the extract solution was filtered through a 0.22 µm membrane filter, and 2 µL was injected. Fig. 6.4.6 shows the chromatogram.

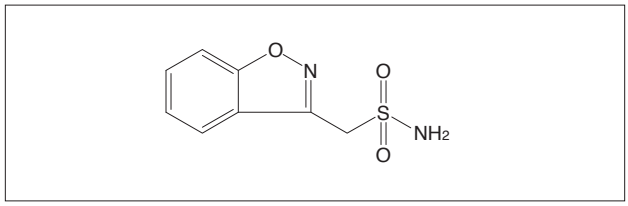


Fig. 6.4.5 Structure of Zonisamide

■Analytical Conditions

Column	: Shim-pack XR-ODS (75 mmL. × 3.0 mm I.D., 2.2 µm)
Mobile Phase	: 10 mmol/L KH_2PO_4 aq. Solution /Acetonitrile = 100/37 (v/v)
Flowrate	: 0.8 mL/min
Column Temp.	: 45 ºC
Injection Volume	: 2 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 242 nm
Flow Cell	: Semi-micro Cell

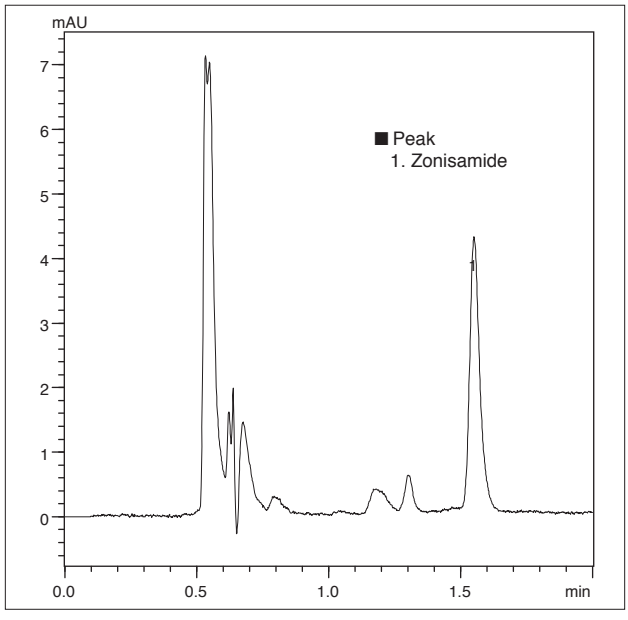


Fig. 6.4.6 Chromatogram of Zonisamide in Serum Sample

* The published data was not acquired using an instrument registered by Japanese pharmaceutical affairs law.

6.5 High Speed, High Resolution Analysisof Clobazam and Cibenzoline in Serum (1) - LC

■Explanation

HPLC is an important technique used for the analysis of drugs in the blood. However, faster analysis is often required to improve sample throughput and productivity at sites handling many specimens. Here, we introduce an example of ultra-high-speed analysis of clobazam and cibenzoline in serum using the Nexera UHPLC (Ultra High Performance Liquid Chromatography) system and the Shim-pack XR-ODS Ⅲ high-speed separation column.

■Analysis of Clobazam

Clobazam is a benzodiazepine type of antiepilepsy drug. *N*-desmethylclobazam, one of the substances formed when clobazam is metabolized in the body, displays activity similar to that of clobazam. Fig. 6.5.1 shows the structures of these substances. A serum sample was analyzed after cleanup by liquid-liquid extraction. Analysis was conducted using 2 different columns, the Shim-pack VP-ODS (particle size: 4.6 µm) for conventional analysis, and the Shimpack XR-ODS Ⅲ (particle size: 1.6 µm) for ultra-highspeed analysis. Fig. 6.5.2 shows the chromatograms.
Conducting ultra-high-speed analysis with the Shim-pack XR-ODS Ⅲ allowed the analysis time to be shortened to about 1/12 that by conventional analysis.
The system back pressure in this analysis was about 85 MPa.

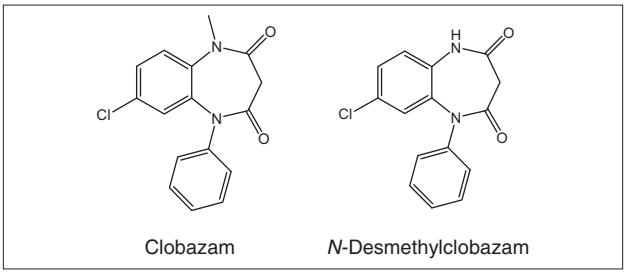


Fig. 6.5.1 Structures of Clobazam and *N*-Desmethylclobazam

■Analytical Conditions

Column	: Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D., 4.6 µm) Shim-pack XR-ODS Ⅲ (50 mmL. × 2.0 mm I.D., 1.6 µm)
Mobile Phase	: 10 mmol/L NaH_2PO_4 aq. Solution /Acetonitrile = 2/1 (v/v)
Flowrate	: 1.0 mL/min (VP-ODS) 0.9 mL/min (XR-ODS Ⅲ)
Column Temp.	: 40 ºC
Injection Volume	: 50 µL (VP-ODS) 10 µL (XR-ODS Ⅲ)
Detection	: UV-VIS Absorbance Detector SPD-20AV at 230 nm
Flow Cell	: Conventional Cell (VP-ODS) Semi-micro Cell (XR-ODS Ⅲ)

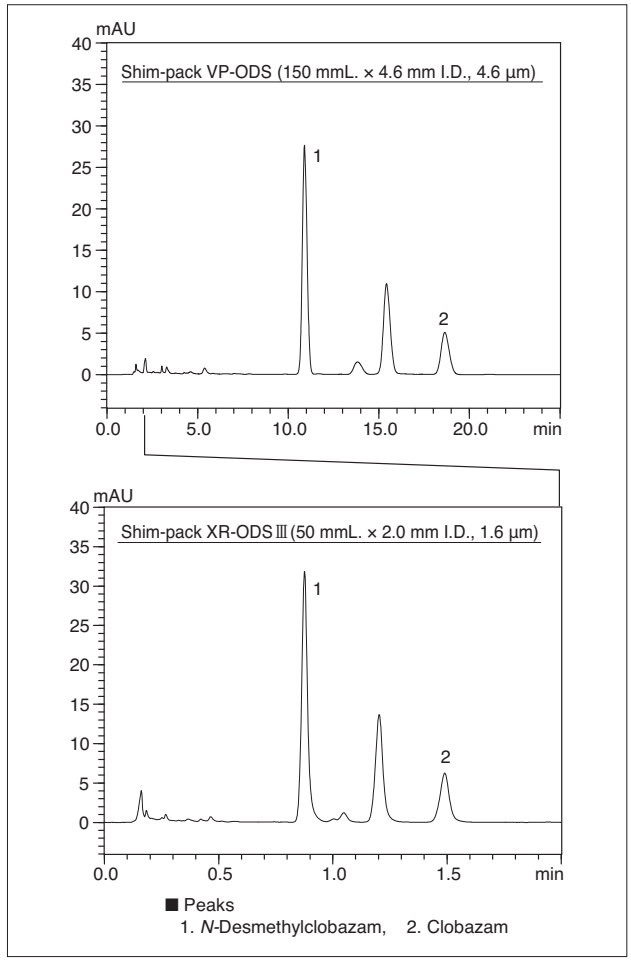


Fig. 6.5.2 Chromatograms of Clobazam and *N*-Desmethylclobazam in Serum Sample
(Upper : Shim-pack VP-ODS, Lower : Shim-pack XR-ODS Ⅲ)

6.5 High Speed, High Resolution Analysisof Clobazam and Cibenzoline in Serum (2) - LC

■Analysis of Cibenzoline

Cibenzoline (Fig. 6.5.3) is a type of antiarrhythmic drug. A serum sample was analyzed using the Shim-pack XR-ODS Ⅲ (particle size : 1.6 µm) after cleanup by liquid-liquid extraction. Fig. 6.5.4 shows the chromatogram. Conducting analysis using these conditions allowed the analysis time to be shortened to about 1/10 that by conventional analysis, which took about 15 minutes. The system back pressure during the high-speed analysis was about 77 MPa.

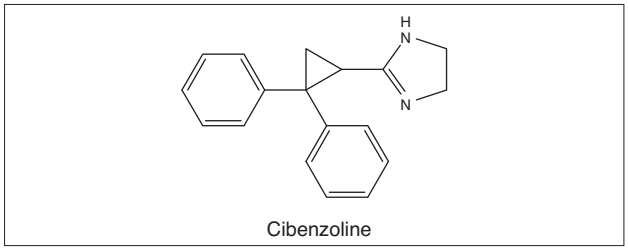


Fig. 6.5.3 Structure of Cibenzoline

■Analytical Conditions

Column	: Shim-pack XR-ODS Ⅲ (50 mmL. × 2.0 mm I.D., 1.6 µm)
Mobile Phase	: Phosphate Buffer/Acetonitrile /Methanol = 20/5/4 (v/v/v)
Flowrate	: 0.7 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 10 µL
Detection	: UV-VIS Absorbance Detector SPD-20AV at 225 nm
Flow Cell	: Semi-micro Cell

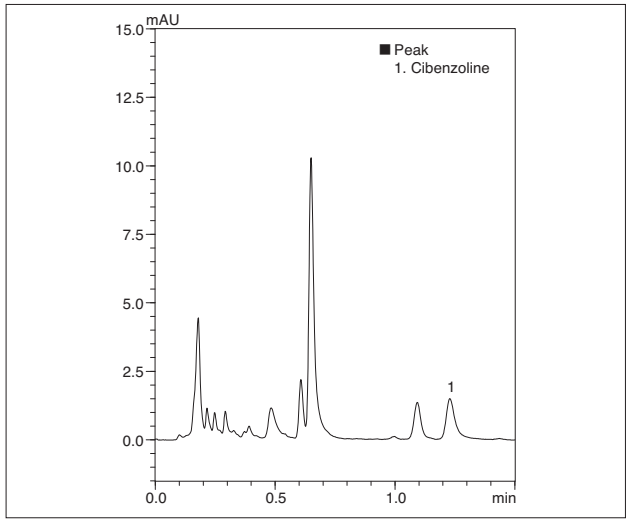


Fig. 6.5.4 Chromatogram of Cibenzoline in Serum Sample

■Reducing the Total Analysis Time with Overlapping Injection

HPLC productivity is improved when the overall analysis time is shortened. This includes decreasing the run time and reducing the autosampler injection cycle time between samples. The Nexera SIL-30AC autosampler is equipped with an overlapping injection feature that, when enabled, loads the next sample while the current analysis is in progress. This feature, combined with the world's fastest and cleanest injection performance, greatly shortens the overall analysis time. Fig. 6.5.5 shows the results of overlapping 10 injections of the Cibenzoline sample from Fig. 6.5.4. Ten analyses were completed in 15 minutes.

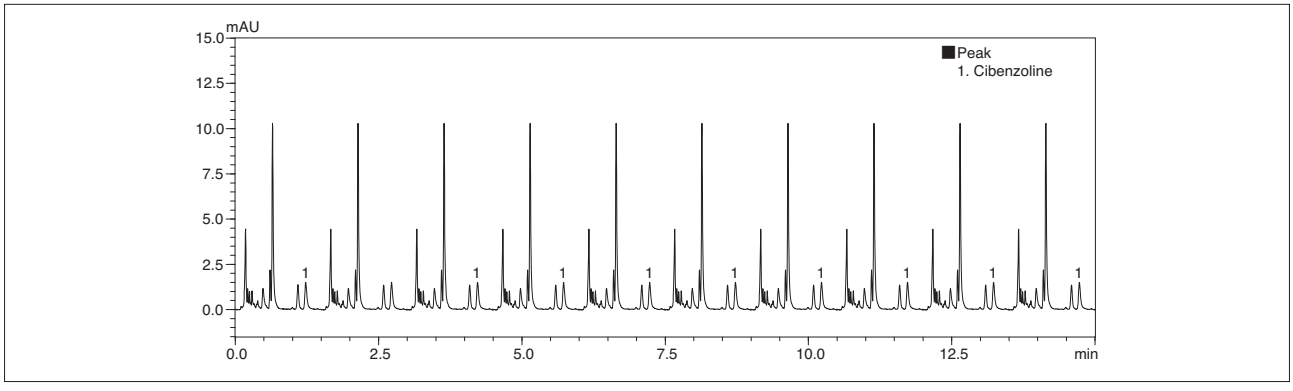


Fig. 6.5.5 Chromatograms of Cibenzoline in Serum Sample Using Overlapping Injection (10 Repetitions)

* The published data was not acquired using an instrument registered by Japanese pharmaceutical affairs law.

6.6 Verification of Viagra (Sildenafil Citrate) - LC/MS

■Explanation

Viagra (sildenafil citrate), which is sold in the U.S., is a drug used to treat male erectile dysfunction. A viagra pill was pulverized and dissolved and the presence of sildenafil citrate was verified. In cases where standard samples are hard to get hold of, mass spectrometry, which uses information about the molecular mass, is particularly effective. Sildenafil citrate was eluted after a retention time of 6.3 minutes under the analytical conditions given below.

■Analytical Conditions

Column	: ODS (150 mmL. × 1.5 mm I.D.)
Mobile Phase	: 50 % Acetonitrile-Water containing 10 mmol/L Ammonium Acetate
Flowrate	: 0.1 mL/min
Column Temp.	: 40 ˚C
Probe Voltage	: + 2.5 kV (ESI-Positive Mode)
Nebulizing Gas Flow	: 4.5 L/min
CDL Temp.	: 230 ˚C
CDL Voltage	: -25 V
DEFs Voltage	: +50 V
Scan Range	: *m/z* 100 – 600 (2.0 sec/scan)

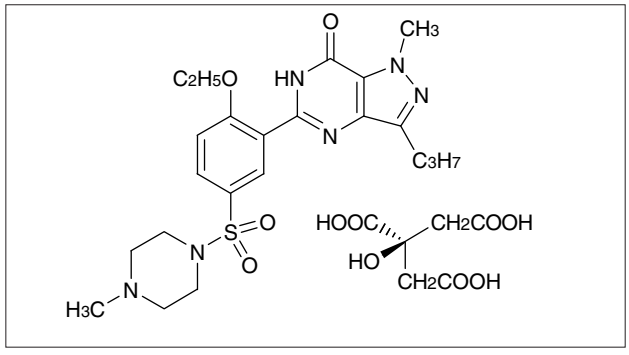


Fig. 6.6.1 Structure of Viagra (sildenafil citrate)

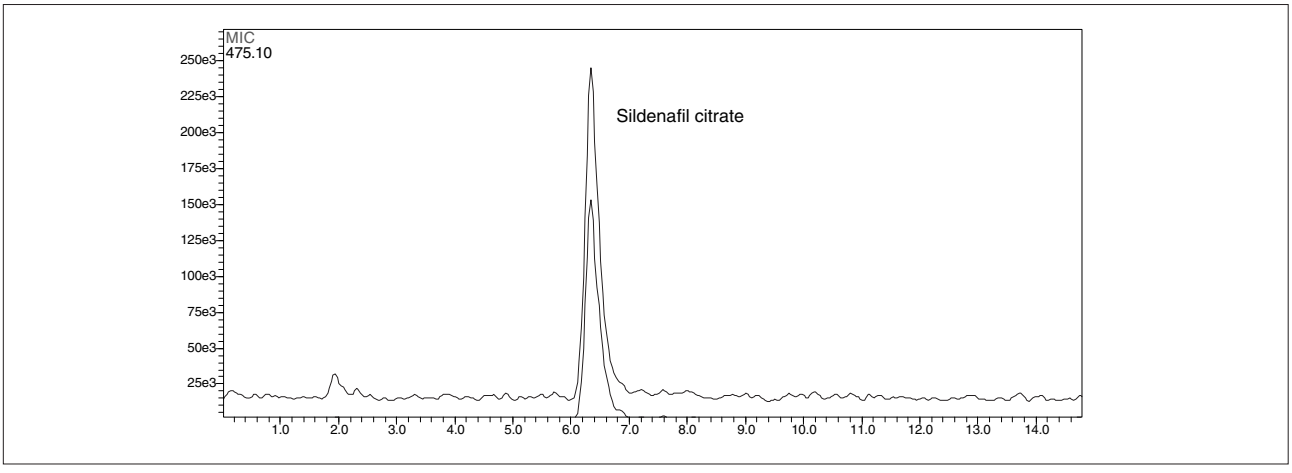


Fig. 6.6.2 Mass Chromatogram for Viagra (sildenafil citrate)

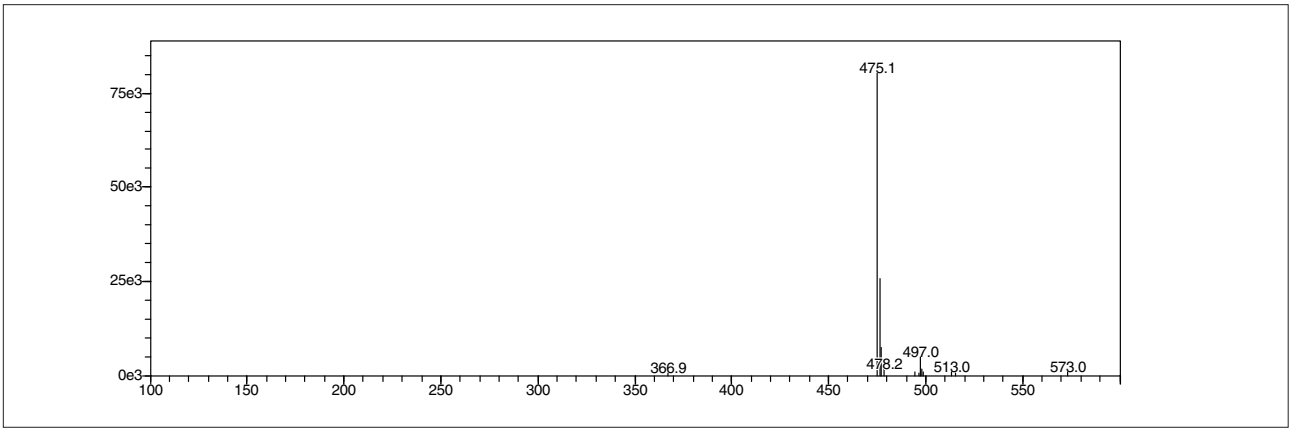


Fig. 6.6.3 Mass Spectrum for Viagra (sildenafil citrate)

6.7 Analysis of a Benzodiazepine-Based Drug (1) - GC/MS

■Explanation

Benzodiazepine drugs are commonly used in sleeping aids and tranquilizers, and sometimes in crimes or suicide. Therefore, these chemical substances are often analyzed by forensic laboratories for criminal or academic investigations. Here we show the results from using GC/MS to measure 9 types of benzodiazepine drugs.

■Analytical Conditions

Instrument : GCMS-QP2010 Ultra
Column : Rxi®-5Sil MS (30 m × 0.25 mm I.D., df = 0.25 µm)
Glass Insert : Silanized splitless insert

[GC]

Injector Temp.	: 260 °C
Column Temp.	: 60 °C (2 min) → 10 °C/min → 320 °C (10 min)
Injection Method	: Splitless Injection
Sampling Time	: 1 min
High Pressure Injection Method	: 250 kPa (1.5 min)
Carrier Gas	: He
Control Mode	: Linear velocity (45.6 cm/sec)
Purge Flowrate	: 3.0 mL/min
Injection Volume	: 1.0 µL

[MS]

Interface Temp.	: 280 °C
Ion Source Temp.	: 200 °C
Solvent Elution Time	: 2.0 min
Measurement Mode	: Scan
Mass Range	: *m/z* 35-600
Event Time	: 0.3 sec
Emission Current	: 150 µA (high sensitivity)

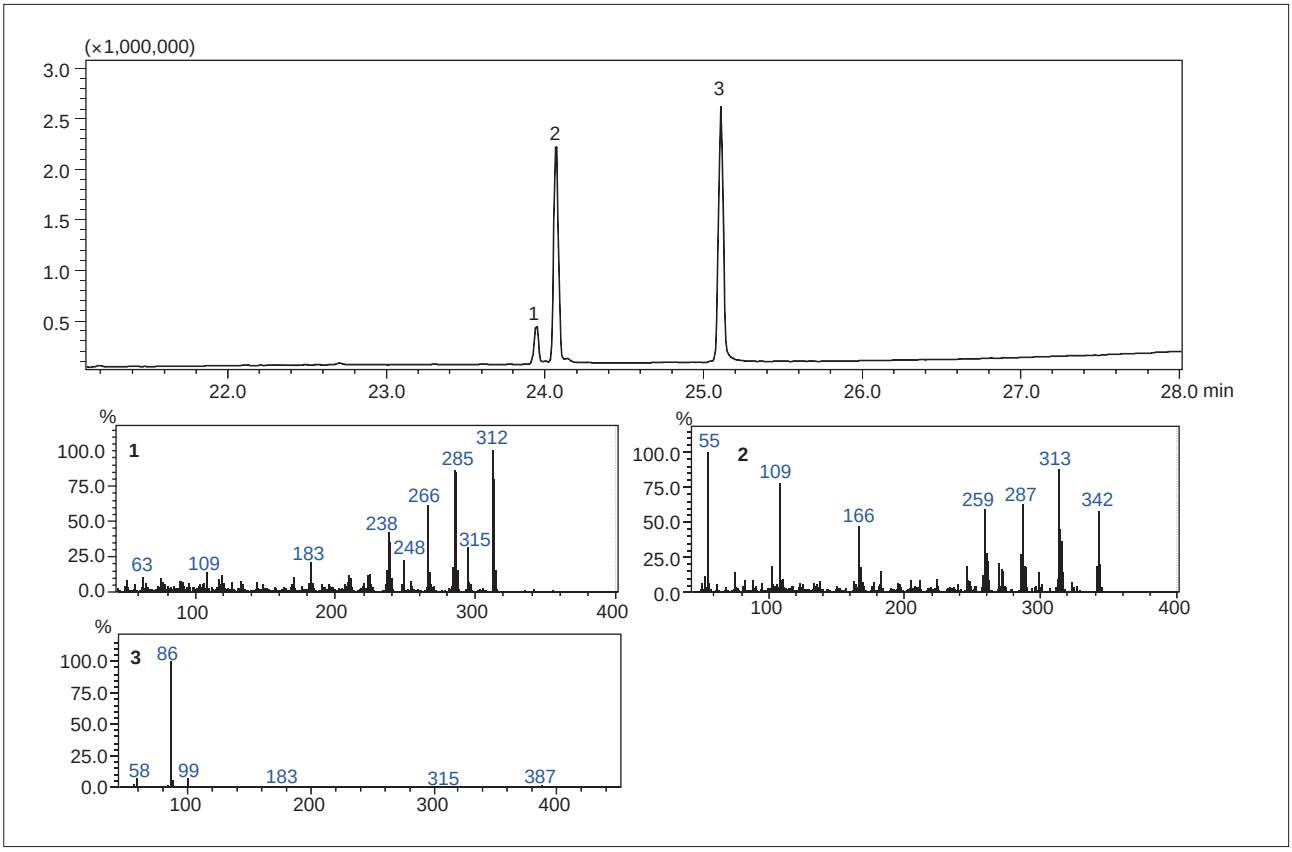


Fig. 6.7.1 Total Ion Current Chromatogram and Mass Spectra
1. Flunitrazepam, 2. Flutoprazepam, 3. Flurazepam

6.7 Analysis of a Benzodiazepine-Based Drug (2) - GC/MS

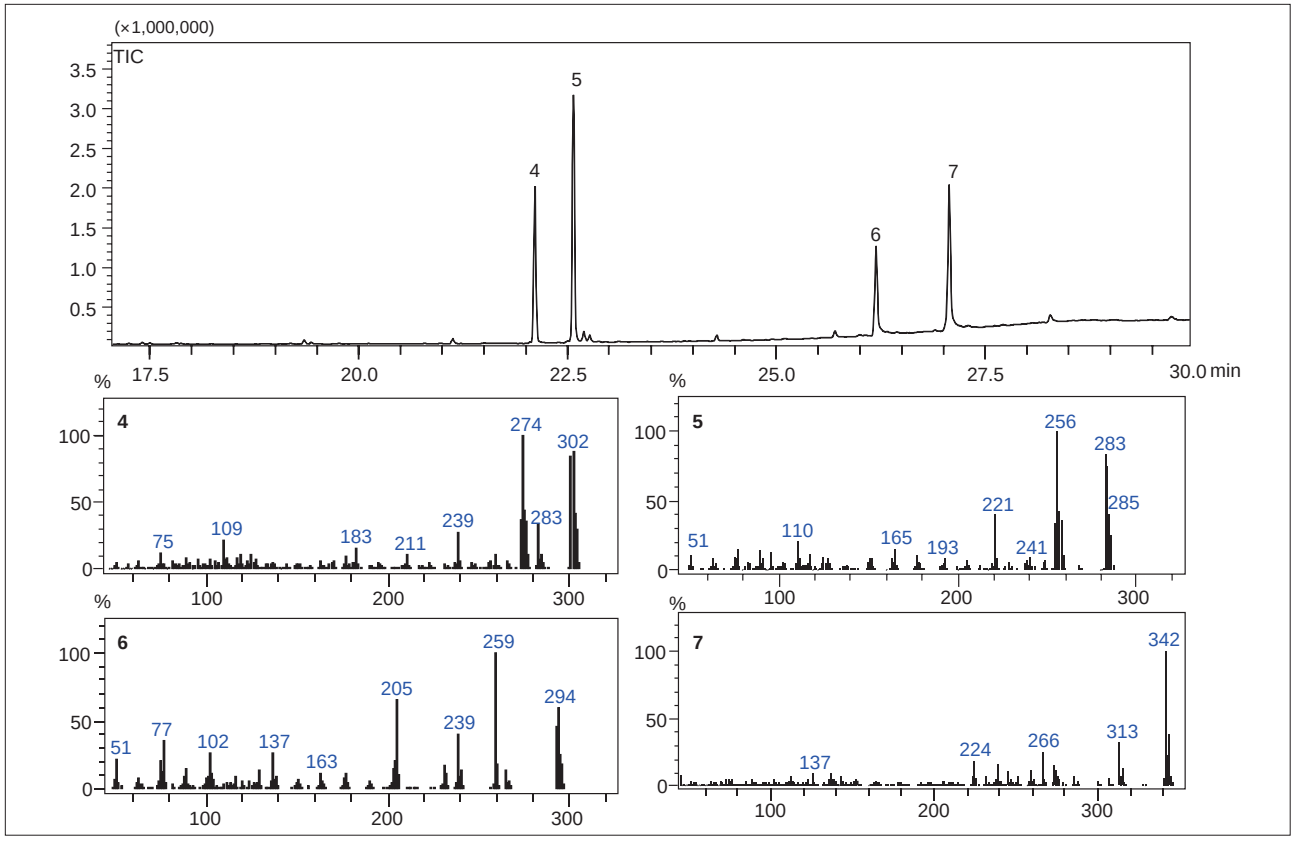


Fig. 6.7.2 Total Ion Current Chromatogram and Mass Spectra
4. Fludiazepam, 5. Diazepam, 6. Estazolam, 7. Etizolam

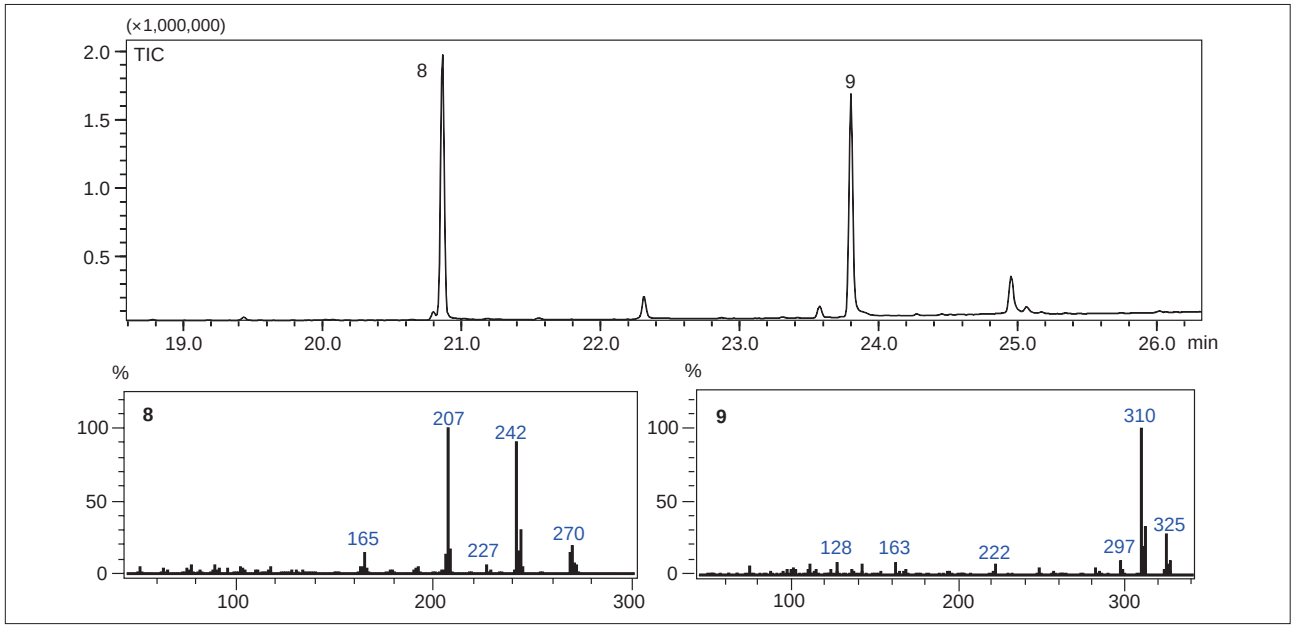


Fig. 6.7.3 Total Ion Current Chromatogram and Mass Spectra
8. Medazepam, 9. Midazolam

6.8 Screening Techniques in Doping Analysis by GC/MS (1) - GC/MS

■Explanation

Sports doping is not only contradictory to the concept of fair play, but it has a negative impact on the health of athletes as well as society in general. For these reasons, drug doping testing is conducted based on regulations imposed by the World Anti-Doping Agency (WADA). Table 6.8.1 lists the sports doping screening techniques. The quadrupole GC/MS is used for analysis of difficult-to-volatilize drugs (Screening Method No. 2), diuretics (No. 5), and *β*-blocker agents (No. 7). Here we introduce an example of the analysis of a difficult-to-volatilize drug (Screening Method No. 2) obtained with the cooperation of MITSUBISHI KAGAKU BIO-CLINICAL LABORATORIES, INC., officially recognized as a WADA testing agency.

Table 6.8.1 Classification of Screening Methods in Sport Doping Analysis

Screening No.	Classification	Drug Example	Analytical Instrument
1	Volatile drugs	Amphetamine	GC-NPD
2	Difficult to volatilize drugs	Cocaine metabolites	GC/MS (Scan)
3	Thermally decomposed substances	Dexamethasone	Q-TOF LC/MS
4	Designer steroids	Testosterone	GC/MS (SIM)
	Anabolic steroids	Stanozolol	GC/HRMS (SIM)
5	Diuretics	Furosemide	GC/MS (SIM)
6	Steroid hormones	Androstenedione	GC/C/IRMS
7	*β*-blocker agents	Metoprolol	GC/MS (Scan)
8	Peptide hormones	EPO, hCG	EIA, immunoblotting

■Analytical Procedures

The pretreatment flow chart for Screening Method No. 2 are shown in Fig. 6.8.1. In the pretreatment procedure, 6 M of hydrochloric acid was added to 5 mL of urine, and this was heated for 30 minutes at 105 ºC to conduct hydrolysis. After washing with diethyl ether, 2-methyl-2-propanol and internal standards were added to the liquid phase, and after adjusting the pH to 9.6 ±0.1, extraction was conducted with diethyl ether. The extract was dried under a stream of nitrogen gas, and after adding methyl orange/acetonitrile/TFA solution, MSTFA was added until the solution turned yellow, after which the solution was heated for 5 minutes at 80 ºC. Then, MBTFA was added, and the solution was heated for 10 minutes at 80 ºC to conduct N-TFA-O-TMS derivatization.

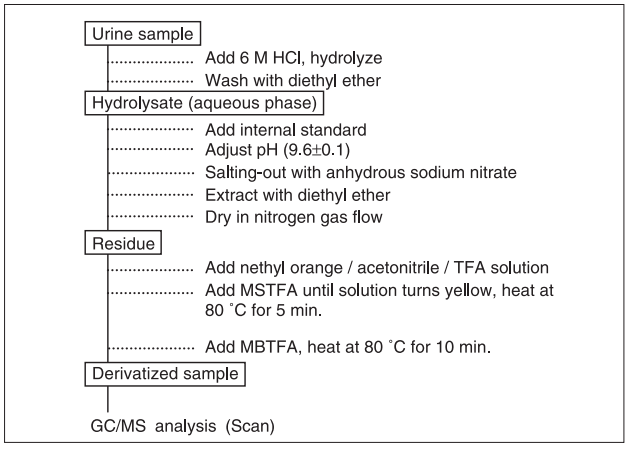


Fig. 6.8.1 Pretreatment Flow for Screening Method No. 2

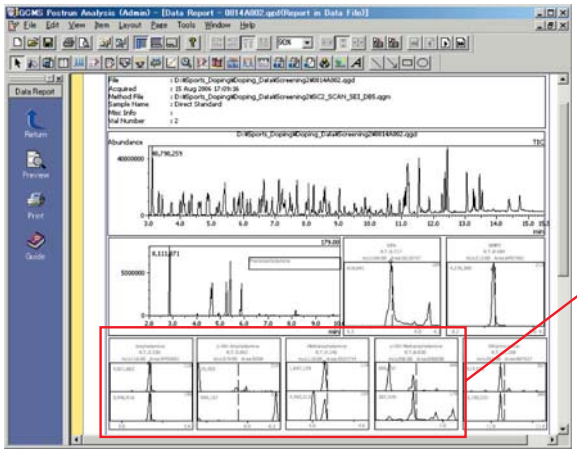
■Analytical Conditions

Instrument : GCMS-QP2010
Workstation : GCMSsolution Ver.2.5
Column : DB-5 (15 m × 0.25 mm I.D. df = 0.25 μm)
-GC-
Column Temp. : 100 ºC (1 min) -16 ºC/min-300 ºC (2 min)
Carrier Gas : He (Constant Linear Velocity Mode)
Linear Velocity : 51.8 cm/sec
Injector Temp. : 280 ºC
Injection Method : Split Injection
Split Ratio : 1:11
-MS-
Interface Temp. : 300 ºC
Ion Source Temp. : 200 ºC
Scan Range : *m/z* 50-550
Scan Interval : 0.3 sec

6.8 Screening Techniques in Doping Analysis by GC/MS (2) - GC/MS

■Sports Doping Test Report Format

In order to present test results in the most effective manner, the results of each analyte must be arranged in an easy-to-view format. For instance, the report must be as compact as possible, displaying chromatograms of the drug and its metabolites side-by-side for easy viewing. GCMSsolution allows the reporting items to be pasted to the screen and freely positioned to easily generate highly effective doping test reports. (Fig. 6.8.2)



Amphetamine and *p*-OH-amphetamine, and methamphetamine and *p*-OH-methamphetamine are drug and metabolite pairs. Placing their chromatograms side-by-side enables easy confirmation in the report.

Fig. 6.8.2 Report Creation Screen

■Analytical Procedures

To ensure data reliability, WADA requires various confirmation tests. In the case of Screening Method No. 2, a Minimum Required Performance Limit (MRPL) of 0.5 µg/mL (strychnine only, 0.2 µg/mL) is set to verify the GC/MS sensitivity.*1 In addition, analysis of a control sample, consisting of drug-free urine, and a blank sample is required to ensure the reliability of the pretreatment procedure and system blank. Fig. 6.8.3 shows these testing results in a report formatted using GCMSsolution. The chromatograms of the analyte target ions and their identifying ions are positioned one above the other, enabling convenient judgment of the presence or absence of the compound at a glance.

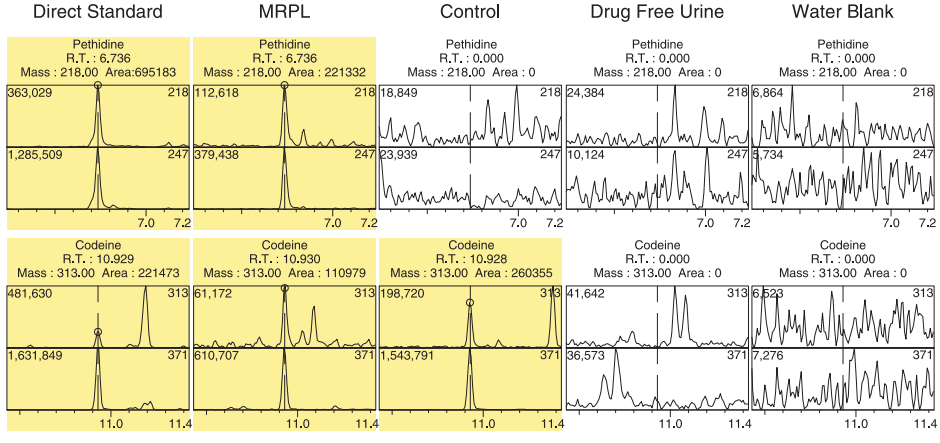


Fig. 6.8.3 Example of Report Format for Sports Doping Test

[Reference]

*1: MINIMUM REQUIRED PERFORMANCE LIMITS FOR DETECTION OF PROHIBITED SUBSTANCES - WADA Technical Document TD2004MRPL

* The published data was not acquired using an instrument registered by Japanese pharmaceutical affairs law.

6.9 GC/MS Forensic Toxicological Database (1) - GC/MS

■Explanation

In recent years, abuse of stimulants and other illegal drugs, particularly hallucinogens shows no signs of abating; crime and poisoning events due to medicines and agricultural medicines, including psychotropic drugs, are also on the increase, presenting a growing and serious social problem. In crime laboratories and forensic research laboratories at universities, these chemicals are analyzed using a gas chromatograph mass spectrometer. The use of libraries of recorded retention indices and mass spectra is effective for identification and detection of these chemical substances. Shimadzu has developed its own proprietary "GC/MS Forensic Toxicological Database" containing more than 1000 retention indices and mass spectra for chemical substances*, including psychotropic drugs, narcotics, stimulants, and pesticides, including common derivatives. With emphasis on chemical substances of abuse, difficult-to-obtain standard samples of chemical substances and metabolites are registered in the database, making this library ideal for forensic toxicological analysis. Moreover, method files containing compound information according to category are also included in this database, thereby eliminating such complicated tasks as investigating analytical conditions and settings. Here we introduce the usefulness of the forensic substance library in this GC/MS Forensic Toxicological Database.

* Includes derivatized compounds.

■Information Contained in Forensic Toxicological Database

The library in the GC/MS Forensic Toxicological Database contains not only mass spectra, but the retention indices obtained in analysis using the standard analytical conditions. Additional information, including the compound name, CAS number, molecular weight, molecular formula and structural formula are also included.

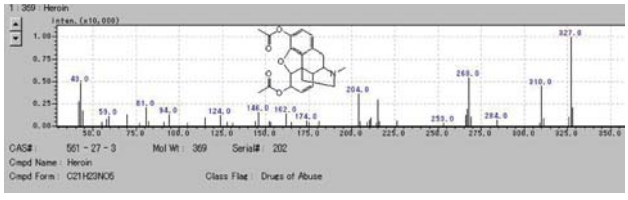


Fig. 6.9.1 Information Included for Registered Compounds

■Standard Analytical Conditions

Instrument	: GCMS-QP2010 Series
Workstation	: GCMSsolution Ver.2.5 or later
Column	: DB-5ms (30 m × 0.25 mm I.D., df = 0.25 µm) or Rxi®-5Sil MS (30 m × 0.25 mm I.D., df = 0.25 µm)
-GC-	
Column Temp.	: 60 ºC (2 min) -10 ºC/min-320 ºC (10 min)
Carrier Gas	: He (Constant Linear Velocity Mode)
Linear Velocity	: 45.6 cm/sec
Injection Method	: Splitless Injection
-MS-	
Interface Temp.	: 280 ºC
Ion Source Temp.	: 200 ºC
Scan Interval	: 0.3 sec

■Effectiveness of Library Search Using Mass Spectra and Retention Indices

Since a typical library search is based solely on the mass spectrum, it is difficult to distinguish among compounds that present similar mass spectra, such as positional isomers and homologs. It is therefore not unusual for multiple compounds to be listed at higher similarity indices in any given library search result. Using the GCMSsolution software (Ver.2.5 or later), sorting of library match compounds can be conducted not only on the basis of mass spectrum, but on retention index as well.

■Library Search for Isomers

Fig. 6.9.2 (a) shows the results of a library search based on the data obtained from analysis of *m*-methoxyamphetamine. Since *o*-, *m*-, and *p*-isomers exist for methoxyamphetamine, these isomers appear in the library search results list with similarity indices of 90 or greater. Fig. 6.9.2 (b) shows the search results in which the retention index (permissible width ±10) is added as a condition to the initial search conditions. It can be seen that there are no search results for the other (*o*- and *p*-) isomers, because these isomers elute outside the retention index window of ±10 RI units. Thus, the reliability of the mass spectral library search result can be improved by using the mass spectrum together with the retention index.

6.9 GC/MS Forensic Toxicological Database (2) - GC/MS

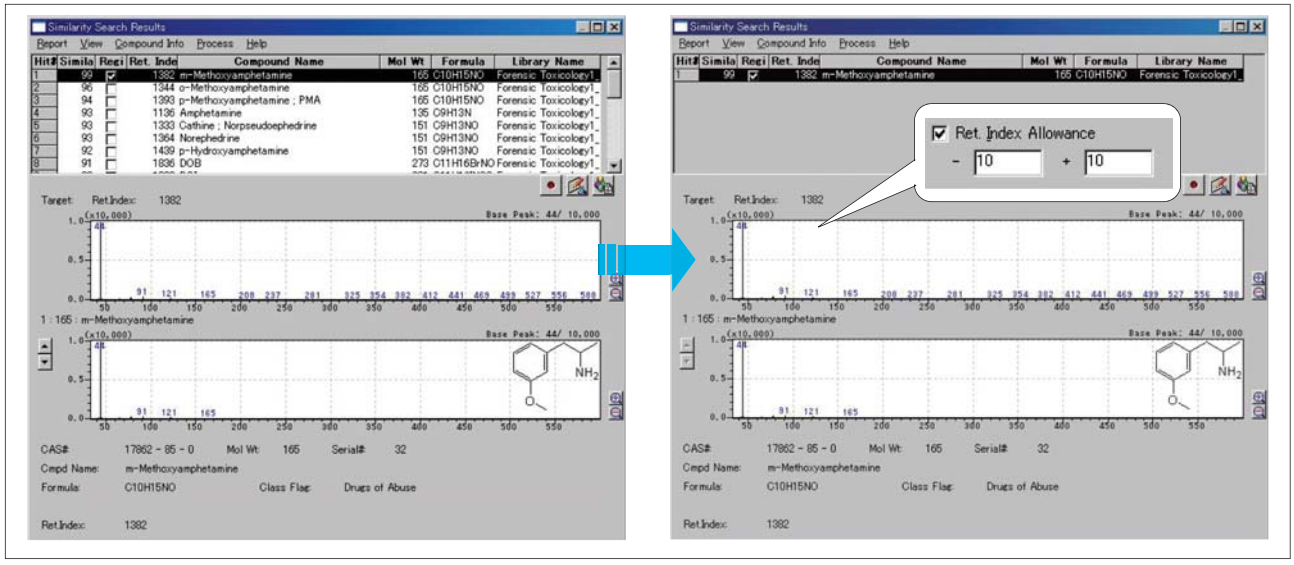


Fig. 6.9.2 Library Search Result for *m*-Methoxyamphetamine
(a) Using only mass spectrum, (b) Using mass spectrum and retention index

■Library Search for Phenethylamines

Fig. 6.9.3 (a) shows the results of the library search based on data obtained in analysis of methamphetamine. Phenethylamines include many compounds that present similar mass spectra, and as in the isomer search results, many compounds appear in the search results list with similarity indices of 90 or greater. Fig. 6.9.3 (b) shows the search results in which the retention index (permissible width ±10) is added as a condition to the initial search conditions, demonstrating that the compounds with a similarity index of 90 or greater are considerably diminished. These results show that not only in the case of isomers, but also when there are many compounds with similar mass spectra, highly reliable search results can be obtained by using a library search that combines retention index in the search conditions.

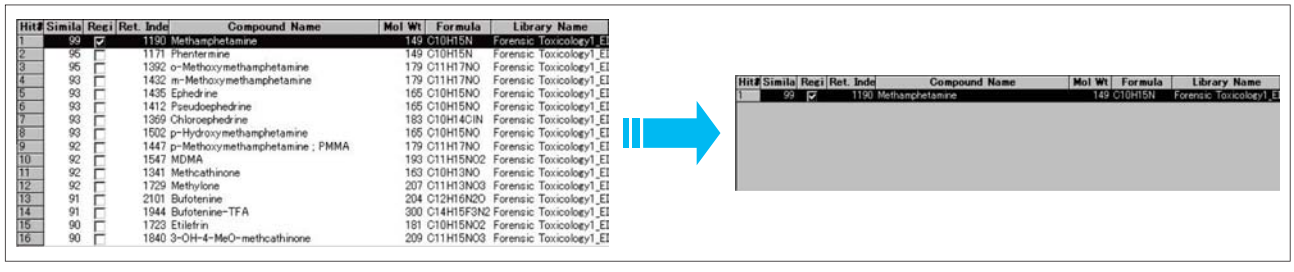


Fig. 6.9.3 Library Search Result for Methamphetamine
(a) Using mass spectrum, (b) Using mass spectrum and retention index

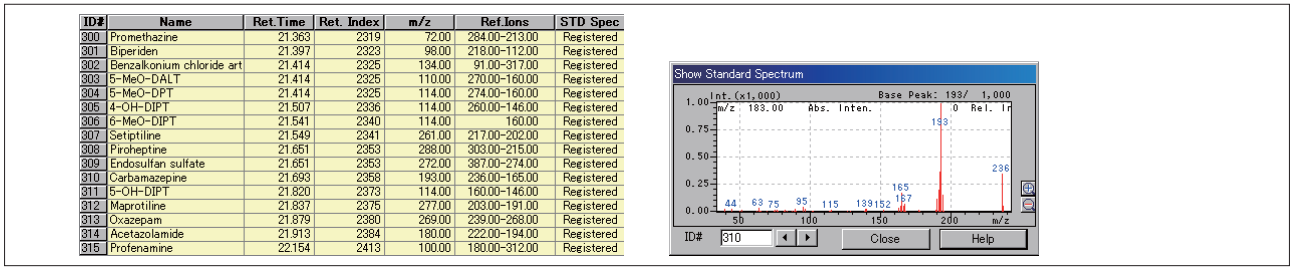
■Explanation

"GC/MS Forensic Toxicological Database" contains a library of mass spectra, method files containing the names, quantitation and reference ions, standard mass spectra, and retention indices specific to each of these drugs of abuse. Here we introduce quick and easy techniques for detecting and conducting semi-quantitation of harmful drugs using method files.

■Content of Forensic Toxicological Method Files

Fig. 6.9.4 shows a view of the information contained in the GC/MS Forensic Toxicological Database, including chemical names, quantitation and reference ions, standard mass spectra, and retention indices.
Furthermore, relative response factors are also included for specific toxic substances, which can be used to calculate rough estimates of their concentrations in real-world samples.

6.9 GC/MS Forensic Toxicological Database (3) - GC/MS



ID#	Name	Ret.Time	Ret. Index	m/z	Ref.Ions	STD Spec
300	Promethazine	21.363	2319	72.00	284.00-213.00	Registered
301	Biperiden	21.397	2323	98.00	218.00-112.00	Registered
302	Benzalkonium chloride art	21.414	2325	134.00	91.00-317.00	Registered
303	5-MeO-DALT	21.414	2325	110.00	270.00-160.00	Registered
304	5-MeO-DPT	21.414	2325	114.00	274.00-160.00	Registered
305	4-OH-DIPT	21.507	2336	114.00	260.00-146.00	Registered
306	6-MeO-DIPT	21.541	2340	114.00	160.00	Registered
307	Setiptiline	21.549	2341	261.00	217.00-202.00	Registered
308	Piroheptine	21.651	2353	288.00	303.00-215.00	Registered
309	Endosulfan sulfate	21.651	2353	272.00	387.00-274.00	Registered
310	Carbamazepine	21.693	2358	193.00	236.00-165.00	Registered
311	5-OH-DIPT	21.820	2373	114.00	160.00-146.00	Registered
312	Maprotiline	21.837	2375	277.00	263.00-191.00	Registered
313	Oxazepam	21.879	2380	269.00	239.00-268.00	Registered
314	Acetazolamide	21.913	2384	180.00	222.00-194.00	Registered
315	Profenamine	22.154	2413	100.00	180.00-312.00	Registered

Fig. 6.9.4 Registered Information in Method File

■Peak Detection Using Mass Chromatograms and Predicted Retention Time

Fig. 6.9.5 shows the screen used to analyze the data obtained from analysis of a drug-spiked urine sample using this method. When conducting actual analysis, it is very difficult to confirm the target peak associated with the drug of abuse because it is buried in the total ion current chromatogram (TICC), as shown in the upper part of the figure. Use of this method file allows easy detection of the drug of abuse even in such cases of difficult-to-pinpoint target drug chromatographic peaks. First, the retention time of the target substance is predicted using the GCMSsolution AART (Automatic Adjustment of Retention Time) feature, based on the retention times of *n*-alkanes measured beforehand, in addition to the registered retention index for each of the toxic substances. Then, the target drug substance is detected from the mass chromatogram (shown at the lower left of Fig. 6.9.5) of the quantitation and reference ions in the vicinity of the predicted retention time. These operations can be performed using the automatic processing function of the GCMSsolution software. Moreover, the final result can be verified through comparison with the standard spectrum, which is registered in the method file; the standard spectrum is shown here in the middle tier.

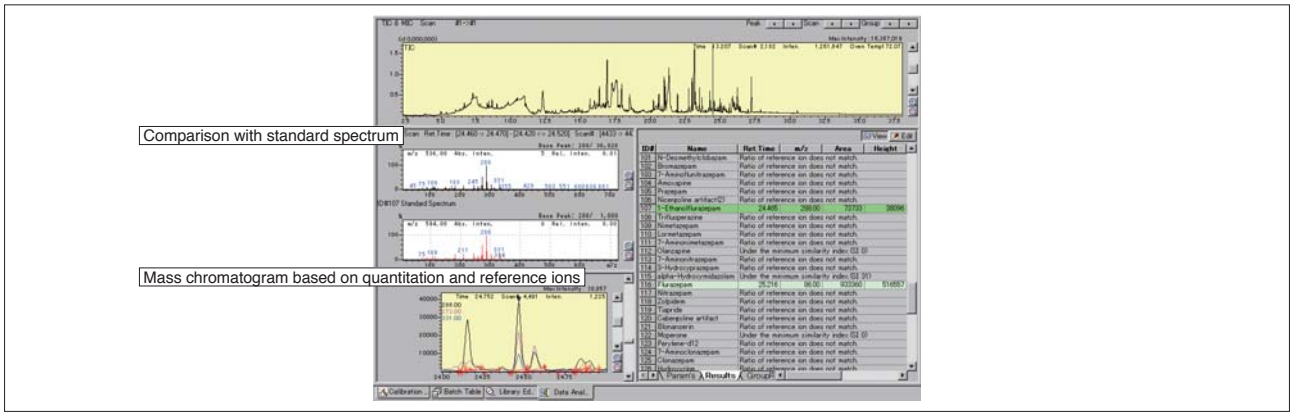
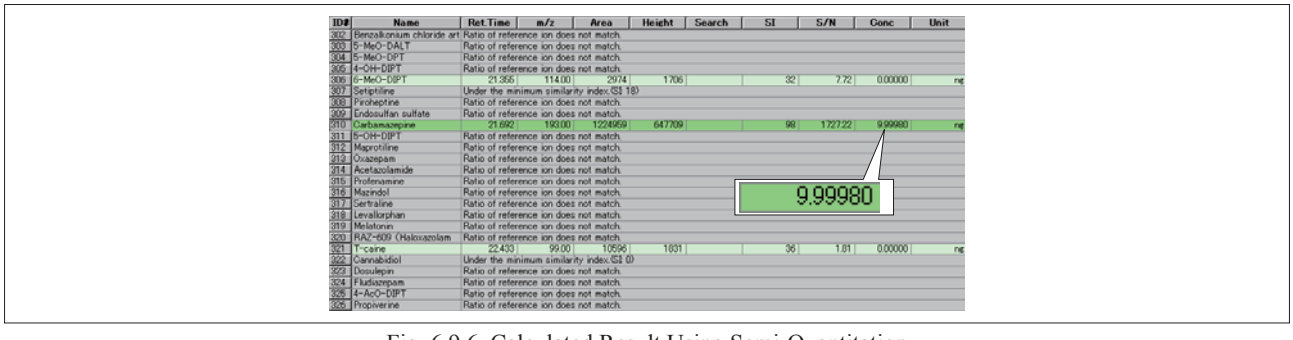


Fig. 6.9.5 Screen Capture of Data Processing Using GCMSsolution

■Semi Quantitation-Calculation

This database method file contains relative response factors which are used for calculating semiquantitative values for specific toxic drugs. As shown in Fig. 6.9.6, the drug concentration can be roughly calculated without generating a calibration curve.



ID#	Name	Ret.Time	m/z	Area	Height	Search	SI	S/N	Conc	Unit
302	Benzalkonium chloride art	Ratio of reference ion does not match.								
303	5-MeO-DALT	Ratio of reference ion does not match.								
304	5-MeO-DPT	Ratio of reference ion does not match.								
305	4-OH-DIPT	Ratio of reference ion does not match.								
306	6-MeO-DIPT	21.366	114.00	2974	1706		32	7.72	0.00000	ng
307	Setiptiline	Under the minimum similarity index (SI 18)								
308	Piroheptine	Ratio of reference ion does not match.								
309	Endosulfan sulfate	Ratio of reference ion does not match.								
310	Carbamazepine	21.692	193.00	1224959	647709		98	1727.22	9.99980	ng
311	5-OH-DIPT	Ratio of reference ion does not match.								
312	Maprotiline	Ratio of reference ion does not match.								
313	Oxazepam	Ratio of reference ion does not match.								
314	Acetazolamide	Ratio of reference ion does not match.								
315	Profenamine	Ratio of reference ion does not match.								
316	Mazindol	Ratio of reference ion does not match.								
317	Sertraline	Ratio of reference ion does not match.								
318	Levallorphan	Ratio of reference ion does not match.								
319	Melatonin	Ratio of reference ion does not match.								
320	RAZ-609 (Haloxazolam	Ratio of reference ion does not match.								
321	T-caine	22.433	99.00	10696	1831		36	1.81	0.00000	ng
322	Cannabidiol	Under the minimum similarity index (SI 0)								
323	Dosulepin	Ratio of reference ion does not match.								
324	Fludiazepam	Ratio of reference ion does not match.								
325	4-AcO-DIPT	Ratio of reference ion does not match.								
326	Propiverine	Ratio of reference ion does not match.								

Fig. 6.9.6 Calculated Result Using Semi-Quantitation

6.10 Analysis of Psychotropic Drugs in Whole Blood Utilizing Simultaneous Scan/MRM Measurements (1) - GC/MS

■Explanation

When analyzing medicinal toxicants using GC/MS, the presence of fatty acids and cholesterol, which exist in large quantities in whole blood, can interfere with detection. In fact, profiles for triazolam and etizolam, benzodiazepine psychotropic drugs, overlap with the cholesterol chromatogram, making data analysis difficult with a single GC-MS system. Furthermore, the retention indices for triazolam and etizolam are adjacent, and both have characteristic *m/z* ratios of 313 and 342, respectively (Fig. 6.10.1), making it even more difficult to distinguish these compounds. Consequently, there are high expectations for utilizing GC-MS/MS. Here we introduce an example of simultaneous scan/MRM measurements for the mass separation of cholesterol from triazolam and etizolam in whole blood.

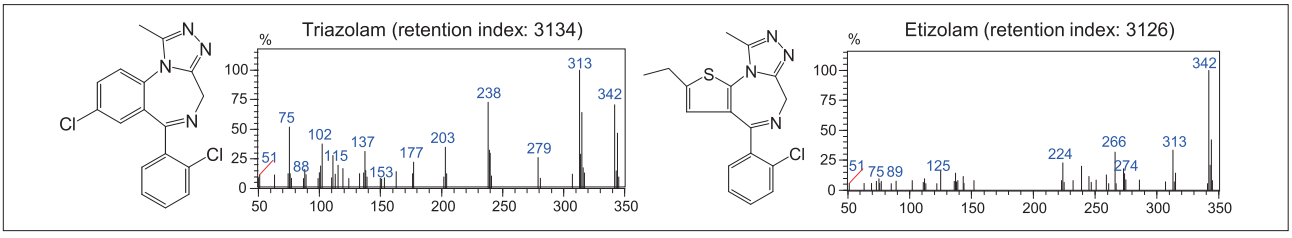


Fig. 6.10.1 Scan Mass Spectra and Retention Indices for Triazolam and Etizolam

■Sample Pretreatment

The liquid-liquid extraction method via EXtrelut®NT3 was used for pretreatment of the whole blood sample. For both the acidic fraction and basic fraction, 1 mL of the collected whole blood sample was measured, and each was then diluted with 1 mL of Milli-Q water. The acidic fraction was adjusted to a pH of 5 using 10 % perchloric acid, and the basic fraction was adjusted to a pH of 9 with 10 % ammonia water. Each solution was poured into EXtrelut®NT3, and after leaving to stand 30 minutes, eluted with a 10 mL mixed solution of chloroform and isopropanol (3 :1). Afterwards, the extracted acidic fraction and basic fraction liquids were mixed, and following desiccation with silica gel, dried and hardened under a nitrogen gas flow. The resulting sample was then re-dissolved with a 200 µL mixed solution of chloroform and isopropanol (3 :1). In order to calculate semi-quantitative values utilizing the "GC/MS Forensic Toxicological Database", the custom internal standard (P/N: 560294, from Shimadzu GLC), which contains 8 PAH-d isomers, was adjusted to a concentration of 1 µg/mL for use as an internal standard sample. The adjusted extracted sample and the internal standard sample were injected simultaneously into the GC-MS/MS system using the AOC-20i+s solvent flush mode.

■Analytical Conditions

Simultaneous scan/MRM measurements were performed on the extracted sample. The MRM measurement targeted the triazolam and etizolam, and the scan data was used simultaneously screen for other medicinal toxicants utilizing the "GC/MS Forensic Toxicological Database".

Instrument : GCMS-TQ8030
Column : Rxi®-5Sil MS (30 m × 0.25 mm I.D. df = 0.25 µm)
Glass Insert : Splitless insert with glass wool

[GC]
Injector Temp. : 260 °C
Column Temp. : 60 °C (2 min) → 10 °C /min → 320 °C (10 min)
Injection Method : Splitless Injection
Flow Control Mode : Linear velocity (45.6 cm/sec)
Injection Volume : 1 µL

[MS]
Interface Temp. : 280 °C
Ion Source Temp. : 200 °C
Acquisition Mode : Scan//MRM
Scan Event Time : 0.15 sec
Scan Mass Range : *m/z* 45 - 700
Scan Speed : 5,000 u/sec

MRM Monitoring *m/z*

Compound Name	Retention Time	Quantitative Transition		Qualitative Transition 1		Qualitative Transition 2	
		Precursor > Product	CE (V)	Precursor > Product	CE (V)	Precursor > Product	CE (V)
Etizolam	27.149	342.00 > 272.00	24	342.00 > 245.00	33	342.00 > 266.00	20
Triazolam	27.171	313.00 > 277.00	25	313.00 > 278.00	18	313.00 > 242.00	35

6.10 Analysis of Psychotropic Drugs in Whole Blood Utilizing Simultaneous Scan/MRM Measurements (2) - GC/MS

■Results

Figs.6.10.2 and 6.10.3 show the mass chromatograms obtained from scan/MRM measurements of the whole blood extracted sample (blank), which did not contain etizolam or triazolam, and the sample created by adding etizolam and triazolam to the blank sample in order to reach a concentration of 500 ng/mL. In the scan mass chromatogram, the cholesterol is detected at the same retention time as etizolam and triazolam, making it difficult to determine the presence of these psychotropic drugs. However, 2-stage mass separation via MRM enables separation from the cholesterol, making selective detection of etizolam and triazolam possible. In the scan measurement, similar mass spectra are indicated for etizolam and triazolam, but in the MRM chromatogram, they could be separated and confirmed without mutual influences.

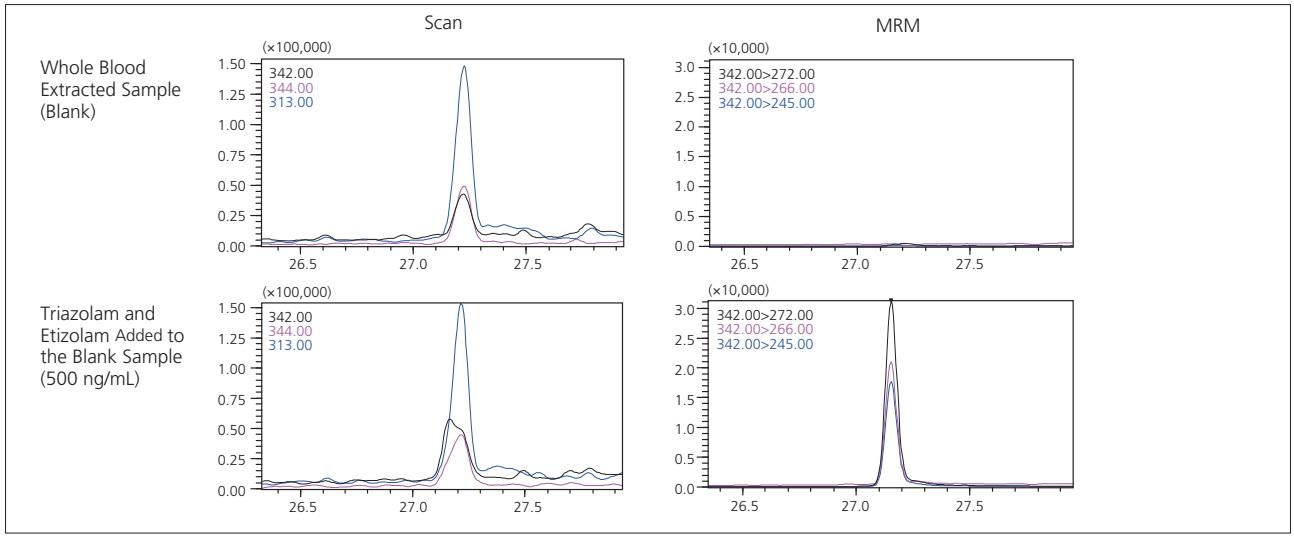


Fig. 6.10.2 Scan and MRM Mass Chromatograms of Etizolam in a Whole Blood Sample
(Left: Scan, Right: MRM, Top: Whole blood extracted sample (Blank),
Bottom: Sampled created by adding triazolam and etizolam to the blank sample (500 ng/mL))

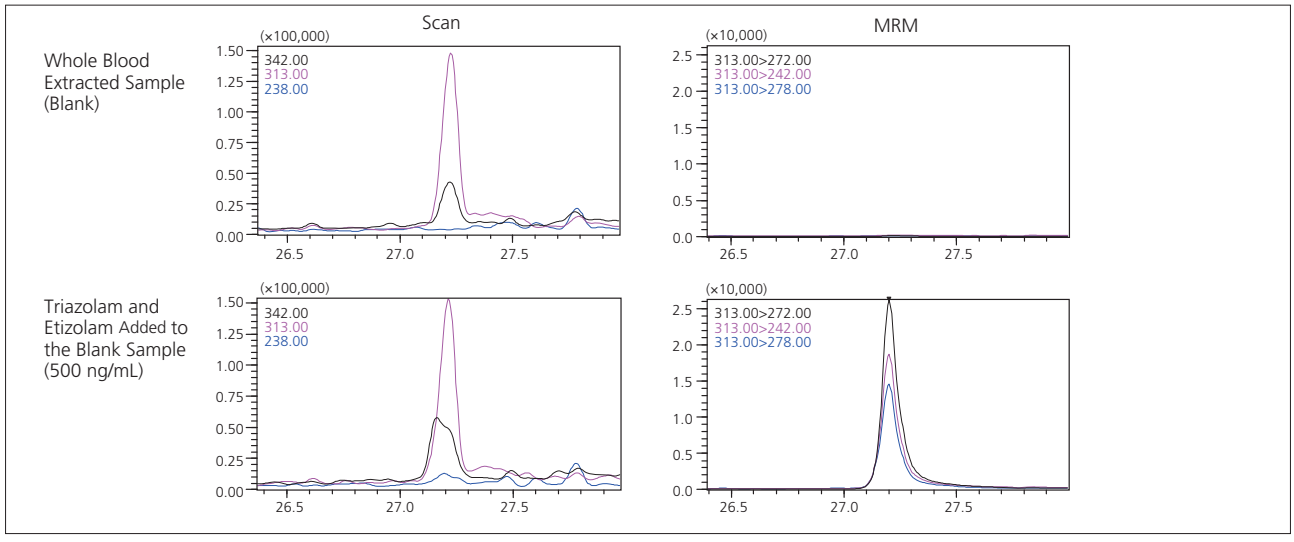


Fig. 6.10.3 Scan and MRM Mass Chromatograms of Triazolam in a Whole Blood Sample
(Left: Scan, Right: MRM, Top: Whole blood extracted sample (Blank),
Bottom: Sampled created by adding triazolam and etizolam to the blank sample (500 ng/mL))

6.10 Analysis of Psychotropic Drugs in Whole Blood Utilizing Simultaneous Scan/MRM Measurements (3) - GC/MS

■Explanation

In GC-MS/MS MRM mode, the MRM transition and collision energy (CE) must be optimized. In addition, when performing a comprehensive analysis of medicinal toxicants in biological samples, optimization of these parameters for each relevant medicinal toxicant is an extremely laborious process. The GCMS-TQ8030 Triple Quadrupole Gas Chromatograph Mass Spectrometer (GC-MS/MS) achieves simultaneous scan/MRM measurements through high-speed scan and high-speed MRM data sampling techniques. Comprehensive analysis of medicinal toxicants is achieved by combining the MRM mode, used to measure toxicants requiring high sensitivity or that are prone to overlap with foreign materials, with the scan mode, which is used to measure toxicants for which sensitivity is sufficient. The "GC/MS Forensic Toxicological Database" contains the retention indices, characteristic *m/z* (quantitative ions and reference ions) mass chromatograms, and mass spectral data for approximately 500 medicinal toxicant components. As a result, screening can be performed by applying this information to the scan data from simultaneous scan/MRM analysis. Here we introduce the application of scan data from simultaneous scan/MRM measurement to the Forensic Toxicological Database, leading to comprehensive screening for medicinal toxicants in a whole blood sample.

■GC/MS Forensic Toxicological Database

The "GC/MS Forensic Toxicological Database" contains the names of medicinal toxicants, as well as their quantitative and reference ions, retention indices, and standard mass spectra. In terms of the retention times for the registered medicinal toxicants, reliable simultaneous estimation of everything from low boiling point to high boiling point components can be performed using the measurement data from *n*-alkane mixed standard samples (C9-C33, provided by Restek Corporation, P/N: 560295) via the GCMSsolution AART function.

The presence or absence of medicinal toxicant content can be determined easily from the registered quantitative and reference ion mass chromatograms.

In addition, relative response factors utilizing internal standards have been registered for medicinal toxicants often involved in cases of abuse, enabling the calculation of approximate concentration values (semi quantitative values).

■Experimental

The liquid-liquid extraction method via EXtrelut®NT3 was used for pretreatment of the whole blood sample. Simultaneous scan/MRM measurements were performed on the extracted sample. The MRM measurement targeted triazolam and etizolam, and the scan data was used for simultaneous screening for medicinal toxicants utilizing the "GC/MS Forensic Toxicological Database".

In order to calculate semi-quantitative values, the custom internal standard (provided by Restek Corporation P/N: 560294), which contains 8 PAH-d isomers, was adjusted to a concentration of 1 µg/mL. The extracted sample and the adjusted internal standard sample were injected simultaneously into the GC-MS/MS system using the AOC-20i+s solvent flush mode.

Instrument : GCMS-TQ8030
Column : Rxi®-5Sil MS (30 m × 0.25 mm I.D. df = 0.25 µm)
Glass Insert : Splitless insert with glass wool (P/N: 221-48876-03)

[GC]
Injector Temp. : 260 °C
Column Temp. : 60 °C (2 min) → 10 °C /min → 320 °C (10 min)
Injection Method : Splitless Injection
Flow Control Mode : Linear velocity (45.6 cm/sec)
Injection Volume : 1 µL

[MS]
Interface Temp. : 280 °C
Ion Source Temp. : 200 °C
Acquisition Mode : Scan//MRM
Scan Event Time : 0.15 sec
Scan Mass Range : *m/z* 45 - 700
Scan Speed : 5,000 u/sec

MRM Monitoring *m/z*

Compound Name	Retention Time	Quantitative Transition		Qualitative Transition 1		Qualitative Transition 2	
		Precursor > Product	CE (V)	Precursor > Product	CE (V)	Precursor > Product	CE (V)
Etizolam	27.149	342.00 > 272.00	24	342.00 > 245.00	33	342.00 > 266.00	20
Triazolam	27.171	313.00 > 277.00	25	313.00 > 278.00	18	313.00 > 242.00	35

6.10 Analysis of Psychotropic Drugs in Whole Blood Utilizing Simultaneous Scan/MRM Measurements (4) - GC/MS

■Results

Fig. 6.10.4 shows the scan chromatogram for the extracted whole blood sample, measured in the simultaneous scan/MRM analysis. By applying the Forensic Toxicological Database to the scan data, it was possible to identify the benzodiazepine psychotropic drugs (diazepam and desmethyldiazepam). To date, the method utilized for compound identification has involved a library search of the peak mass spectrum detected with the total ion current chromatogram (TICC). However, the method utilizing the Forensic Toxicological Database uses the estimated retention times and characteristic *m/z* mass chromatogram (MC) for detection as shown in Fig. 6.10.5. Accordingly, it enables quick and easy determination of the presence or absence of low-concentration medicinal toxicants that cannot be confirmed with the conventional method. In addition, the mass spectra of the detected medicinal toxicants can be compared to registered standard mass spectra, thereby improving the reliability of the data analysis. By performing simultaneous scan/MRM analysis, it becomes possible to simultaneously screen for components not targeted by MRM measurements.

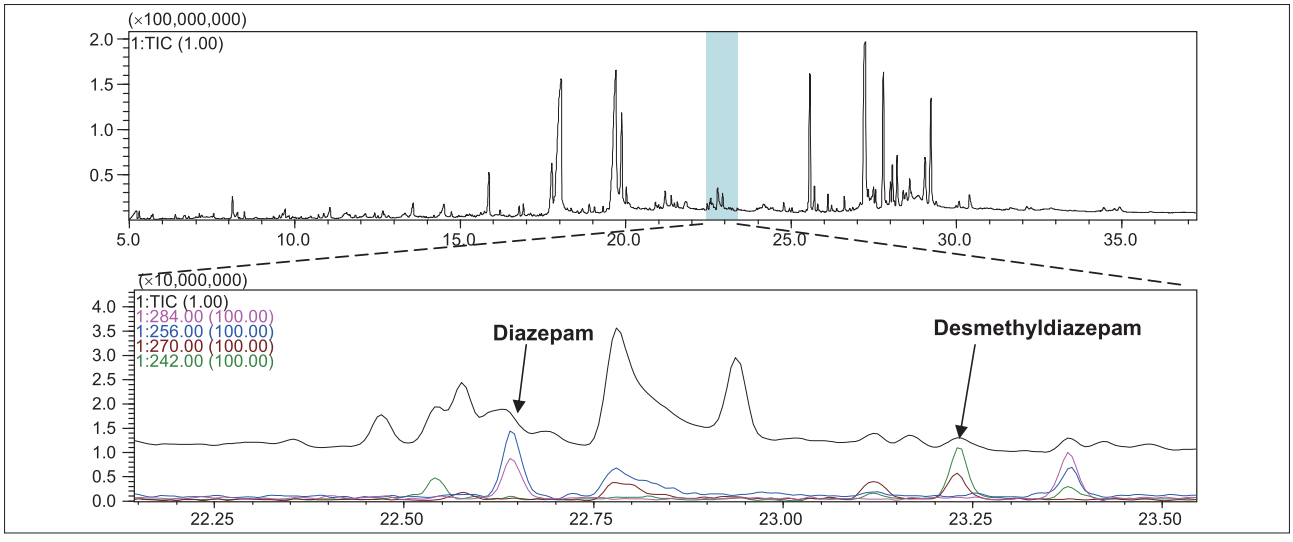


Fig. 6.10.4 Scan Chromatogram for Measured Whole Blood Sample
(Top: Scan TICC; Bottom: Enlarged TICC and MC for detected medicinal toxicants)

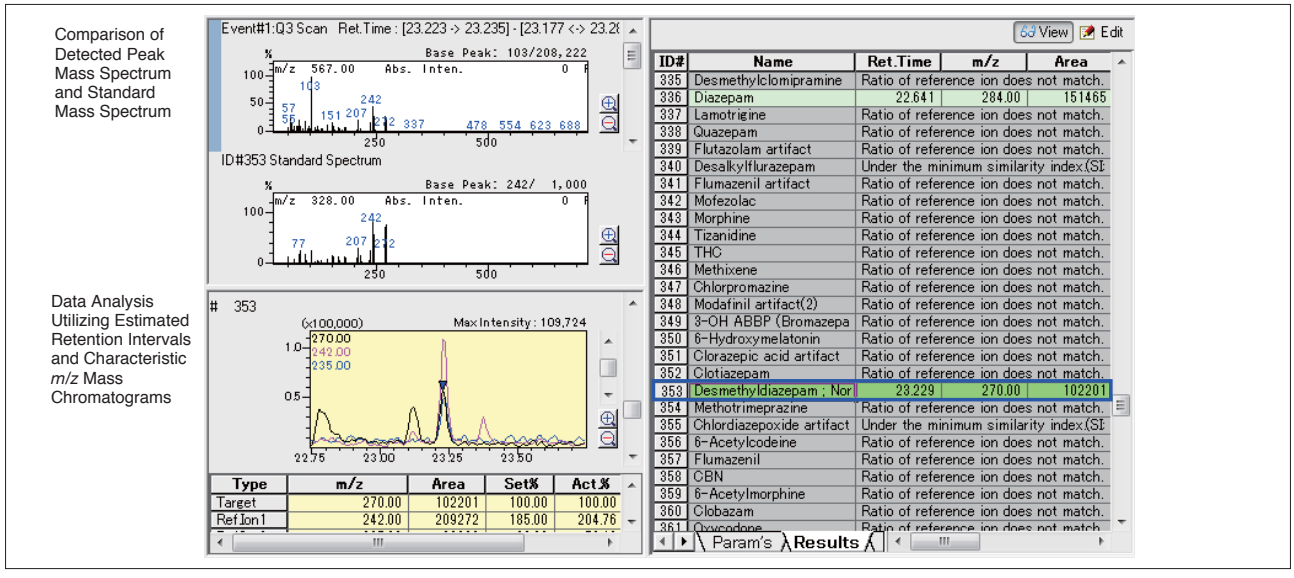


Fig. 6.10.5 Scan Data Analysis Window Using the "GC/MS Forensic Toxicological Database"

7. Pharmaceutical Related

7.1 Introduction of Performance Validation Software for Improved GLP/GMP Support (1) - UV

■Explanation

Presently, validation of analytical instruments is a requirement in various regulations and standards, however, conducting instrument validation manually is a cumbersome task. This software conforms to the JIS K0115 General Rules for Molecular Absorptiometric Analysis, as well as testing stipulated by the Japanese Pharmacopoeia (JP), European Pharmacopoeia (EP), and US Pharmacopeia (USP). Thus, performance validation tasks involving condition settings and other complex operations are greatly facilitated with this software.

■Validation

The manufacturing control and quality control in manufacturing sites for quality management of drugs and quasi-drugs is referred to as GMP (Good Manufacturing Practice), and in Japan, according to the "Ministerial Ordinance Concerning Standards for Manufacturing Control and Quality Control of Drugs and Quasi-Drugs (Ministry of Health, Labour and Welfare Ordinance No.179, December 24,2004), it is stated that ["Validation" means to verify and document that the buildings and facilities of the manufacturing site, procedures, processes, and other procedures of the manufacturing control and quality control (hereinafter referred to as "manufacturing procedure, etc.") provide the anticipated results.] Regarding analytical equipment, it states that "Validation refers to the periodic inspection and maintenance (including calibration) which must be conducted according to previously documented procedures, and that such records of inspection and maintenance must be retained." The software for this validation has been incorporated as standard in the UV-2600/2700. Table 7.1.1 shows the Performance Validation Software inspection items and which standards require which items of inspection. This software supports all of the necessary items. Of these, 9 items related to "instrument performance testing" of spectrophotometers are described in the General Rules for Molecular Absorptiometric Analysis. However, there is no clear reference to the range of compliance. Even in the US Pharmacopeia, while the required inspection items are discussed, there is no clear specification of the range of compliance. On the other hand, the required tests and compliance ranges are both specified in the Japanese Pharmacopoeia and European Pharmacopoeia, International harmonization of Japanese, the United States and European drug regulations is currently in progress however, there is not yet any common validation requirements for spectrophotometers.

Table 7.1.1 Inspection Items of Performance Validation Software

Inspection Item	JIS	JP	EP	USP
Wavelength accuracy	○	○	○	○
Wavelength repeatability	○	○		
Photometric accuracy	○	○	○	○
Photometric repeatability	○	○		
Resolution	○		○	
Stray light	○		○	
Noise level	○			
Baseline flatness	○			
Baseline stability	○			

■Japanese Pharmacopoeia (JP)

Spectrophotometric wavelength and transmittance testing is described in the "Apparatus and Adjustment" section of the "General Tests, Processes and Apparatus –Ultraviolet-visible Spectrophotometry" of 16th Edition of the Japanese Pharmacopoeia.

Wavelength testing is to be conducted using either a commercially available wavelength calibration optical filter or a low-pressure mercury lamp or a deuterium discharge lamp. Among these, wavelength testing is typically conducted using the 2 bright lines (486.00 nm, 656.10 nm) of the deuterium discharge lamp provided as standard with the instrument. On the other hand, in the case of the low-pressure mercury lamp, 4 bright lines are used (253.65 nm, 365.02 nm, 435.84 nm and 546.07 nm), permitting wavelength testing over a wide range, including the ultraviolet region. Thus, we used the optional low-pressure mercury lamp unit for the UV-2600/2700. Fig. 7.1.1 shows an external view of the low-pressure mercury lamp unit installed in the UV-2600.

When bright lines are used, the acceptable wavelength range for wavelength accuracy* is within ±0.3 nm, and for wavelength repeatability*, it is within ±0.2 nm of the mean value. When the filter is used, the wavelength accuracy* must be within ±0.5 nm, and the wavelength repeatability* must be within ±0.2 nm of the mean value. In addition, for transmittance and absorbance, testing must be conducted using a commercially available transmittance calibration filter.

Regarding the acceptable ranges of transmittance or absorbance, for photometric accuracy*, it must be within the upper limit and low lower limit plus 1 %, respectively, of the relative accuracy indicated in the transmittance calibration filter test results document. For photometric repeatability*, when the absorbance is 0.500 or less, it must be within ±0.002 of the mean value, and when the absorbance exceeds 0.500, it must be within ±0.004 of the mean value. Also, if multiple transmittance calibration filters having different transmittances are used together, it is advisable to verify the linearity.

7.1 Introduction of Performance Validation Software for Improved GLP/GMP Support (2) - UV

Setting the various test conditions for these validation items, conducting the measurements, and then manually calculating the measurement values is extremely complex work. That is what makes the Performance Validation Software so useful. With this software, all that is required is to select the test item, and validation is easily conducted.

Fig. 7.1.1 Low-Pressure Mercury Lamp Unit

■European Pharmacopoeia (EP)

In the "Absorption spectrophotometry, ultraviolet and visible" section of the 7th Edition of the European Pharmacopoeia, there are requirements related to wavelength accuracy*, photometric accuracy*, resolution*, and stray light* for ultraviolet-visible spectrophotometers. There are no items of resolution* or stray light* in the Japanese Pharmacopoeia. Additionally, there is a description regarding slit width setting and the optical path length of the measurement cell. Regarding wavelength accuracy*, this can be checked using any of holmium perchlorate solution absorption bands, or the bright lines of a hydrogen-discharge tube, deuterium-discharge tube, or low-pressure mercury lamp, whichever is appropriate. The acceptable range is within ±1 nm in the ultraviolet region, and ±3 nm in the visible region. It is stated that photometric accuracy* can be checked using a suitable absorption filter or potassium dichromate solution. The acceptable range is within ±0.010 of the absorbance. Regarding stray light*, this can be checked using an absorption filter or 12 g/L potassium chloride solution. At 198 nm, absorbance must be 2.0 or greater (with transmittance, 1 % or less). Resolution* is to be checked using hexane solution containing 0.02 % (V/V) of toluene. The ratio of the maximum absorption value at 269 nm and the minimum absorption value at 266 nm must be checked. The Performance Validation Software supports both the stray light and resolution items of the European Pharmacopoeia, permitting easy validation of all the specified items.

■The United States Pharmacopeia (USP)

In the "spectrophotometry and light-scattering" section of the United States Pharmacopeia, there are requirements related to wavelength accuracy* and photometric accuracy* for ultraviolet-visible spectrophotometers. First, regarding wavelength accuracy*, it is stated that this can be checked using the bright lines of a low-pressure mercury lamp, the bright lines of a hydrogen-discharge tube, or an appropriate didymium or holmium, etc. glass filter having absorption in the ultraviolet region. It is stated that photometric accuracy* can be checked using a standard glass filter or a solution of known transmittance, such as potassium dichromate. However, specific acceptable ranges are not mentioned for these items. In the Performance Validation Software, the strictest values within the acceptable ranges of other regulations and standards are set as recommended values.

Note) The descriptions associated with the regulations and specifications are those that were current as of September, 2011.
Note) Items indicated with an asterisk (*) are the names of items that are supported in the Performance Validation Software.

■Example of Instrument Validation

Fig. 7.1.2 shows the Performance Validation Software screen. The screen is divided broadly into 4 panes.

(1) File Information Pane
This is where information is entered for automatic saving of files following measurement. Testing can not start unless the required items are entered to prevent omissions of necessary items. Also, measured data is saved without fail, so there is no forgetting to record data.

(2) Inspection Items Pane
This is where inspection items are verified. The items are displayed in an easy-to-understand list. Pressing the Conditions setting button in the toolbar displays the Inspection Conditions setting screen, where items to be displayed can be added or removed. Fig. 7.1.3 shows the screen for setting the inspection conditions. The items required to be inspected can easily be selected using the checkboxes. Displaying the detailed conditions makes it easy to modify the acceptable ranges and perform settings according to recommended values.

(3) System State Pane
This pane displays the remaining inspection time and inspection progress status (inspection log).

7.1 Introduction of Performance Validation Software for Improved GLP/GMP Support (3) - UV

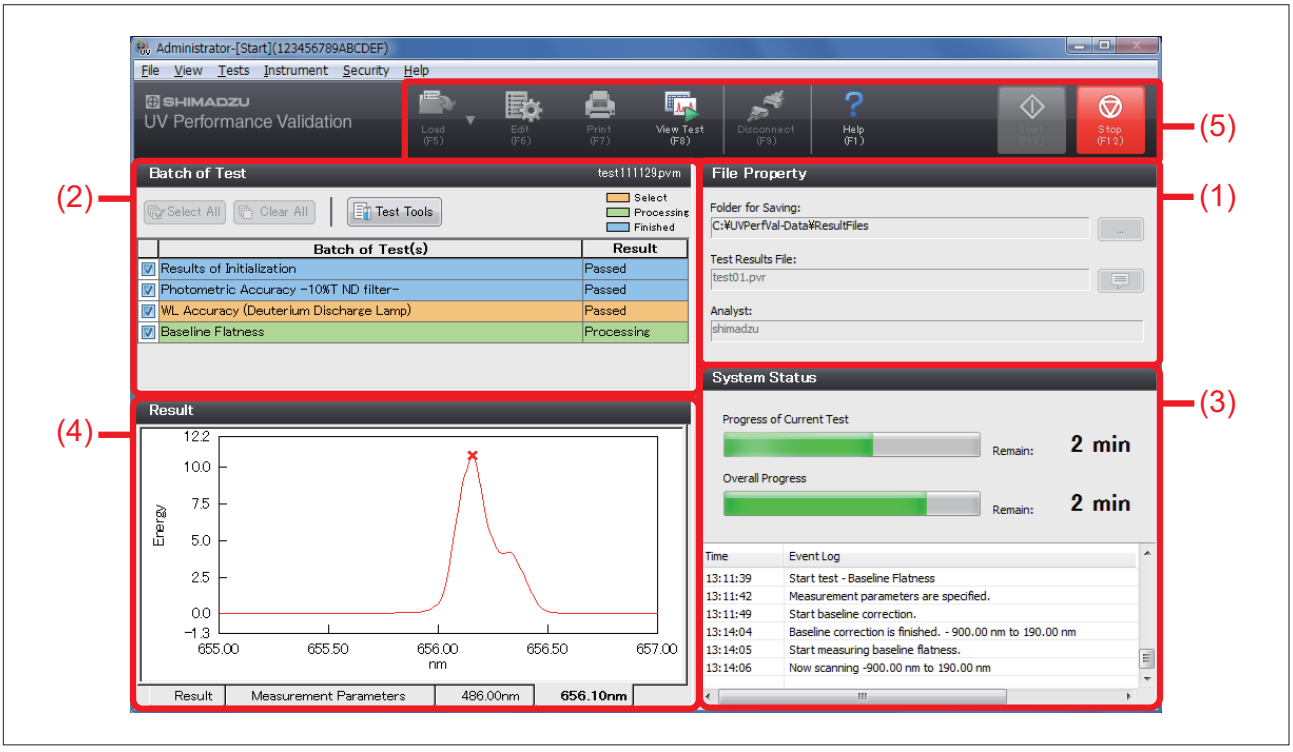


Fig. 7.1.2 Performance Validation Software Screen

(4) Inspection Results Pane
The data of the inspection currently in progress or the results of selected inspection items are displayed. Also, switching between tabs at the bottom of the screen allows viewing of measurement data and conditions.

(5) Toolbar
Functions that are used often are displayed as tool buttons.

After verifying the items to be included in the inspection, the inspection is started by pressing the Start button. Since the instrument conditions and other settings are conducted automatically by the software, the operation is nearly effortless for the analyst. All that is required is to check that the sample compartment is empty and to insert or remove filters according to the on-screen directions.
When the inspection is completed, a pass/fail assessment result is displayed for each inspection item in pane (2). To print all of the inspection results, all that is required is to press the Print button. The printout contains the details and the pass/fail assessment result for each of the inspection items. Moreover, the measurement results are automatically saved as an electronic file. Printout examples of wavelength accuracy (low-pressure mercury lamp) and resolution (toluene/hexane method) inspection results newly supported in the Performance Validation Software are shown in Fig. 7.1.4 and Fig. 7.1.5.

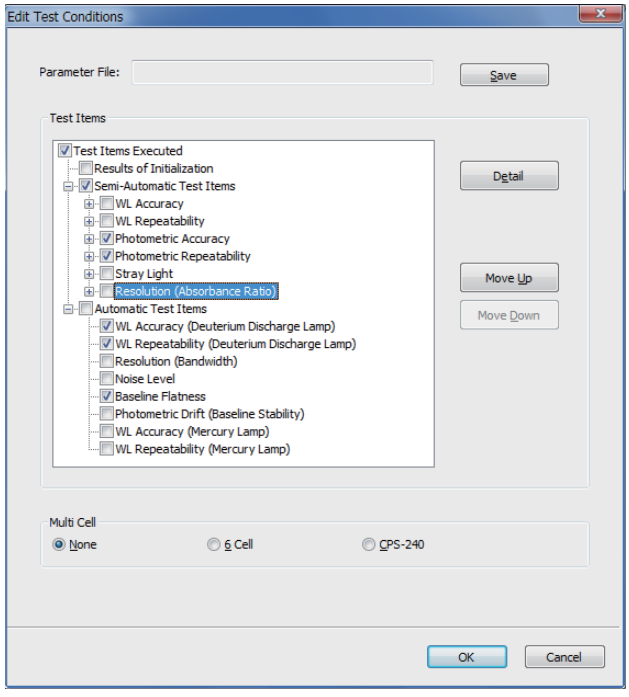
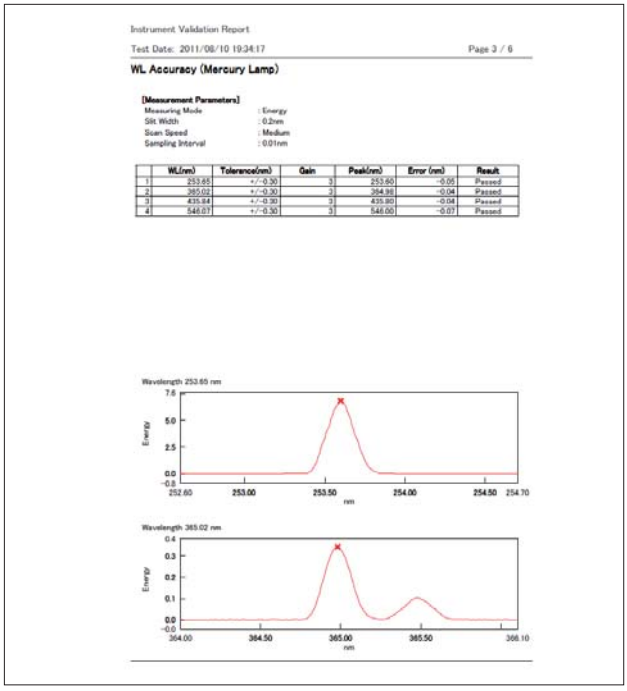
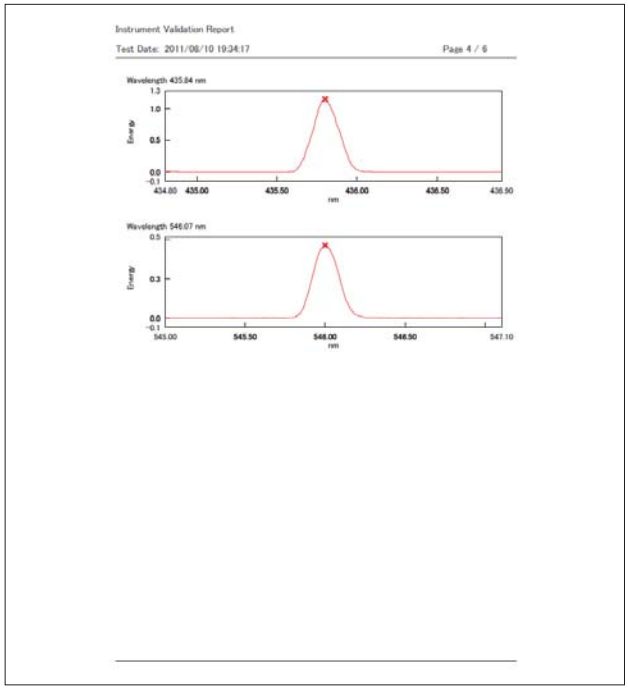


Fig. 7.1.3 Screen for Setting of Inspection Items

7.1 Introduction of Performance Validation Software for Improved GLP/GMP Support (4) - UV

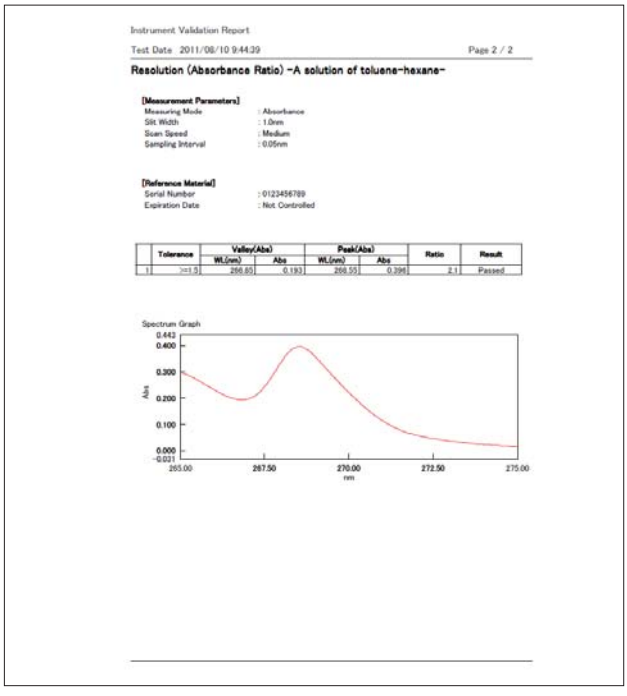


Wavelength Accuracy (Low-Pressure Mercury Lamp) First Page



Wavelength Accuracy (Low-Pressure Mercury Lamp) Second Page

Fig. 7.1.4 Printout Results (1)



Resolution (Toluene/Hexane Method)

Fig. 7.1.5 Printout Results (2)

■Conclusion

Using this Performance Validation Software makes it easy to conduct an entire series of required regulation and standards-related operations; from the setting of validation conditions, to data measurement, pass/fail judgment, results printout and file storage.

7.2 Quantitation Limit of Pharmaceuticals (1) - UV

■Explanation

Quality control and securing product safety are important in the manufacture of pharmaceutical products, so it is essential that substances other than those specifically approved are not included with the specified ingredients. Cleaning validation is one of the measures specified in the GMP standard, because cleaning of manufacturing equipment is essential for preventing contamination and cross contamination of pharmaceutical products. This means that contaminants from the environment must not become mixed in with the product ingredients, and residual substances adhering to the manufacturing equipment must not contaminate the next product to be processed by that equipment. To verify these requirements, the cleaning itself must be evaluated, and the analytical instruments typically used for cleaning validation are the ultraviolet-visible spectrophotometer, the total organic carbon analyzer, and the high-performance liquid chromatograph. The quantitation limit of an analytical instrument is the limit value at which residual sample can be quantitated. To determine whether or not the analytical instrument to be used for conducting cleaning validation can quantitate down to the permissible level of the residual substance, it is important to determine the quantitation limit. Here we introduce the determination of quantitation limit for the Shimadzu UV-1800 ultraviolet-visible spectrophotometer by absorption photometry, with samples consisting of detergent A used for cleaning in the pharmaceutical field, and the typically used pharmaceutical materials acetylsalicylic acid and isopropylantipyrine, presented along with the calculation method.

■Determining the Quantitation Limit

One method of obtaining the quantitation limit is to determine the concentration value that corresponds to the absorbance which is 10 times the noise level. The actual measurement method involves first measuring the absorption spectrum of a standard sample, and noting the wavelength of the greatest absorption peak. Next, measure the absorbance values at the wavelength of the greatest absorption peak using several samples of known concentration. The slope of the calibration curve is determined from the relationship between the concentrations of the samples and the respective absorbance values. Lastly, repeat measurement of a blank sample (dilute solvent) is conducted and the standard deviation is obtained. The quantitation limit is calculated from the slope of the calibration curve and the value equivalent to 10 times the standard deviation. Determination of the quantitation limits of detergent A, acetylsalicylic acid and isopropylantipyrine according to this method are respectively presented below.

■Analytical Conditions

Instrument	: UV-1800
Measurement Wavelength Range	: 190 to 300 nm (detergent A) 250 to 350 nm (acetylsalicylic acid and isopropylantipyrine)
Scan Speed	: Medium
Sampling Pitch	: 1 nm
Photometric Value	: Absorbance
Slit Width	: 1 nm
Lamp Switching Wavelength	: 340 nm
Cell	: 10 mm quartz cell

■Quantitation Limit of Detergent A

Fig. 7.2.1 shows the absorption spectrum of detergent A. The sample concentrations are 100 mg/L and 10 mg/L. Fig. 7.2.2 shows the calibration curve at a measurement wavelength of 225 nm, and Table 7.2.1 shows the results of 10 repeat measurements of a blank sample and the standard deviation σ. The quantitation limit for detergent A is determined to be 0.00096 ÷ 0.00599, ~ to 0.16 mg/L by 10 σ = 0.00096 Absorbance, and calibration curve formula Absorbance = 0.00599 Conc.

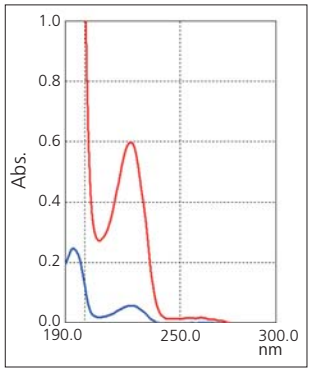


Fig. 7.2.1 Absorption Spectra of Detergent A

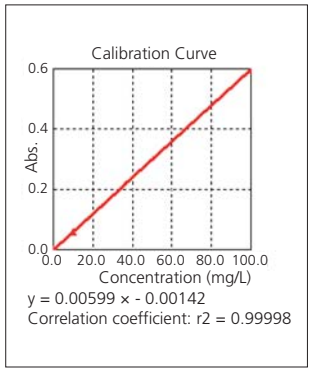


Fig. 7.2.2 Calibration Curve of Detergent A

Table 7.2.1 Absorbance of Blank Solution Measured Ten Times for Detergent A and Standard Deviation σ

Sample ID	WL225.0
1	0.00009
2	0.00020
3	0.00008
4	0.00011
5	0.00018
6	0.00012
7	0.00021
8	0.00034
9	0.00000
10	0.00006
Standard deviation σ	0.000096

7.2 Quantitation Limit of Pharmaceuticals (2) - UV

■Quantitation Limit of Acetylsalicylic Acid

Fig. 7.2.3 shows the absorption spectrum of acetylsalicylic acid methanol solution. The sample concentrations from higher-to-lower absorbance values are 400, 160, 80, 40, 20, and 8 mg/L. Fig. 7.2.4 shows the calibration curve at a measurement wavelength of 276 nm, and Table 7.2.2 shows the results of 10 repeat measurements of a blank sample and the standard deviation σ. The quantitation limit for acetylsalicylic acid is determined to be 0.42 mg/L.

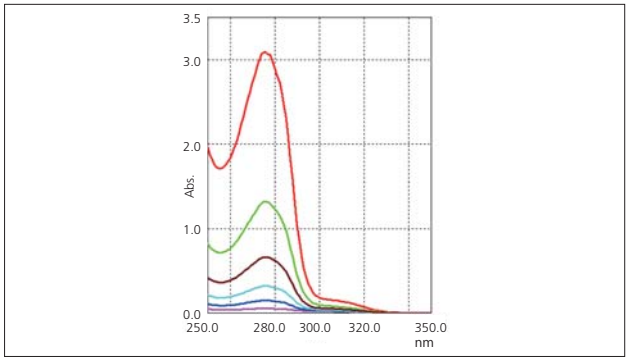


Fig. 7.2.3 Absorption Spectra of Acetylsalicylic Acid Methanol Solution

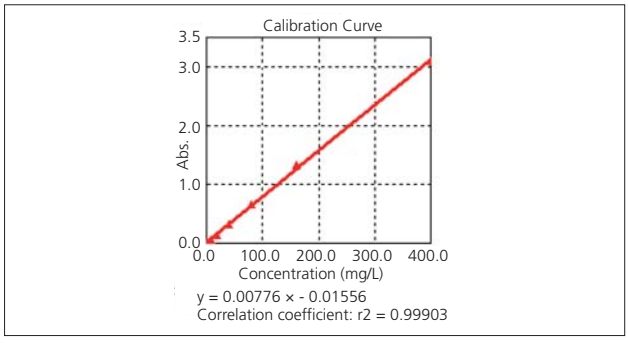


Fig. 7.2.4 Calibration Curve of Acetylsalicylic Acid

Table 7.2.2
Absorbance of Blank Solution Measured Ten Times for Acetylsalicylic Acid and Standard Deviation σ

Sample ID	WL276.0
1	0.00009
2	-0.00018
3	-0.00024
4	0.00018
5	0.00067
6	0.00079
7	0.00037
8	0.00024
9	0.00021
10	0.00035
Standard deviation σ	0.000325

■Quantitation Limit of Isopropylantipyrine

Fig. 7.2.5 shows the absorption spectrum of isopropylantipyrine methanol solution. The sample concentrations from higherto lower absorbance values are 80, 32, 16, 8, 4 and 1.6 mg/L. Fig. 7.2.6 shows the calibration curve at a measurement wavelength of 273 nm, and Table 7.2.3 shows the results of 10 repeat measurements of a blank sample and the standard deviation σ. The quantitation limit for isopropylantipyrine is determined to be 0.092 mg/L.

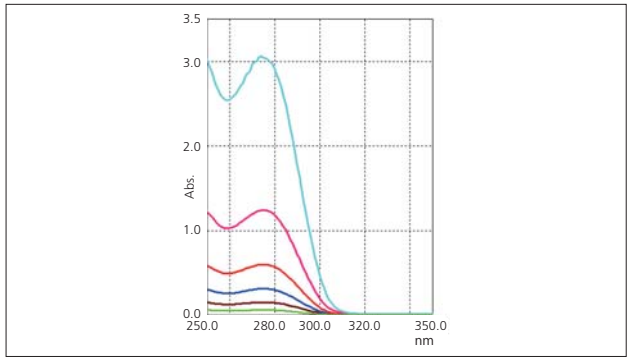


Fig. 7.2.5 Absorption Spectra of Isopropylantipyrine Methanol Solution

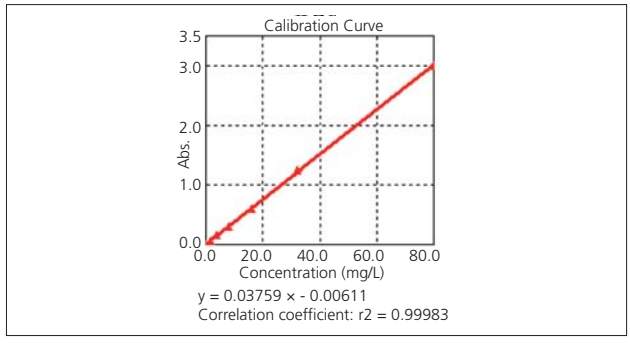


Fig. 7.2.6 Calibration Curve of Isopropylantipyrine

Table 7.2.3
Absorbance of Blank Solution Measured Ten Times for Isopropylantipyrine and Standard Deviation σ

Sample ID	WL273.0
1	0.00000
2	0.00005
3	0.00032
4	-0.00024
5	-0.00020
6	0.00023
7	-0.00011
8	0.00095
9	0.00032
10	0.00006
Standard deviation σ	0.000347

■Conclusions

The measurement results for detergent A, acetylsalicylic acid, and isopropylantipyrine were determined to illustrate the method of calculating quantitation limits based on measurements conducted using an UV-VIS spectrophotometer. Determination of the quantitation limit makes it possible to verify the lower limit of residual substances and residual detergent that can be quantitated. The validity of cleaning can be verified from various viewpoints using not just a UV-VIS spectrophotometer, but together with other types of instruments as well, including total organic carbon analyzers, high-performance liquid chromatographs, and high-performance liquid chromatograph mass spectrometers, for cleaning validation.

7.3 Analysis of Colored Contaminant on Surface of Tablet Using Infrared Microscope (1) - FTIR

■Explanation

Discoloration of tablet surface may be due to discoloration of the tablet itself, or by contamination of foreign colored substances. The analysis presented here is of the partial yellow discoloration of a tablet surface shown in the photograph of Fig. 7.3.1. The discolored area appears to be a stain in the tablet itself. In this case, the tablet surface irregularity makes direct measurement difficult by the use of microscopic ATR method, which requires close contact. Therefore, scrapings from the discolored portion were taken in the pretreatment process, and were analyzed using two different techniques, i.e., single reflection ATR and transmission microscopy.

■Measurement by Single Reflection ATR Method

Scrapings from the discolored and non-discolored areas of the tablet were analyzed using the single reflection ATR accessory (MIRacle). The obtained spectra of the discolored and non-discolored areas are shown in overlay fashion in Fig. 7.3.2. Because the spectral intensity of the discolored sample is considerably lower than that of non-discolored sample due to the small quantity of discolored sample available for analysis, the spectra shown in Fig. 7.3.2 were adjusted so that their spectral intensities are adjusted and their peaks are nearly aligned. This makes it clear that the spectrum of the non-discolored sample is overlaid on that of the discolored sample. In this case, a subtraction spectrum of the two must be calculated.

The resultant subtraction spectrum is shown in Fig. 7.3.3. In addition, a spectral search conducted on the basis of the spectrum in Fig. 7.3.3 yielded the spectrum shown in Fig. 7.3.4. The result indicates that the substance causing the discoloration of the tablet is carnauba wax. Carnauba wax is a naturally occurring wax that is widely used in lipstick, foundation and as an additive in foods. It is normally quite difficult to scrape away a portion of a discolored section without including some of the normal section. In addition, since the single reflection ATR method produces an averaged spectrum of the sample on the prism, the spectra of the discolored and non-discolored substances overlap. However, as shown here, the spectrum of discolored portion can be obtained by generating a subtraction spectrum with respect to the normal spectrum.

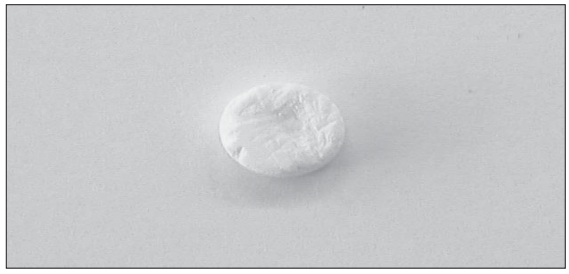


Fig. 7.3.1 Photograph of Contaminant on Tablet Surface

■Analytical Conditions

Resolution : 4 cm^{-1}
Accumulation : 60
Detector : DLATGS

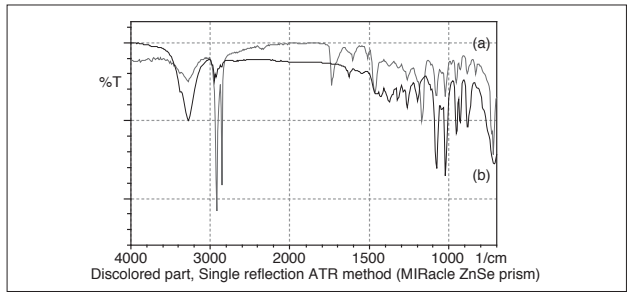


Fig. 7.3.2 Spectra of Discolored (a) and Non-Discolored (b) Parts on Tablet by Single Reflection ATR Method

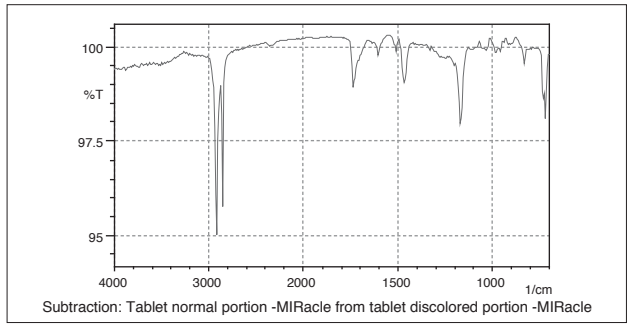


Fig. 7.3.3 Subtraction Spectrum of Discolored and Normal Parts of Tablet Surface

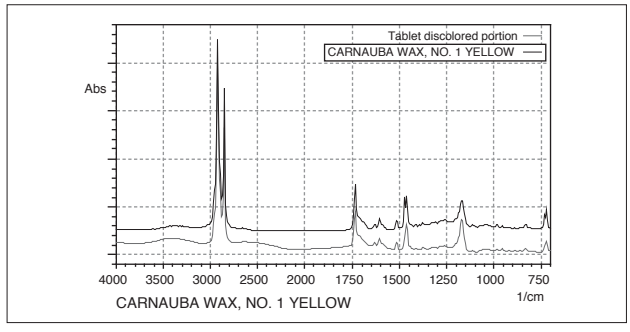


Fig. 7.3.4 Search Result

7.3 Analysis of Colored Contaminant on Surface of Tablet Using Infrared Microscope (2) - FTIR

■Measurement by IR Transmission Microscopy

The same contaminant was taken from the tablet and spread thinly on a diamond cell. The sample was then measured by IR transmission microscopy. Fig. 7.3.5 shows an enlarged photograph of the discolored scraping after it was spread thinly on the diamond cell. Fig. 7.3.6 shows the obtained spectrum of a 30 by 30 micron area of the sample. In contrast with the spectrum obtained using the single reflection ATR method (Fig. 7.3.2), it is evident that the spectrum of the normal section does not overlap with that of the discolored one. It is common in microscope measurement for the spectrum of a normal section to overlap the spectrum of a discolored section, but in this measurement, the spectrum of only discolored site was obtained without conducting subtraction spectrum processing.

■Analytical Conditions

Resolution : 8 cm^{-1}
Accumulation : 60
Detector : MCT

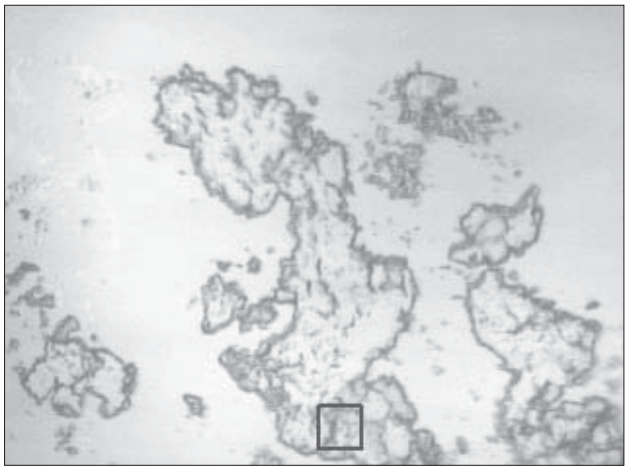


Fig. 7.3.5 Enlarged Photograph of Scrapped Contaminant

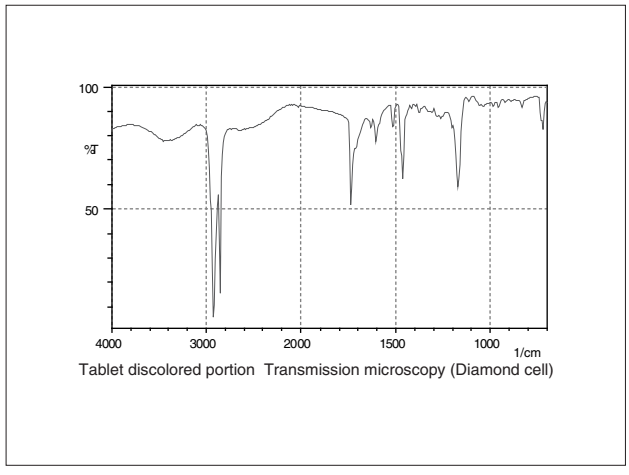


Fig. 7.3.6 Spectrum of Discolored Part on Tablet by IR Transmission Microscopy

7.4 Analysis of Dimethicone Based on USP-Specified Method (1) - FTIR

■Explanation

Quantitative analysis of liquid solutions by spectroscopy is often conducted by the "solution method," in which a fixed pathlength cell is utilized. However, when the solutions are viscous in nature, not only is cleaning of the cell a time-consuming and tedious task, injection can be difficult depending on the sample, and measurement can also be problematic due to the formation of bubbles. In such cases, the ATR method is effective because the close contact required between the sample and prism is easily achieved by merely applying a droplet of sample onto the prism, thereby permitting simple measurement.

Here, using a viscous solution of silicone oil, dimethicone (dimethyl polysiloxane) as the sample, we introduce an example of ATR measurement in which excellent repeatability was obtained. This analysis of dimethicone is based on the method specified in the United States Pharmacopeia (referred to below as USP).

■Dimethicone Analysis Method

The non-toxic, powerful defoaming agent silicone oil is widely used in foods, cosmetics and various commodities with a broad array of characteristics that are dictated by the structure, molecular weight, side chains and end groups[1]. Wide-ranging analyses are conducted on the various functional groups, binding modes, degree of polymerization, degeneration and presence or absence of blending, etc.

Here we consider dimethicone, a pharmaceutical that is prescribed to reduce flatulence, the production of excessive gas in the gastrointestinal tract. The structure of dimethicone is shown in Fig. 7.4.1. According to the USP, the ATR method is used for analysis of dimethyl siloxane $[-(CH_3)_2SiO-]_n$ in dimethicone[2]. Using this method, the infrared spectrum of the sample is measured, and the absorbance value at 1259 cm^{-1} due to the bending vibration of CH_3 is determined, thereby allowing management of dimethyl siloxane content (portion within broken line area of Fig. 7.4.1).

The formula below is used to calculate the content of dimethyl siloxane.

$100\ (A_U/A_S)(D_S/D_U)$....(1)

Here, A_U is the absorption of dimethicone at 1259 cm^{-1}, A_S is the absorption at 1259 cm^{-1} using USP standard, D_U is the specific gravity of dimethicone, and D_S is the specific gravity of the USP standard.

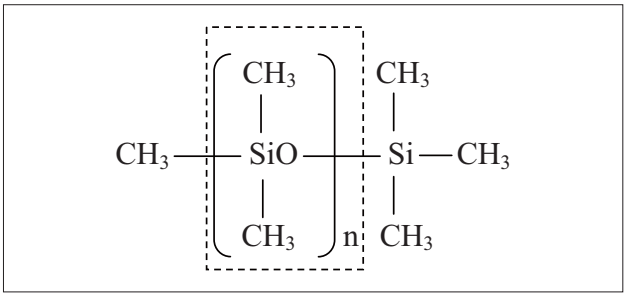


Fig. 7.4.1 Structure of Dimethicone (Dimethyl Siloxane Within Broken Line Area)

■Measurement of Dimethicone by ATR Spectroscopy

ATR measurement devices include single reflection and multiple reflection accessories, in which the greater the number of reflections, the greater the absorption peak obtained. However, even a single reflection ATR accessory can be used to obtain a detectable absorption peak at 1259 cm^{-1} generated by the bending vibration of CH_3. Furthermore, measurement can be conducted using just a small quantity of sample, while the cleaning of the prism between analyses is simple as well. Thus, the MIRacle A single reflection ATR accessory shown in Fig. 7.4.2 was used here, together with a Ge prism.

The prism was cleaned after each of the 3 repeat measurements to investigate the repeatability of ATR measurement.

Fig. 7.4.3 shows the overlaid ATR spectra obtained from measurement of a USP standard sample.

Fig. 7.4.2 MIRacle A Single Reflection ATR Accessory

■Analytical Conditions

Instruments : IRPrestige-21, MIRacle A (Ge prism)
Resolution : 4 cm^{-1}
Accumulation : 45 scans
Detector : DLATGS

7.4 Analysis of Dimethicone Based on USP-Specified Method (2) - FTIR

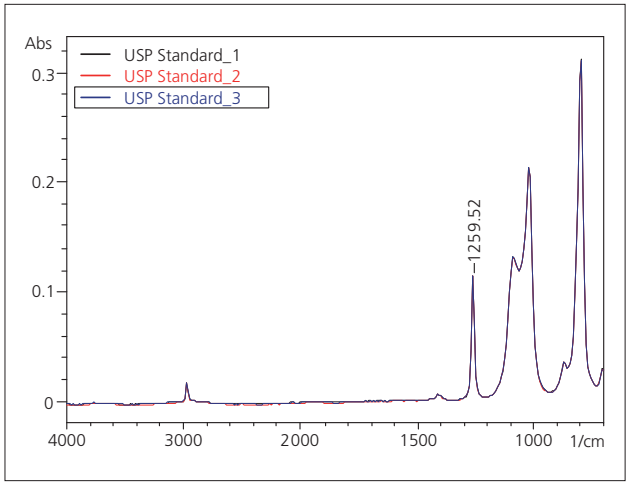


Fig. 7.4.3 ATR Spectra of USP Standard Sample

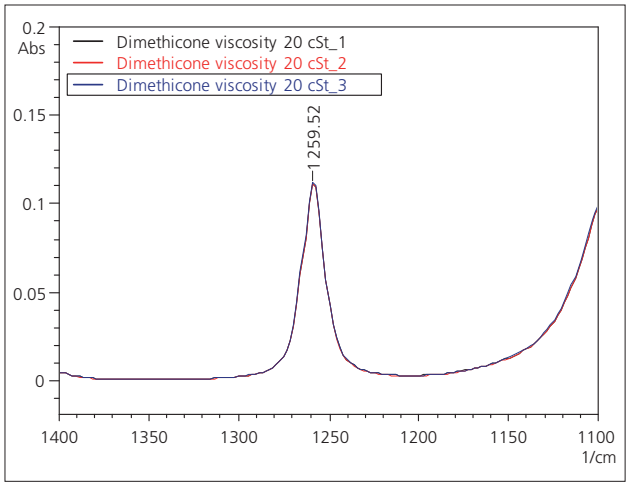


Fig. 7.4.4 ATR Spectra of Dimethicone with Viscosity of 20 cSt

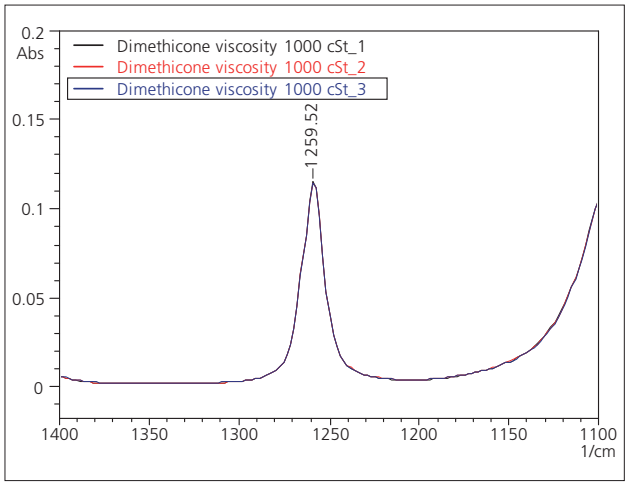


Fig. 7.4.5 ATR Spectra of Dimethicone with Viscosity of 1000 cSt

■Determination of the Quantity of Dimethyl Siloxane

Measurement was conducted using two dimethicone samples of viscosity 20 cSt and 1000 cSt, respectively (both from Sigma-Aldrich Corporation). The magnified ATR spectra with the characteristic peak in the vicinity of 1259 cm^{-1} are shown in Fig. 7.4.4 and Fig. 7.4.5. In addition, the absorbance values at 1259 cm^{-1} for the respective samples are shown in Table 7.4.1. Good repeatability was clearly obtained for all the samples. The quantity of dimethyl siloxane in the dimethicone was then determined using the equation (1) on the previous page and the average value of these absorbance values. The calculation yielded results of 98.89 % -101.07 % for the 20 cSt viscosity sample, and 98.89 % for the 1000 cSt viscosity sample, with both samples within the 97 % -103 % range of the USP standard values. Regarding the specific gravities of the samples, the maximum and minimum values specified in the USP were used.

Table 7.4.1 Peak Absorbances at 1259 cm^{-1}

Sample	Repeat Measurement	Absorbance
USP Standard	1	0.11277
	2	0.11282
	3	0.11265
	Average	0.11275
	Standard Deviation	0.00009
	CV Value (%)	0.08
20 cSt	1	0.10876
	2	0.10883
	3	0.10964
	Average	0.10908
	Standard Deviation	0.00049
	CV Value (%)	0.45
1000 cSt	1	0.11188
	2	0.11144
	3	0.11117
	Average	0.11150
	Standard Deviation	0.00036
	CV Value (%)	0.32

■Conclusion

Viscous dimethicone samples were analyzed by ATR spectroscopy, and excellent repeatability was obtained. Quantitative analysis by the solution method has the advantage of high sensitivity with the ability to detect low-level absorption through appropriate selection of a solid cell. However, for analysis of samples having some degree of viscosity, as those presented here, the ATR method with its simple measurement and easy cleaning of the prism, is very effective.

[References]

1) High Polymer Analysis Handbook [A New Edition] Edited by Polymer Analysis and Research Round-Table Conference, The Japan Society for Analytical Chemistry
2) USPC Official Monographs: Dimethicone

7.5 Analysis of Tablet Coating Layer by Raman Microscopy (1) - RM

■Explanation

Tablets, depending on the type, have a surface coating layer, and further, may also be layered internally. Raman microscopy can be as effective, or even more effective than FTIR microscopy for examining the structure of the layers. Coating agents may be broadly classified as being either organic or inorganic in nature. A typical inorganic coating agent is titanium oxide, a substance which generates extremely strong Raman scattering. Here we introduce an example of analysis of a coating layer using a section of titanium oxide coating that was cut out from the coated tablet using a microtome.

The measurement was conducted using Kaiser Raman Spectrophotometer Series 5000 (laser: 532 nm, microscope: Olympus BX60). For the sample, a commercial tablet (antidiarrheal drug) was used, and after a section was cut out using a microtome, the section was secured to the microscope stage so as to orient the cut surface horizontally. When observing the section surface, a white-colored portion of several µm in size was observed in the approximately 50 µm thick coating layer (see Fig. 7.5.1), and mapping measurement was conducted centered on this portion.

■Coating Layer Section Mapping

Fig. 7.5.2 shows Raman spectra of the coating layer, in which (a) is the spectrum of the white-colored portion, and (b) is that of the normal portion. From this, it is evident that that peak at 387 cm^{-1} seen in the spectrum of normal portion is absent in the spectrum of the white portion. Fig. 7.5.3 shows the mapping measurement results, in which the peak areas of the peaks included in the 406 to 355 cm^{-1} region are indicated on the longitudinal axis of the 3D display. Since there is no corresponding peak in the white region, it appears in an inverted state. In addition, area mapping was also conducted for the peaks in the region of 890 to 520 cm^{-1}, as shown in the results of Fig. 7.5.4. Both parts contain the same peaks, but since the peak intensities are several times greater in the white-colored portion, the white part is markedly displayed.

■Analytical Conditions

Laser	: 532 nm
Exposure Time	: 20 sec
Accumlation	: 1
Magnification	: × 50
Datapoints	: 13 × 11 = 143

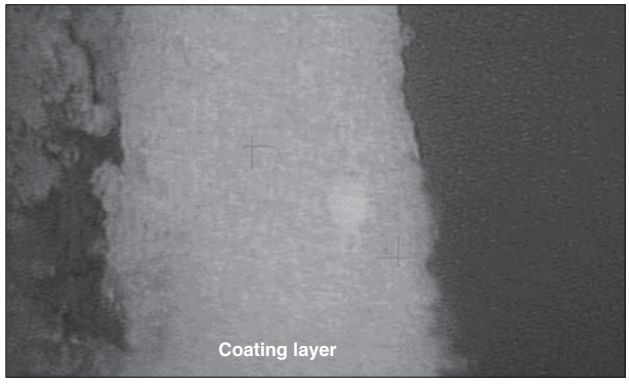


Fig. 7.5.1 Enlarged Photograph of Tablet Section

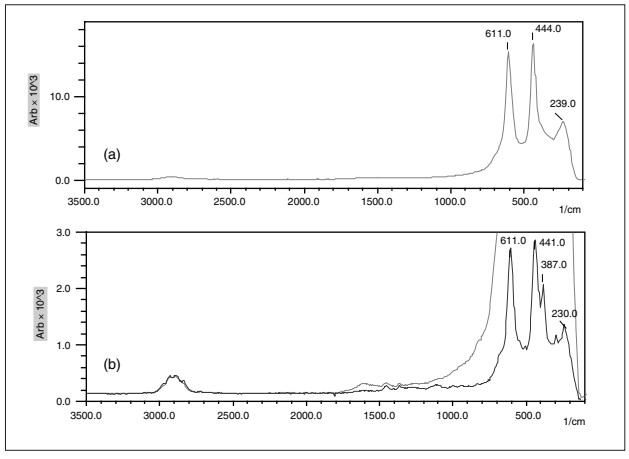


Fig. 7.5.2 Raman Spectra of Coating Layer
(a) White-colored part, (b) Normal part

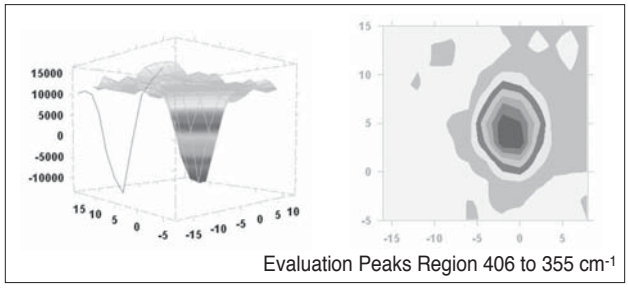


Fig. 7.5.3 Mapping Results 3D Display (Evaluation Peaks: 406 to 355 cm^{-1})
Evaluation Peak Region: 406 to 355 cm^{-1}

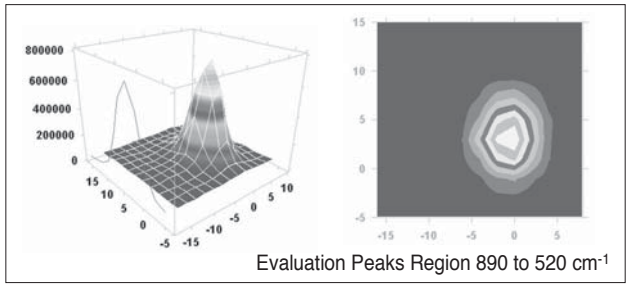


Fig. 7.5.4 Mapping results 3D Display (Evaluation peaks: 890 to 520 cm^{-1})
Evaluation Peak region: 890 to 520 cm^{-1}

7.5 Analysis of Tablet Coating Layer by Raman Microscopy (2) - RM

■Raman Spectrum of Titanium Oxide

There are two kinds of titanium oxide minerals used industrially, rutile and anatase, which differ according to their crystal structure. The spectra for these are shown in Fig. 7.5.5. From these, it is clear that the spectrum of the white-colored portion shown in Fig. 7.5.2 matches well with that of rutile. The spectra of Fig. 7.5.5 were excerpted from the Research Information Database (http://riodb.ibase.aist.go.jp/rasmin/index.html) of the National Institute of Advanced Industrial Science and Technology, accessible to the public.

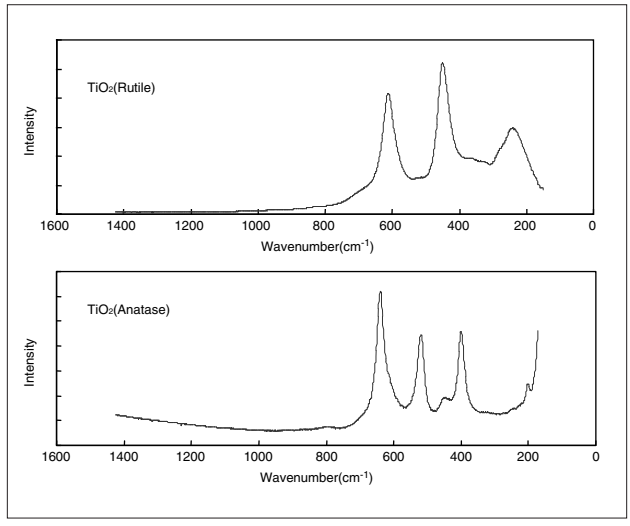


Fig. 7.5.5 Raman Spectra of Titanium Oxide

■Coating Layer Analysis by X-Ray Fluorescence Analysis and X-Ray Diffraction

The Raman spectrum revealed that the white-colored substance is TiO_2 (rutile), but since the peak at 387 cm^{-1} in the spectrum of the normal portion could not be ascertained, we conducted X-ray fluorescence analysis. The Shimadzu µEDX-1200, an instrument capable of narrowing the measurement spot to 50 µm, was used for the analysis. The results, as shown in Fig. 7.5.6, indicate the detection of Fe in the coating layer. In addition, the results of the coating surface analysis using an X-ray diffraction instrument indicated that its composition was TiO_2 (rutile) and Fe_2O_3. This suggests that the peak at 387 cm^{-1} appeared in the spectrum of the normal portion in Fig. 7.5.2 is due to Fe_2O_3.

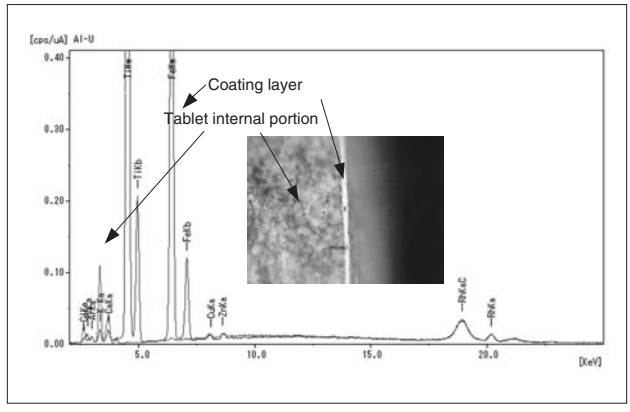


Fig. 7.5.6 Results of X-Ray Fluorescence Analysis

7.6 Analysis of Pharmaceutical Residual Solvents - GC
USP31-NF26<467>Residual Solvents-Procedure A - (1)

■Explanation

Residual solvents in pharmaceuticals are defined as volatile organic compounds used in or generated from the manufacture of drug substances, pharmaceutical additives, or drug products. They are strictly controlled according to risk classifications from Class 1 to Class 3, which are based on the risk to human health.

Headspace GC methods specified in the USP (U.S. Pharmacopeia), General Chapters <467> Residual Solvents, are commonly used for analysis of residual solvents. These USP methods were created based on the analytical methods specified in the EP (European Pharmacopoeia), in accordance with policies specified by the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use).

Here we show data obtained using the Shimadzu HS-20 Headspace Sampler and Shimadzu GC-2010 Plus Gas Chromatograph, from Class 1 and Class 2 standard solutions, in accordance with Water-Soluble Articles, Procedure A, in USP <467> Residual Solvents.

■Analytical Conditions

HS-20

Oven Temp.	: 80 ºC	Shaking Level	: Off
Equilibrating Time	: 60 min	Sample Pressurization	: 75 kPa
Pressurizing Time	: 1 min	Load Time	: 0.5 min
Injection Time	: 1 min	Needle Flush Time	: 20 min
Sample Line Temp.	: 110 ºC	Transfer Line Temp.	: 120 ºC
Vial Capacity	: 20 mL		

GC-2010 Plus

Column	: Rxi-624SilMS (30 m × 0.32 mm I.D. df = 1.8 µm)	Split Ratio	: 1:5
		Hydrogen	: 40 mL/min
Column Temp.	: 40 ºC (20 min) - 10 ºC/min - 240 ºC (20 min)	Air	: 400 mL/min
Carrier Gas Linear Velocity	: 35 cm/sec (He)		
FID Temp.	: 260 ºC		
Makeup Gas	: 30 mL/min (He)		

■Results

1. Class 1

Fig. 7.6.1 shows the Class 1 standard solution chromatogram. Procedure A requires that the S/N ratio obtained for 1,1,1-Trichloroethane in this chromatogram be 5 or higher. As shown, the S/N ratio was 200. Even for carbon tetrachloride, which had the lowest sensitivity level, the S/N was 10.

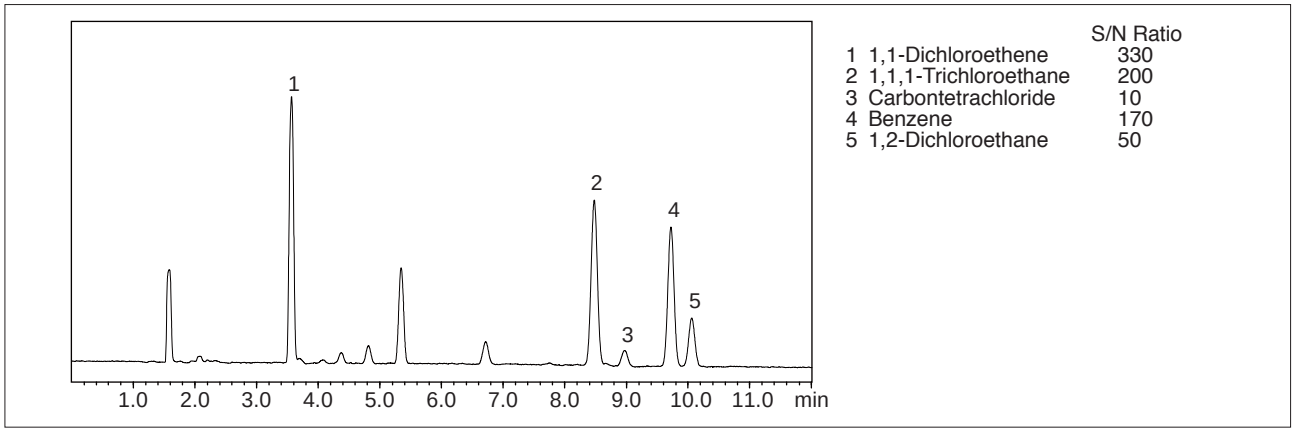


Fig. 7.6.1 Water-Soluble Articles, Procedure A, Class 1 Standard Solution Chromatogram

7.6 Analysis of Pharmaceutical Residual Solvents - GC
USP31-NF26<467>Residual Solvents-Procedure A - (2)

2. Class 2

Due to the large number of components in the Class 2 standard solution, it was separated into two mixtures: A and B. Respective measurement results are shown in Fig. 7.6.2 and Fig. 7.6.3.

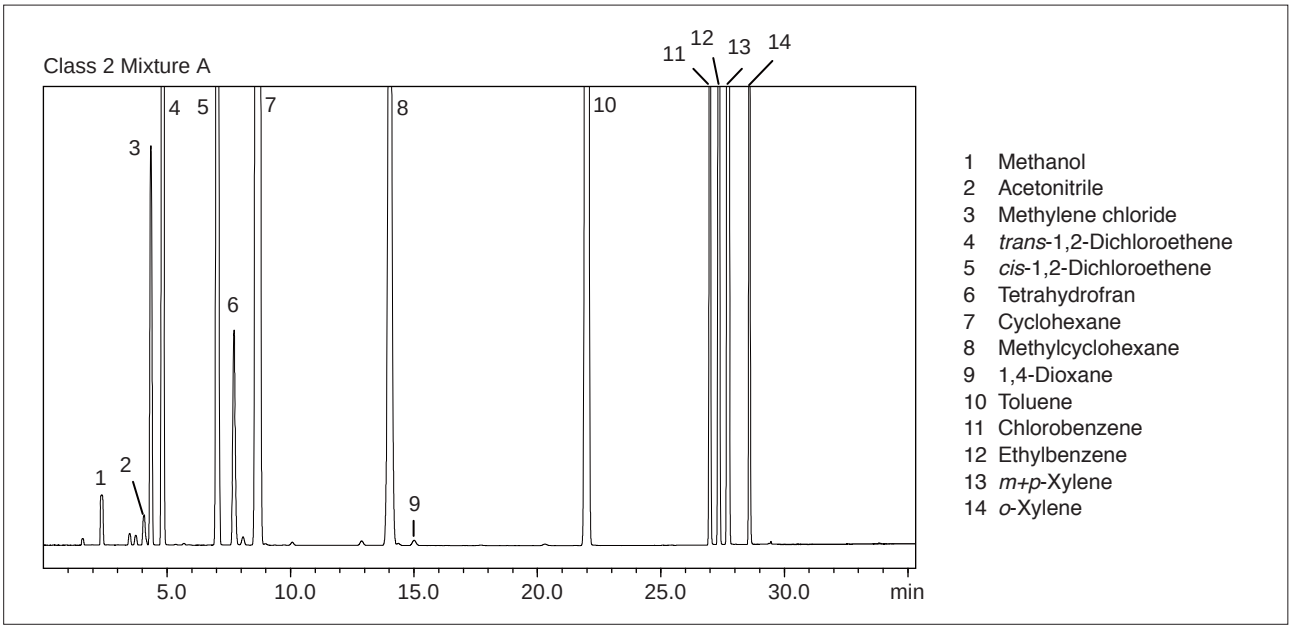


Fig. 7.6.2 Water-Soluble Articles, Procedure A, Class 2 Mixture A Standard Solution Chromatogram

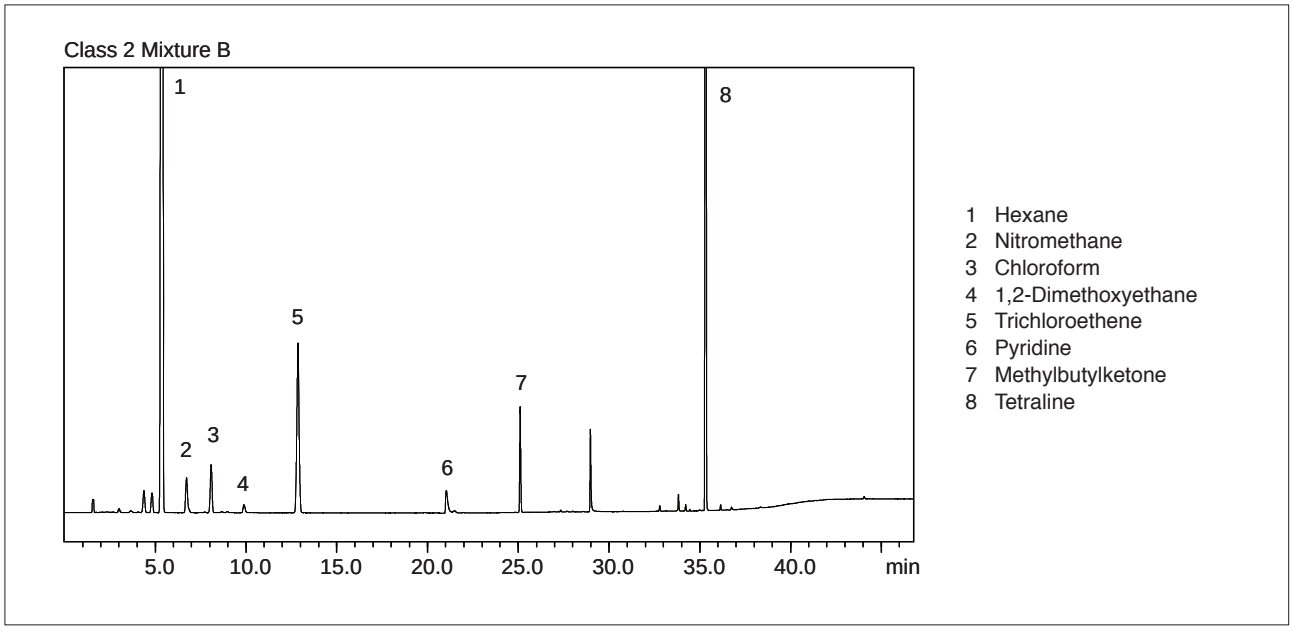


Fig. 7.6.3 Water-Soluble Articles, Procedure A, Class 2 Mixture B Standard Solution Chromatogram

7.6 Analysis of Pharmaceutical Residual Solvents - GC
USP31-NF26<467>Residual Solvents-Procedure A - (3)

Procedure A requires that the resolution for acetonitrile and methylene chloride in the Class 2 standard solution Mixture A chromatogram be 1.0 or greater. Fig. 7.6.4 shows that, using the Restek Rxi-624SilMS low-bleed column, the specified peaks are completely separated, with a resolution of 1.5.

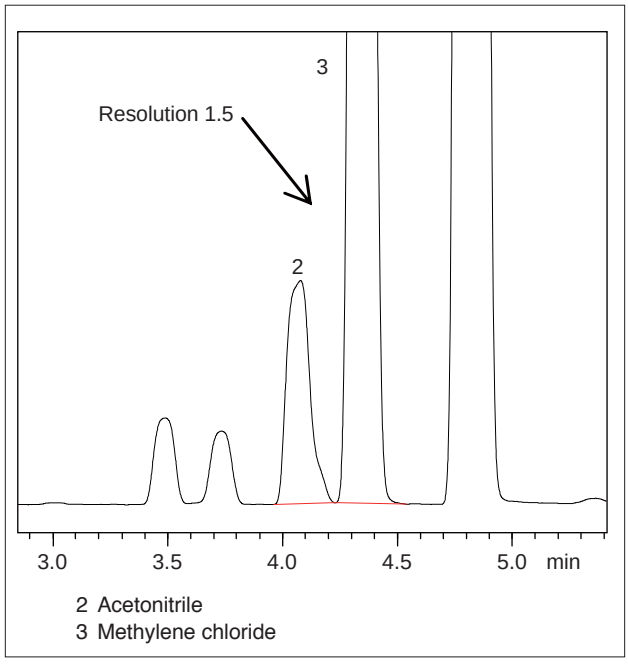


Fig. 7.6.4 Separation Between Acetonitrile and Methylene Chloride

The area repeatability (RSD %) was evaluated by measuring the sample 20 consecutive times. The resulting RSD % value was between 1 % and 3 %, which indicates a higher repeatability than obtained using previous headspace samplers (see Table 7.6.1).

The HS-20 headspace sampler achieves this unprecedented high repeatability by maintaining a uniform temperature distribution within the air tank oven and by using an advanced pressure control (APC) system for precise pressure control.

Table 7.6.1 Peak Area Repeatability of Class 2A and 2B

	RSD % n = 20
Class 2A	
2 Acetonitrile	1.1
3 Methylene chloride	1.7
4 *trans*-1,2-Dichloroethene	2.3
5 *cis*-1,2-Dichloroethene	1.9
6 Tetrahydrofuran	0.6
10 Toluene	2.5
11 Chlorobenzene	2.5
Class 2B	
4 1,2-Dimethoxyethane	3.1
6 Pyridine	2.6

7.6 Analysis of Pharmaceutical Residual Solvents - GC
USP31-NF26<467>Residual Solvents-Procedure B - (1)

■Explanation

Residual solvents in pharmaceuticals are defined as volatile organic compounds used in or generated from the manufacture of drug substances, pharmaceutical additives, or drug products. They are strictly controlled according to risk classifications from Class 1 to Class 3, which are based on the risk to human health.

Headspace GC methods specified in the USP (U.S. Pharmacopeia), General Chapters <467> Residual Solvents, are commonly used for analysis of residual solvents. These USP methods were created based on the analytical methods specified in the EP (European Pharmacopoeia), in accordance with policies specified by the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use).

Here we show data obtained using the Shimadzu HS-20 Headspace Sampler and Shimadzu GC-2010 Plus Gas Chromatograph, from Class 1 and Class 2 standard solutions, in accordance with Water-Soluble Articles, Procedure B, in USP <467> Residual Solvents.

■Analytical Conditions

HS-20

Oven Temp.	: 80 ºC	Shaking Level	: Off
Equilibrating Time	: 60 min	Sample Pressurization	: 75 kPa
Pressurizing Time	: 1 min	Load Time	: 0.5 min
Injection Time	: 1 min	Needle Flush Time	: 20 min
Sample Line Temp.	: 110 ºC	Transfer Line Temp.	: 120 ºC
Vial Capacity	: 20 mL		

GC-2010 Plus

Column	: StabilWAX (30 m × 0.32 mm I.D. df = 0.25 µm)	Split Ratio	: 1:10
		Hydrogen	: 40 mL/min
Column Temp.	: 50 ºC (20 min) - 6 ºC/min - 165 ºC (20 min)	Air	: 400 mL/min
Carrier Gas Linear Velocity	: 35 cm/sec (He)		
FID Temp.	: 250 ºC		
Makeup Gas	: 30 mL/min (He)		

■Results

1. Class 1

Fig. 7.6.5 shows the Class 1 standard solution chromatogram. Procedure B requires that the S/N ratio obtained for benzene in this chromatogram be 5 or higher. In this example, the S/N ratio for benzene was 60.

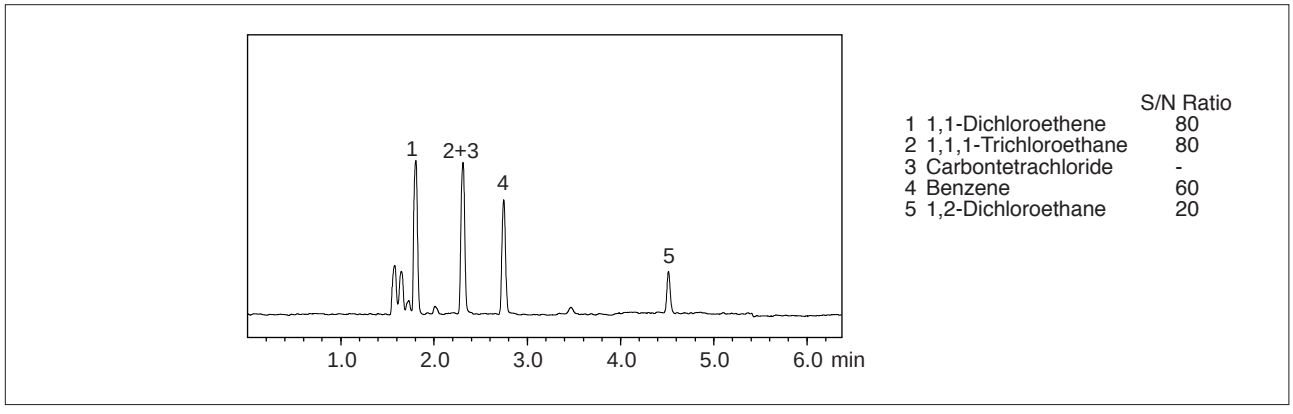
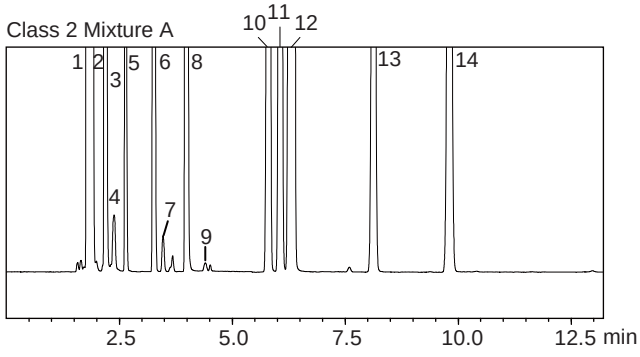


Fig. 7.6.5 Water-Soluble Articles, Procedure B, Class 1 Standard Solution Chromatogram

7.6 Analysis of Pharmaceutical Residual Solvents - GC
USP31-NF26<467>Residual Solvents-Procedure B - (2)

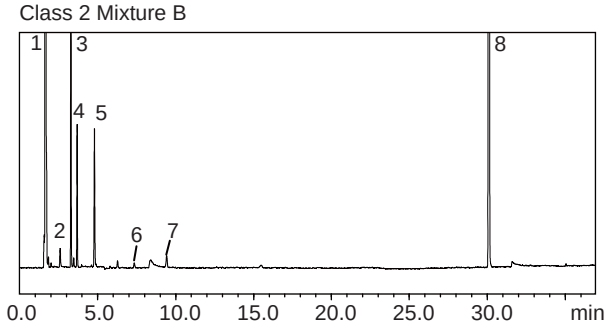
2. Class 2

Due to the large number of components in the Class 2 standard solution, it was separated into two mixtures: A and B. Respective measurement results are shown in Fig. 7.6.6 and Fig. 7.6.7.



1 Cyclohexane
2 Methylcyclohexane
3 *trans*-1,2-Dichloroethene+Tetrahydrofran
4 Methanol
5 Methylene chloride
6 *cis*-1,2-Dichloroethene
7 Acetonitrile
8 Toluene
9 1,4-Dioxane
10 Ethylbenzene
11 *p*-Xylene
12 *m*-Xylene
13 *o*-Xylene
14 Chlorobenzene

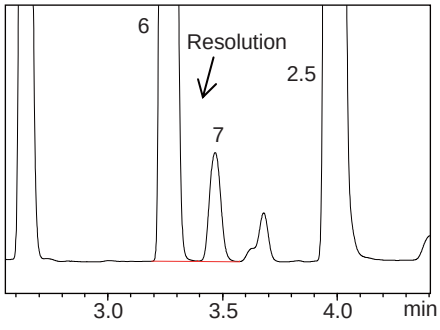
Fig. 7.6.6 Water-Soluble Articles, Procedure B, Class 2 Mixture A Standard Solution Chromatogram



1 Hexane
2 1,2-Dimethoxyethane
3 Trichloroethene
4 Chloraoform
5 Methylbutylketone
6 Nitromethane
7 Pyridine
8 Tetraline

Fig. 7.6.7 Water-Soluble Articles, Procedure B, Class 2 Mixture B Standard Solution Chromatogram

Procedure B requires that the resolution for *cis*-1,2-dichloroethene and acetonitrile in the chromatogram measured from the Class 2 standard solution Mixture A be 1.0 or greater. Fig. 7.6.8 shows that, using the Restek StabilWAX column, the specified peaks are completely separated, with a resolution of 2.5.



6 *cis*-1,2-Dichloroethene
7 Acetonitrile

Fig. 7.6.8 Separation Between *cis*-1,2-Dichloroethene and Acetonitrile

7.7 USP-Specified TOC System Suitability Test (1) - TOC

■Explanation

The United States Pharmacopeia (USP) specifies the use of Total Organic Carbon (TOC) for management of organic impurities in purified water (PW) and water for injection (WFI). According to the USP, the TOC analyzer to be used for these analyses must satisfy the TOC system suitability testing requirement, and must be capable of detecting TOC at concentrations below 0.05 mg/L.Here, using the Shimadzu TOC-LCPH combustion catalytic oxidation type analyzer, we introduce examples of TOC system suitability testing and measurement of TOC at a concentration below 0.05 mg/L.

■TOC System Suitability Test Specified in USP

The TOC system suitability test indicated in the USP specifies the use of two types of USP reference standards (sucrose and 1,4-benzoquinone). Sucrose is used as a test solution standard, and 1,4-benzoquinone is used as the system suitability test solution.In addition, calibration of the TOC analyzer is specified to be conducted using a method that is suitable for that instrument. The test procedure is shown in Table 7.7.1.

Table 7.7.1 TOC System Suitability Test Procedure Specified in USP

TOC system suitability test procedure

(1) Measure the TOC in distilled water (distilled water used for preparing test solution), This value is indicated as r_W.

(2) Measure the TOC in the sucrose standard solution (0.50 mg/L carbon concentration). This value is indicated as r_S.

(3) Measure the TOC by the system suitability test (1,4-benzoquinone solution with 0.50 mg/L carbon concentration). This value is indicated as r_{SS}.

(4) The system suitability test requirement is satisfied if: detection rate = 100 (r_{SS} - r_W) / (r_S - r_W) is 85 % - 115 %

■TOC System Suitability Test Data by USP Method

The TOC system suitability test was conducted using the Shimadzu TOC-LCPH combustion catalytic oxidation type analyzer by the procedure outlined in Table 7.7.1. The instrument was calibrated beforehand using aqueous solutions of potassium hydrogen phthalate with carbon concentrations of 0 and 0.5 mgC/L, respectively. The TOC system suitability test data are shown in Fig. 7.7.1. According to the USP, the detection rate is to be evaluated using the analyzer response values, but here, the measured concentrations were used instead.

The result indicated a 100.1 % detection rate with respect to the system suitability test solution (1,4-benzoquinone aqueous solution), thereby satisfying the system suitability test requirement. (Table 7.7.2)

■Analytical Conditions

Instrument : Shimadzu TOC-LCPH Combustion Catalytic Oxidation Typse Analyzer

Catalyst : High-sensitivity catalyst

Injection Volume : 816 µL

Measurement Item : TOC (=NPOC: TOC by acidification/ sparging)

Calibration Curve : 2-point calibration curve using 0-0.5 mgC/L potassium hydrogen phthalate aqueous solution

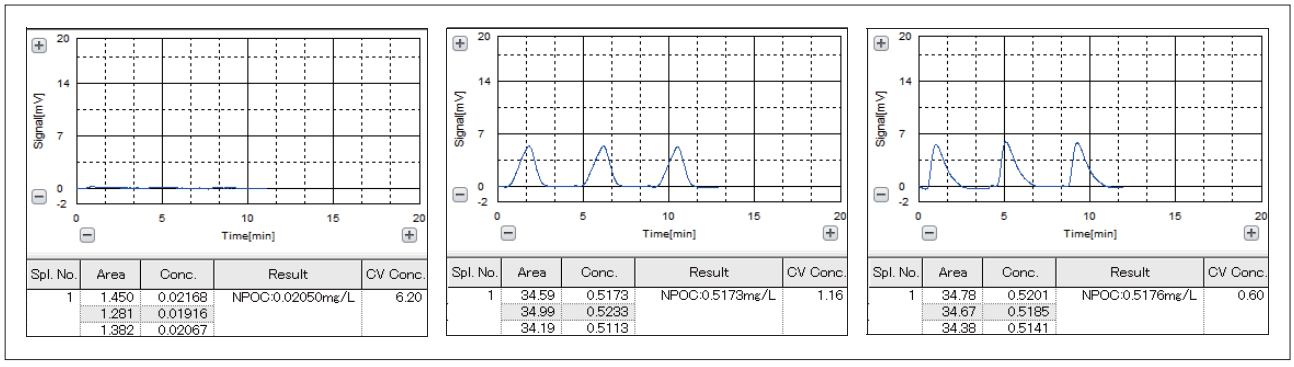


Fig. 7.7.1 TOC System Suitability Test Data

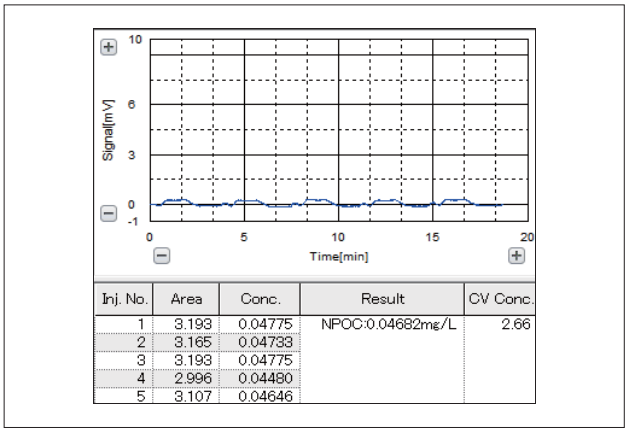
7.7 USP-Specified TOC System Suitability Test (2) - TOC

Table 7.7.2 Results of TOC System Suitability Test

(1)Distilled water TOC value r_w = 0.0205 mg/L
(2)Sucrose standard solution TOC value r_s = 0.5173 mg/L
(3)System suitability test solution (1,4-benzoquinone aqueous solution) TOC value r_{ss} = 0.5176 mg/L
(4)System suitability test solution detection rate
= 100 (r_{ss} - r_w) / (r_s - r_w)
= 100 (0.5176 - 0.0205) / (0.5173 - 0.0205)
= 100.1 %

■TOC Measurement Below 0.05 mg/L

The USP specifies that TOC analyzers to be used must be able to detect TOC at a concentration below 0.05 mg/L. To verify this, we measured a potassium hydrogen phthalate aqueous solution with a TOC concentration of 0.025 mgC/L. The results are shown in Fig. 7.7.2 and Table 7.7.3. Because the distilled water used to prepare the sample contained TOC components as impurities, the measurement resulted in a higher concentration of 0.047 mgC/L, with a resulting coefficient of variation (CV) of 2.66 %. Since the coefficient was within 10 %, a CV value commonly associated with a concentration close to the lower limit of quantitation, the Shimadzu TOC-LCPH combustion catalytic oxidation type analyzer clearly satisfied the USP requirement for TOC measurement of concentrations below 0.05 mg/L.



Inj. No.	Area	Conc.	Result	CV Conc.
1	3.193	0.04775	NPOC:0.04682mg/L	2.66
2	3.165	0.04733		
3	3.193	0.04775		
4	2.996	0.04480		
5	3.107	0.04646		

Fig. 7.7.2 Measurement Data for TOC Concentrations Below 0.05 mg/L (50 µg/L)

■Analytical Conditions

Instrument : Shimadzu TOC-LCPH Combustion Catalytic Oxidation Type Analyzer
Catalyst : High-sensitivity catalyst
Injection Volume : 816 µL
Measurement Item : TOC (= NPOC: TOC by acidification/sparging)
Calibration Curve : 2-point calibration curve using 0-0.5 mgC/L potassium hydrogen phthalate aqueous solution
Sample : 0.025 mgC/L potassium hydrogen phthalate aqueous solution

Table 7.7.3 Measurement Data for TOC Concentrations Below 0.05 mg/L (50 µg/L)

Sample Name	TOC Value [mgC/L]	Coefficient of Variation (CV) [%]
Potassium hydrogen phthalate aqueous solution	0.047	2.66

7.8 Cleaning Validation by TOC Analyzer (1) - TOC

■Explanation

To ensure quality control and safety in manufacturing facilities within the pharmaceutical industry, it is important that cleaning validation be conducted following the cleaning of production-related equipment. Cleaning validation ensures that the quantity of residual substances collected from the surfaces of the equipment is within permissible limit. Depending on the sampling method and measurement method used for this cleaning validation using a TOC analyzer, the following 3 types of methods are available.

(1) Rinse sampling–TOC measurement method
(2) Swab sampling–aqueous extraction–TOC measurement method
(3) Swab sampling–direct combustion carbon measurement method

Here we introduce the features of each of these methods, using the TOC-LCPH total organic carbon analyzer in the measurement of residual pharmaceutical products and their constituent substances.

■Preparation of Residue Measurement Sample

In order to evaluate the cleaning validation sampling methods, residue measurement samples were created by applying various types of pharmaceutical products and their constituents to stainless steel pots. The aqueous and non-aqueous substances that were used are listed in Table 7.8.1. The aqueous substances and non-aqueous substances were dissolved in water and ethanol or acetone, respectively, and the solution concentrations were adjusted to 2000 mgC/L (= carbon concentration of 2000 mg/L). 100 µL of each solution was then applied to a 5 cm by 5 cm squares area on the surface of a stainless steel pot, and the respective solvents were dried out to produce residue measurement samples. Thus, the amount of carbon in the sample at each application site was 200 µg. Among these, Gentacin ointment (aminoglycoside antibiotic) and Rinderon ointment (corticosteroid) were prepared based on determination of their carbon concentrations using the Shimadzu total organic analyzer system including the solid sample combustion unit.

Table 7.8.1 Sample Types

Substance Name	Solubility in Water	Solvent Used in Solution Preparation
Tranexamic acid	Soluble	Water
Anhydrous caffeine	Soluble	Water
Isopropylantipyrine	Insoluble	Ethanol
Nifedipine	Insoluble	Acetone
Gentacin ointment	Insoluble	Ethanol
Rinderon ointment	Insoluble	Acetone

■(1) Rinse Sampling–TOC Measurement Method

The Rinse Sampling – TOC Measurement method is a technique in which the final rinse water used in the cleaning of a production equipment unit is used as the TOC measurement sample. This method is suitable for systems that cannot easily be disassembled, such as CIP (clean-in-place) equipment and narrow tubing. However, sampling is considered to be difficult if the residues are not soluble in water. To evaluate the recovery of the various substances when using the Rinse Sampling – TOC Measurement method, 100 mL of pure water was transferred to the stainless steel pot with the patch of dried sample, and after stirring with a stirrer for 15 minutes to prepare the rinse solution, TOC measurement was conducted. Some of the measurement data are shown in Fig. 7.8.1. Since the carbon content in each of the residue measurement samples is 200 µg, the TOC concentration would be 2 mgC/L if all of the sample were to dissolve in the water. Now, for the blank, measurement was conducted in the same way using water that was transferred to the stainless steel pot, which in this case had no patch of dried sample applied to its surface. The measured blank concentration was subtracted from each TOC concentration, and then compared to the theoretical value of 2 mgC/L to determine the rate of recovery.

The results are shown in Table 7.8.2. Water-soluble tranexamic acid and anhydrous caffeine had high recovery rates as expected. Moreover, water-insoluble isopropylantipyrine and nifedipine had high recovery rates. However, recovery rates of Gentacin ointment and Rinderon ointment were both low, at less than 20 %. From these results, it is clear that evaluation of the rinse water using this method is unreliable due to the variation of recovery of substances which are not readily soluble in water.

■Analytical Conditions

Instrument	: Shimadzu TOC-LCPH Total Organic Carbon Analyzer
Catalyst	: High sensitivity catalyst
Measurement Item	: TOC (=TOC by acidification sparge processing)
Calibration Curve	: 2-point calibration curve using 0-3 mgC/L potassium hydrogen phthalate aqueous solution
Injection Volume	: 500 µL

7.8 Cleaning Validation by TOC Analyzer (2) - TOC

Table 7.8.2 Measurement Results for Rinse Sampling – TOC Measurement Method

Substance Name	TOC Concentration [mgC/L]	Recovery Rate, [TOC Conc. – Blank / Theoretical Conc.]
Blank	0.030	-
Tranexamic acid	2.14	105 %
Anhydrous caffeine	2.19	108 %
Isopropylantipyrine	2.20	109 %
Nifedipine	2.17	107 %
Gentacin ointment	0.117	4.35 %
Rinderon ointment	0.333	15.2 %

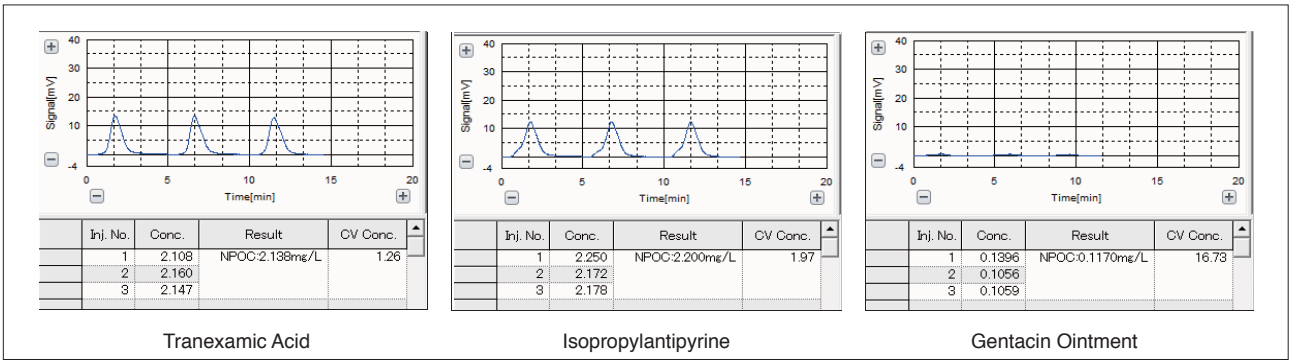


Fig. 7.8.1 Measurement Data Using Rinse Sampling – TOC Measurement Method

■(2)Swab Sampling–Water Extraction–TOC Measurement Method

The Swab Sampling –Water Extraction–TOC Measurement method, as illustrated in Fig. 7.8.2, consists of wiping the inside surface of the production apparatus with a fibrous swab material, extracting the adhering material with water, and conducting TOC measurement of the extract solution. Since the residue is physically wiped off from a fixed area of the surface of the apparatus using the swab material, and then analyzed, the sampling efficiency is high. However, because water is used for extraction of the residue, residues that are insoluble in water are difficult to extract. Accordingly, cleaning evaluation with respect to these residues may be difficult for the same reason as that described with respect to difficultto-dissolve substances in the (1) Rinse Sampling – TOC Measurement method. To evaluate the recovery of the various substances when using the Swab Sampling – Water Extraction –TOC Measurement method, the sample, which was applied to a stainless steel pot, was wiped off with a 5 cm by 5 cm squares piece of fibrous swab material, which was then placed in a glass jar containing 100 mL of pure water. The residue was then extracted by stirring with a stirrer for 1 hour, after which TOC measuremet was conducted. Some of the measurement data are shown in Fig. 7.8.3. Since the fibrous swab material (Alpha 10 obtained from Texwipe Co.) that was used is made of polyester, very little organic material is extracted from the swab itself. Since the carbon content in each of the residue measurement samples is 200 µg , the TOC concentration in the extraction solution would be 2 mgC/L if all of the sample were wiped off.

For the blank, measurement was conducted in the same way by wiping the stainless pot which had no sample applied before conducting extraction. The measured blank concentration was subtracted from each TOC concentration, and then compared to the theoretical value of 2 mgC/L to determine the rate of recovery. The results are shown in Table 7.8.3. Water-soluble tranexamic acid and anhydrous caffeine had high recovery rates as expected. Moreover, water-insoluble isopropylantipyrine and nifedipine had high recovery rates of about 90 %. However, recovery rates of Gentacin ointment and Rinderon ointment were both low, at less than 10 %. From these results, it is clear that evaluation of the rinse water using this method is unreliable due to the variation of recovery of substances which are not readily soluble in water.

■Analytical Conditions

Instrument	: Shimadzu TOC-L_{CPH} Combustion Total Organic Carbon Analyzer
Catalyst	: High sensitivity catalyst
Measurement Item	: TOC (=TOC by acidification sparge processing)
Calibration Curve	: 2-point calibration curve using 0-3 mgC/L potassium hydrogen phthalate aqueous solution
Injection Volume	: 500 µL
Swab Material	: 5 cm by 5 cm squares piece of Texwipe Alpha 10 swab material washed in pure water and dried

7.8 Cleaning Validation by TOC Analyzer (3) - TOC

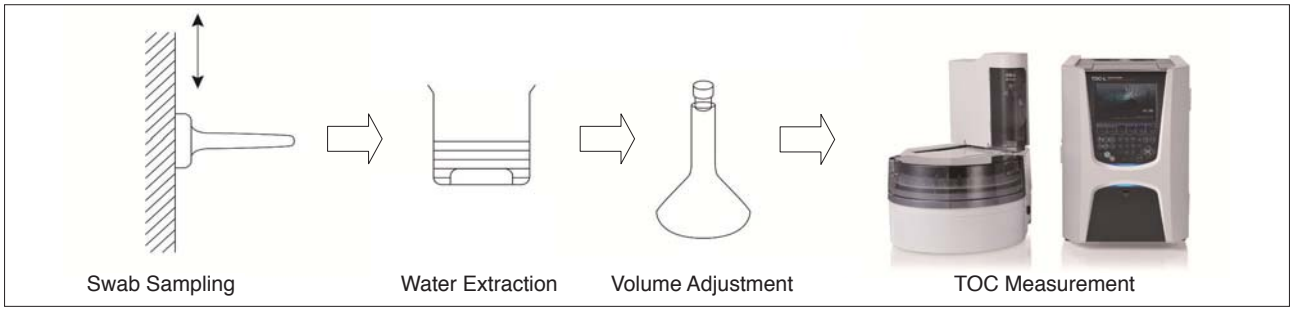


Fig. 7.8.2 Swab Sampling–Water Extraction–TOC Measurement Method

Table 7.8.3 Measurement Results for Swab Sampling–Water Extraction–TOC Measurement Method

Substance Name	TOC Concentration [mgC/L]	Recovery Rate, [TOC Conc. – Blank / Theoretical Conc.]
Blank	0.059	-
Tranexamic acid	2.19	107 %
Anhydrous caffeine	2.23	109 %
Isopropylantipyrine	1.90	92.2 %
Nifedipine	1.86	89.9 %
Gentacin ointment	0.093	1.70 %
Rinderon ointment	0.208	7.45 %

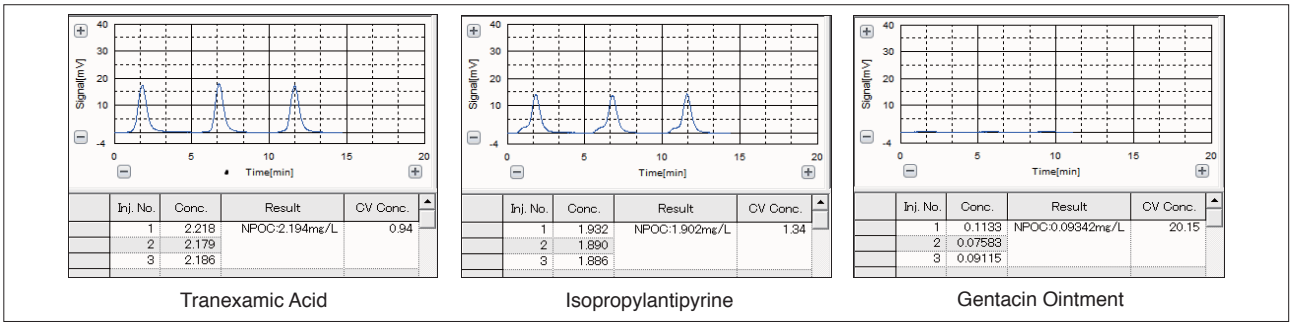


Fig. 7.8.3 Measurement Data Using Swab Sampling–Water Extraction–TOC Measurement Method

■(3)Swab Sampling–Direct Combustion Method

The Swab Sampling – Direct Combustion method, as illustrated in Fig. 7.8.4, consists of wiping the inside surface of the production apparatus with a piece of inorganic quartz glass filter paper swab material, and then conducting measurement using a direct combustion carbon measurement system. The swab material with the adhering residue is merely placed in the sample boat, and the carbon content is measured directly by the TOC analyzer with the connected SSM-5000A Solid Sample Combustion Unit. By using this method, water-insoluble residues that are difficult to extract in water can also be collected, and measurement can be quickly and easily conducted without the need for any pretreatment, such as sample extraction, etc. To evaluate the recovery rate of the different types of substances using the Swab Sampling –Direct Combustion method, we used the quartz glass filter paper swab material to wipe off the sample adhering to the stainless steel pot, placed the swab in the SSM-5000A sample boat, and conducted TC measurement. Some of the measurement data are shown in Fig. 7.8.5. Since the carbon content in each of the residue measurement samples is 200 µg, the TC value would be 200 µg if all of the sample were wiped off.

For the blank, measurement was conducted in the same way by wiping the stainless pot which had no sample applied. The measured blank value was subtracted from each TC value, and then compared to the theoretical value of 200 µg to determine the rate of recovery. The results are shown in Table 7.8.4. A high recovery rate of about 100 % was obtained for all the substances, regardless of whether they were watersoluble or water-insoluble.

■Analytical Conditions

Instrument	: Shimadzu TOC-L_{CPH} Total Organic Carbon Analyzer + SSM-5000A Solid Sample Combustion Unit (IC circuit bypass using system with cell switching valve set)
Cell Length	: Short cell
Ssm Carrier Gas	: 400 mL/min oxygen gas
Measurement Item	: TC
Calibration Curve	: 1-point calibration curve using 30 µL of 1 % C glucose aqueous solution
Swab Material	: Advantec QR-100 quartz glass filter paper (diameter 45 mm) heat-treated at 600 °C for 15 minutes

7.8 Cleaning Validation by TOC Analyzer (4) - TOC

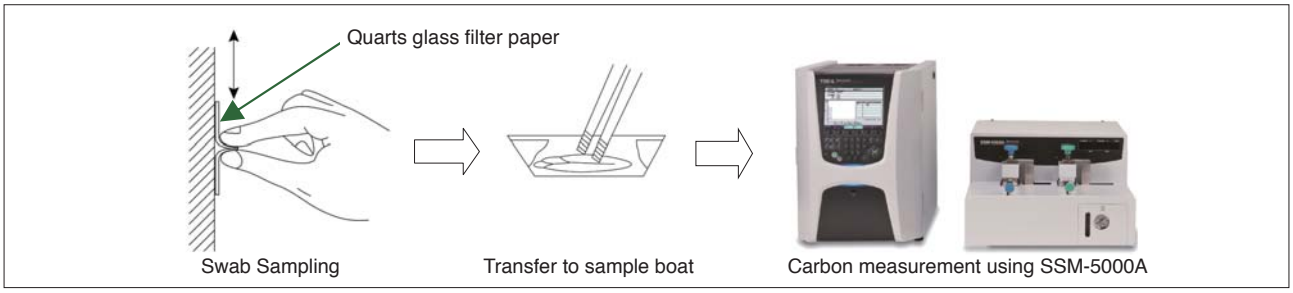


Fig. 7.8.4 Swab Sampling –Direct Combustion Method

Table 7.8.4 Measurement Results for Swab Sampling –Direct Combustion Method

Substance Name	TOC Value [μ C]	Recovery Rate, [TC Value – Blank/Theoretical Value]
Blank	0.00	-
Tranexamic acid	202	101 %
Anhydrous caffeine	201	100 %
Isopropylantipyrine	210	105 %
Nifedipine	212	106 %
Gentacin ointment	200	100 %
Rinderon ointment	209	104 %

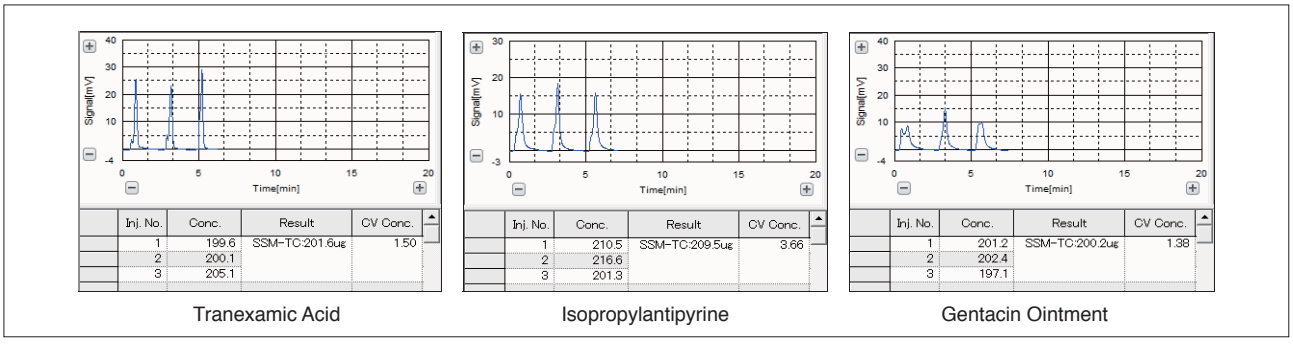


Fig. 7.8.5 Measurement Data Using Swab Sampling–Direct Combustion Method

■Conclusion

The measurement methods used here and their respective recovery rates are summarized in Table 7.8.5. When using the Rinse Sampling–TOC Measurement and the Swab Sampling–Water Extraction–TOC Measurement method, substances that do not easily dissolve in water were found to include those that had high recovery rates, and those that had low recovery rates. This may be due to differences in the strength with which the substances adhere to the stainless steel pot. Accordingly, it is probable that residue evaluation using these methods would be difficult for substances with low recovery rates. In contrast to that, high recovery rates were obtained for all the substances when using the Swab Sampling–Direct Combustion method, regardless of whether the substances were water-soluble or water-insoluble, thereby permitting residue evaluation. Therefore, this method is considered to be an effective measurement method for conducting cleaning validation.

Table 7.8.5 Summary of Measurement Results

Substance Name	Solubility in Water	Recovery Rate		
		Rinse Sampling – TOC Measurement Method	Swab Sampling – WaterExtraction – TOC Measurement Method	Swab Sampling – Direct Combustion Method
Tranexamic acid	Soluble	105 %	107 %	101 %
Anhydrous caffeine	Soluble	108 %	109 %	100 %
Isopropylantipyrine	Insoluble	109 %	92.2 %	105 %
Nifedipine	Insoluble	107 %	89.9 %	106 %
Gentacin ointment	Insoluble	4.35%	1.70 %	100 %
Rinderon ointment	Insoluble	15.2%	7.45 %	104 %

8. Physical Property and Observation

8.1 Sulfathiazole Crystal Polymorphism - TA

■Explanation

It is generally acknowledged that crystal shape and solubility can differ even for the same substance. For that reason, crystal polymorphism is an important problem in the pharmaceutical field. Polymorphism is easily measured using DSC. In Fig. 8.1.1, endothermic peaks can be seen at 168.3 ˚C and 202.2 ˚C, but in the case sulfathiazole, which has been heat-processed up to a temperature of 185 ˚C, the peak at 168.3 ˚C has disappeared, as shown in Fig. 8.1.2. This suggests that the peak at 168.3 ˚C represents the existence of an unstable type of crystal.

■Analytical Conditions

Instrument	: DSC-60
Sample	: Sulfathiazole
Sample volume	: 3.88 mg
Carrier Gas	: Nitrogen
Flowrate	: 30 mL/min
[Temperature Program]	
Heating Rate	: 5 ˚C/min

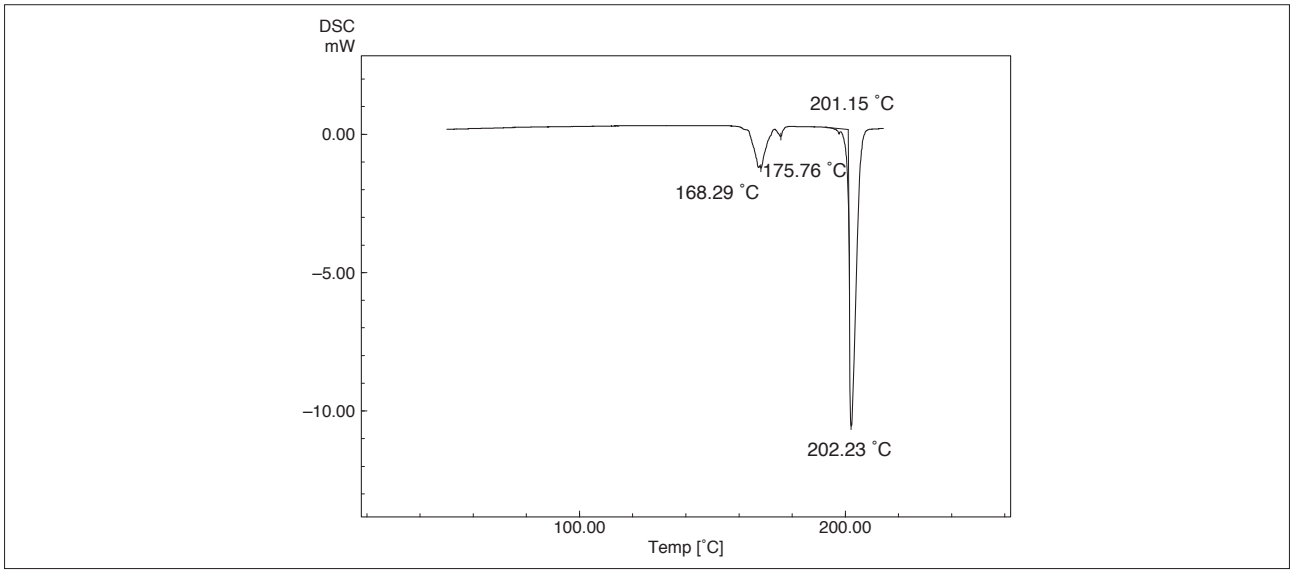


Fig. 8.1.1 DSC Curve of Sulfathiazole (original)

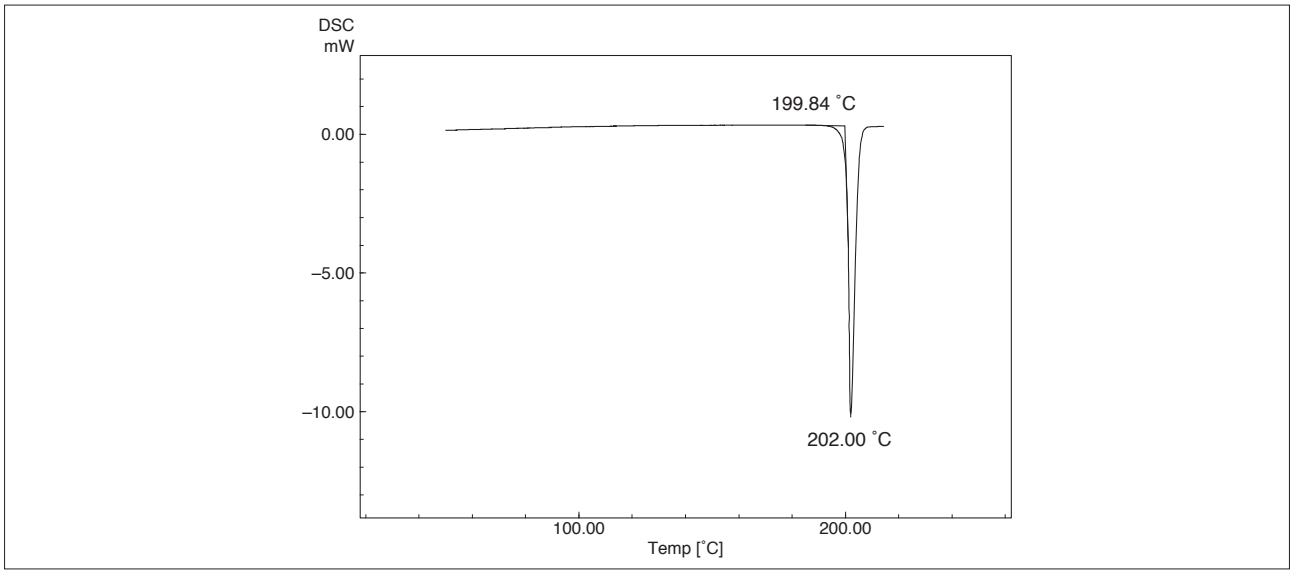


Fig. 8.1.2 DSC Curve of Sulfathiazole (heat-processed up to 185 ˚C)

8.2 Interaction of Benzoic Acid and Magnesium Oxide - TA

■Explanation

Pharmaceutical products are produced with the inclusion of a variety of additives. Verification of whether interaction occurs between such additives to form other substances is easily accomplished by DSC. Here we investigated the interaction of benzoic acid and magnesium oxide by DSC. Fig. 8.2.1 shows the melt characteristics of benzoic acid alone in a sealed cell, and melting is seen to occur at 122.7 ˚C. Magnesium oxide alone was similarly measured, but as can be seen in Fig. 8.2.2, no change was detected. Fig. 8.2.3 shows the results of DSC measurement of a mixture of benzoic acid and magnesium oxide, mechanically mixed at a 1:1 ratio. A pattern completely different from the individual DSC curves is shown, indicating the occurrence of some sort of interaction between the substances.

■Analytical Conditions

Instrument : DSC-60
Sample (Fig. 8.2.1) : Benzoic acid
Sample Volume : 4.83 mg
Sample (Fig. 8.2.2) : Magnesium oxide
Sample Volume : 4.38 mg
Sample (Fig. 8.3.3) : Benzoic acid + magnesium oxide
Sample Volume : 2.18 mg
[Temperature Program]
Heating Rate : 10 ˚C/min

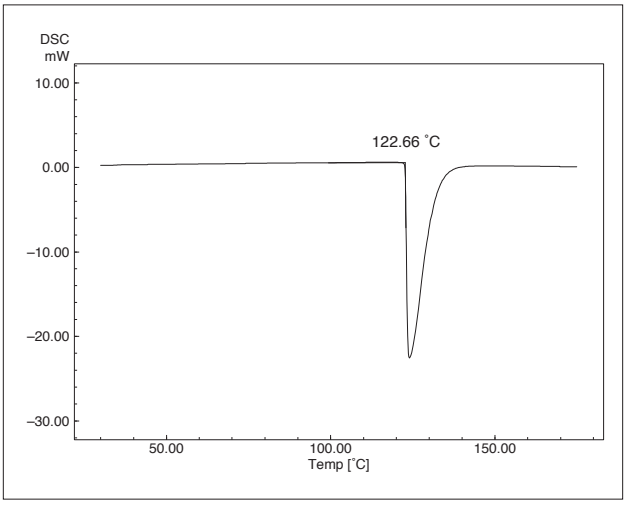


Fig. 8.2.1 DSC Curve of Benzoic Acid Alone

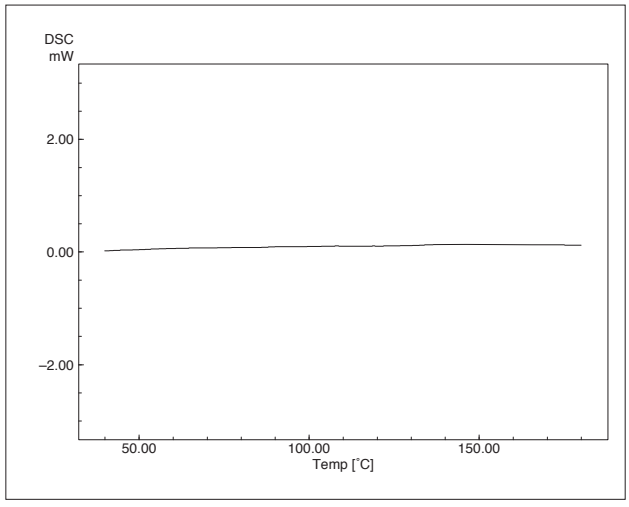


Fig. 8.2.2 DSC Curve of Magnesium Oxide Alone

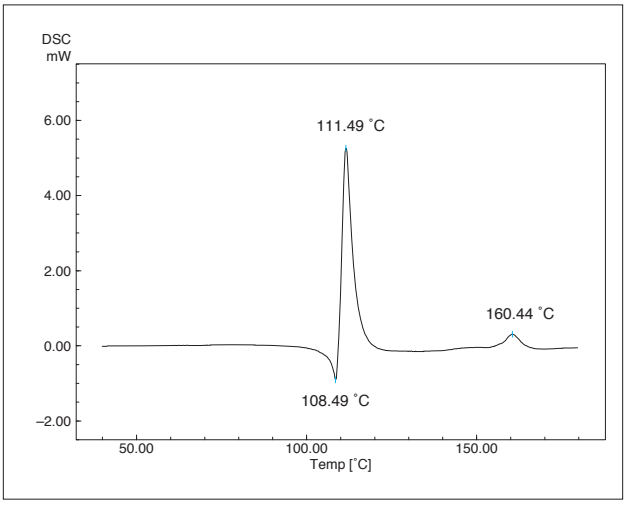


Fig. 8.2.3 DSC Curve of Benzoic Acid and Magnesium Oxide Mixture (1:1 by weight)

8.3 Observation of Capsules Using X-Ray CT System (1) - NDI

■Explanation

Industrial X-ray CT systems have conventionally been widely used to perform inspections and structural analyses of a variety of manufactured products including electronic parts, automotive parts, and resin molded parts. Recently however, they have also proved useful in the medical field for observing the internal structure of pharmaceutical tablets and granules. This article introduces images of granule-filled gelatin capsules, taken with a new X-ray CT system that provides clearer, more distinct data.

■Micro Focus X-Ray CT System

The equipment used to take the images is the new inspeXio SMX-100CT Micro Focus X-ray CT system (Fig. 8.3.1). This CT system is equipped with a sealed tube type micro focus X-ray generator with a maximum output of 100 kV, as well as a high-sensitivity image intensifier, enabling 3-dimensional observations of resins, pharmaceuticals, bones, and other soft materials at high magnification.

Fig. 8.3.1 Overview of the inspeXio SMX-100CT Micro Focus X-Ray CT System

■Observation of Granule-Filled Capsules

Fig. 8.3.3 shows a fluoroscopic image of the packaged granule-filled capsule in Fig. 8.3.2. In this fluoroscopic image, the granules inside the capsule are overlapping, and it is impossible to assess the positional relationship between the individual granules filling the capsules. Fig. 8.3.4 shows the CT image of this capsule as is. Fig. 8.3.4 is a cross-sectional image corresponding to a vertical cut through the capsule near the center, which makes it possible to observe the position of each granule precisely.
Fig. 8.3.5 shows this CT imaging data displayed 3-dimens ionally. When the data is displayed 3-dimensionally it becomes possible to observe the granules inside the capsule even more volumetrically. Furthermore, with the X-ray CT system, the interior can be observed in detail in magnified images such as in Fig. 8.3.6, without pretreating the capsule. In this way, observations with the sample conditions even kept as is revealed that this capsule contains 2 types of granules. We thus proceeded with more detailed observations, with these 2 types of granules removed from the capsule.

Fig. 8.3.2 Overview of a Capsule

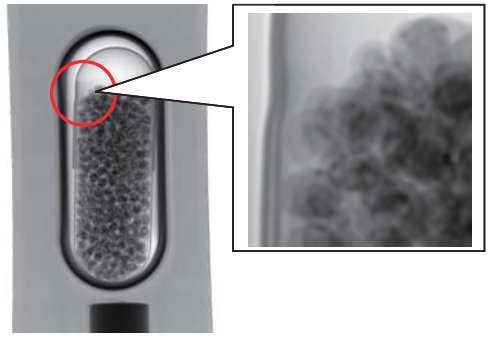


Fig. 8.3.3 Fluoroscopic Image of a Capsule

8.3 Observation of Capsules Using X-Ray CT System (2) - NDI

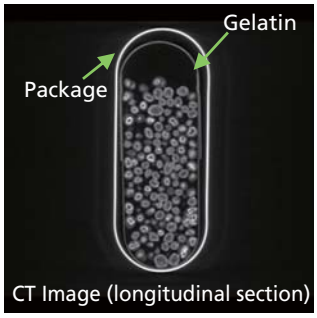


Fig. 8.3.4 CT Sectional Image of a Capsule

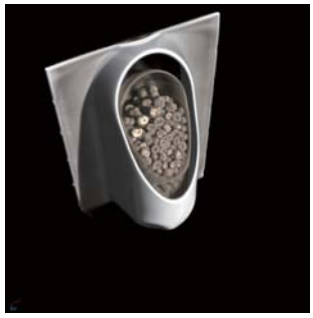


Fig. 8.3.5 3-Dimensional Image of a Capsule

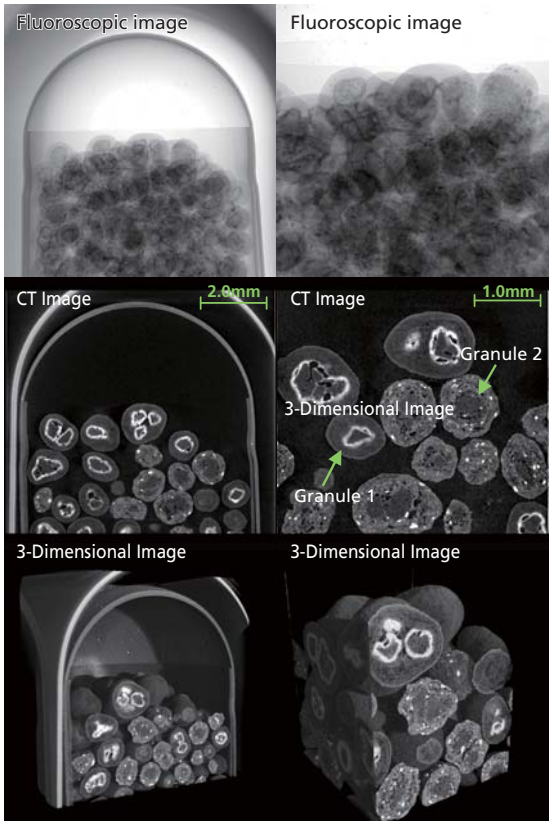


Fig. 8.3.6 Observational Images of a Capsule
Left : FOV size approx. 8 mm
Right : FOV size approx. 4 mm (high magnification)

■Observations of Granules

The fluoroscopic images, CT images, and 3-dimensional images of granules 1 and 2 are shown in Fig. 8.3.7. With granule 1, a 3-layered structure is evident, consisting of a low-density pharmaceutical agent (Fig. 8.3.7 (1)), packed with a separate granule (Fig. 8.3.7 (3)) coated with a slightly-higher-density pharmaceutical agent (Fig. 8.3.7 (2)). In the enlarged CT image, it is possible to observe that there is a void near the boundary between pharmaceutical agents (1) and (2) (Fig. 8.3.7 (1) and (2)).

With granule 2, it is possible to observe a 2-layer structure, consisting of a granule of low-density pharmaceutical agent (Fig. 8.3.7 (4)) coated by a separate, slightly-higher-density pharmaceutical agent (Fig. 8.3.7 (5)). In addition, it is evident that the granule (Fig. 8.3.7 (4)) contains a void similar to that in granule 1.

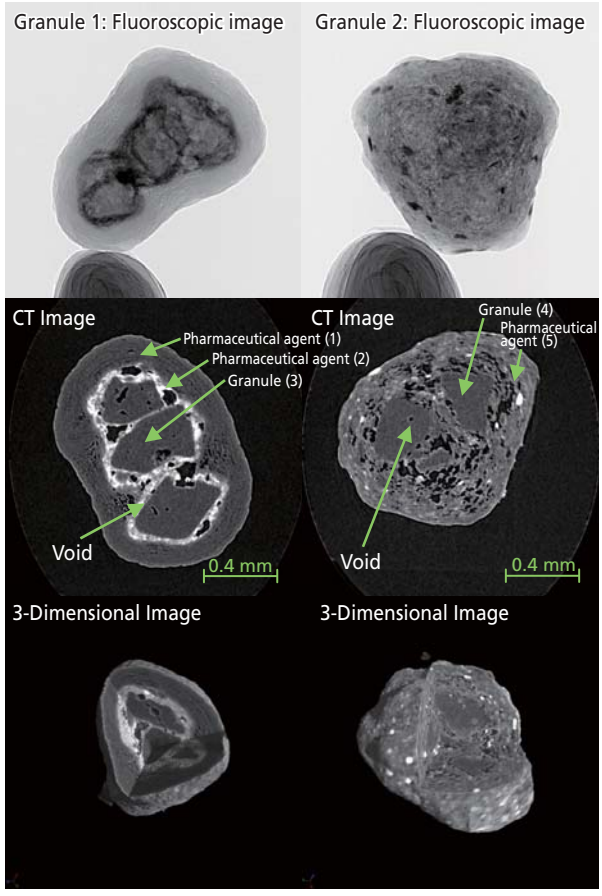


Fig. 8.3.7 Observational Images of Granules
Left : Granule 1
Right : Granule 2

■Conclusion

In this way, with the new inspeXio SMX-100CT, detailed observations of internal structure, all the way from the overall capsule image to the individual granules inside the capsule, can be obtained quickly and without complicated processing or treatments. In addition, this is a very useful system for observing not only capsules, but also tablet cracks, pharmaceutical agent distributions, and even coating layers.

8.4 Measurement of Press-Through Package Force and Tablet Break Force (1) - TM

■Explanation

Many tablets and capsules are packaged in thin metallic (aluminum, etc.) and plastic materials. This type of package is referred to as a PTP (Press-Through Package) or PTP sheet, and serves to protect the contained tablets and capsules and facilitate handling of the contents. Therefore, quality control is necessary to ensure that the package does not peel excessively, and that the tablet is not too difficult to dispense. Here, using the Shimadzu EZTest compact table-top universal testing machine and the Trapezium X material testing software, we introduce an example of the adhesive strength of the PTP package, in addition to the force required to break a tablet in half.

Fig. 8.4.1 PTP Packaged Tablets

Fig. 8.4.2 Testing Machine (EZ Test)

■Press-Dispense Test for PTP Packaged Tablets

As shown in Fig. 8.4.3, the test was performed by pushing the tablet out of the PTP package using an upper ϕ10 mm spherical press jig at a test speed of 50 mm/min, and a fixing platform below. In this case, the result obtained was a maximum test force mean value of 24.3 N. The judgment as to whether or not the tablet is easy to push out can be obtained from the maximum test force, which is applicable to product development and quality control.

Fig. 8.4.3 View of Testing Machine During Measurement

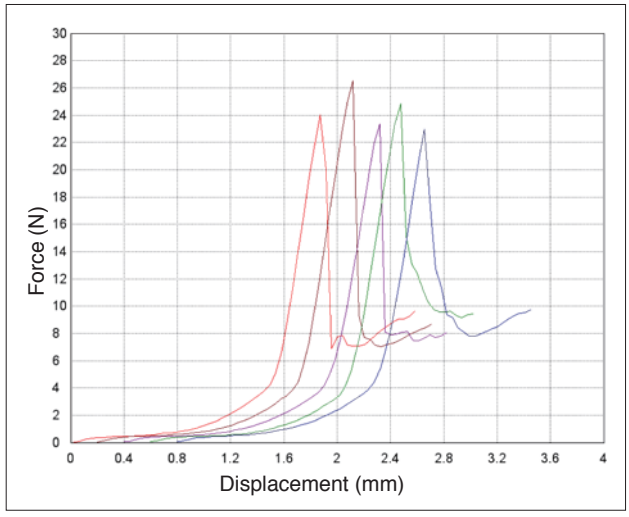


Fig. 8.4.4 Results of Press-Dispense Test

8.4 Measurement of Press-Through Package Force and Tablet Break Force (2) - TM

■Peel Test for PTP Packages

As shown in Fig. 8.4.5, using a 100 N pantograph type jigs above and fixing platform below, we performed the test by peeling the aluminum foil off from the underside of the PTP package at a test speed of 50 mm/min. Peeling began at about 0.3 N, and at the package edge the force was about 3 N.

Fig. 8.4.5 View of Testing Machine During Measurement

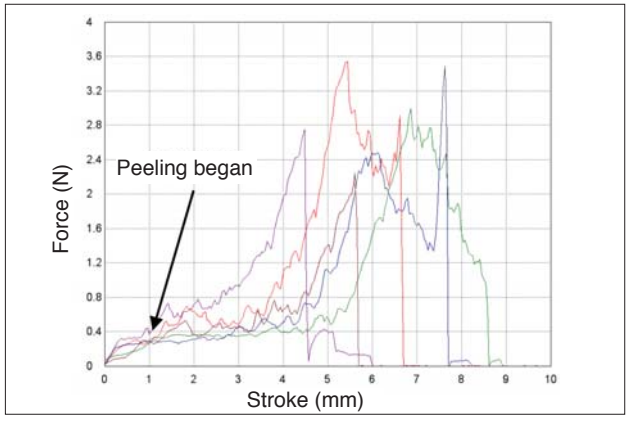


Fig. 8.4.6 Results of Peel Test

■Break Test for Tablets

Some tablets have a groove in the center, allowing each to be taken whole by adults or in a half-dose by children. Accordingly, it must be able to be broken in half using some suitable degree of force. As a suitable method of simulating this breaking-in-half, the tablet was placed above the bend test jig supports (5 mm distance between supports), and measurement was conducted at a test speed of 0.5 mm/min until the tablet broke. As shown in the measurement result graph of Fig. 8.4.8, the force required to break the tablet in half (mean value of maximum test force) was about 24 N. By using this kind of measurement, the depth of the tablet center groove can be optimized so that the tablet is easily and accurately broken in half.

Fig. 8.4.7 View of Testing Machine During Measurement

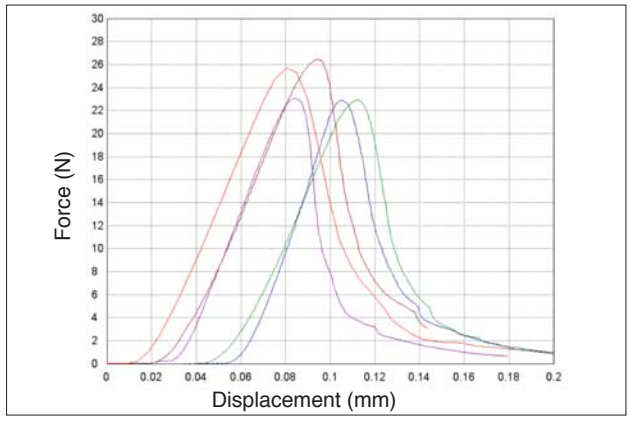


Fig. 8.4.8 Results of Break Force Test

Thus, by combining the Shimadzu EZ-Test compact table-top universal testing machine with the abundant selection of test jigs, test force evaluation of drug products including the packaging material can be conducted easily and efficiently.

9. Life Science

9.1 Analysis of Carbonylated Proteins (1) - MALDI-TOF MS

■Explanation

Using the AXIMA MALDI-TOF MS system for end-point analysis of two-dimensional electrophoresis, we analyzed carbonylated proteins in the CA1 area of a monkey hippocampus affected by ischemia-reperfusion. It is known that reactive oxygen species, or oxygen radicals (ROS), negatively impact a great many diseases, including cancer, cardiac disease and cerebral stroke, which are the leading causes of death among the Japanese, in addition to such lifestyle diseases as diabetes and arteriosclerosis. Reactive oxygen species are known to include hydrogen peroxide, the superoxide anion radical, and the hydroxyl radical, etc., and all of these cause nonphysiological posttranslational modifications in nucleic acids, lipids, proteins and other types of biological molecules. Protein carbonylation is a type of protein damage resulting from oxidative stress in cells, and these carbonylated proteins are used as markers for oxidative stress to proteins. Oxidative carbonylation occurs when an aldehyde is formed on the side chains of amino acids such as arginine and lysine, which comprise proteins (Fig. 9.1.1).

It is suggested that selective neuronal cell death occurs in the hippocampus CA1 area due to transient cerebral ischemia, resulting in memory impairment. Here, using a sample consisting of the hippocampus CA1 area affected by transient cerebral ischemia, two-dimensional electrophoresis was conducted, and spots thought to be carbonylated proteins were excised and analyzed by LC-MALDI. The results confirmed the carbonylation of the 469th arginine of the Heat shock-70 kDa protein 1 (Hsp70-1), which plays a role in regulating cell death (Fig. 9.1.2, 3).

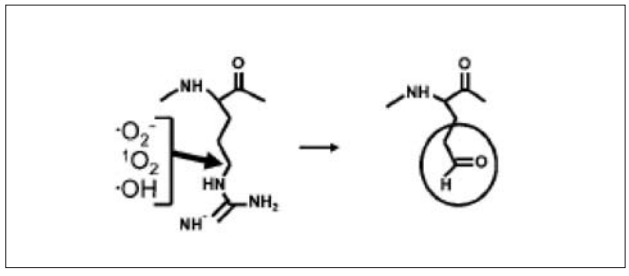


Fig. 9.1.1 Carbonylation of Arginine

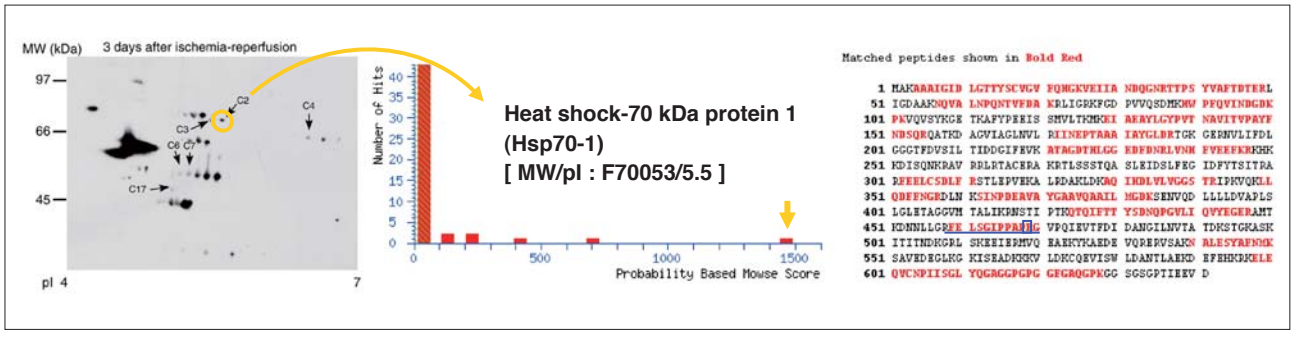


Fig. 9.1.2 Results of Identification of Spot C2 (Hsp70-1) by LC-MALDI

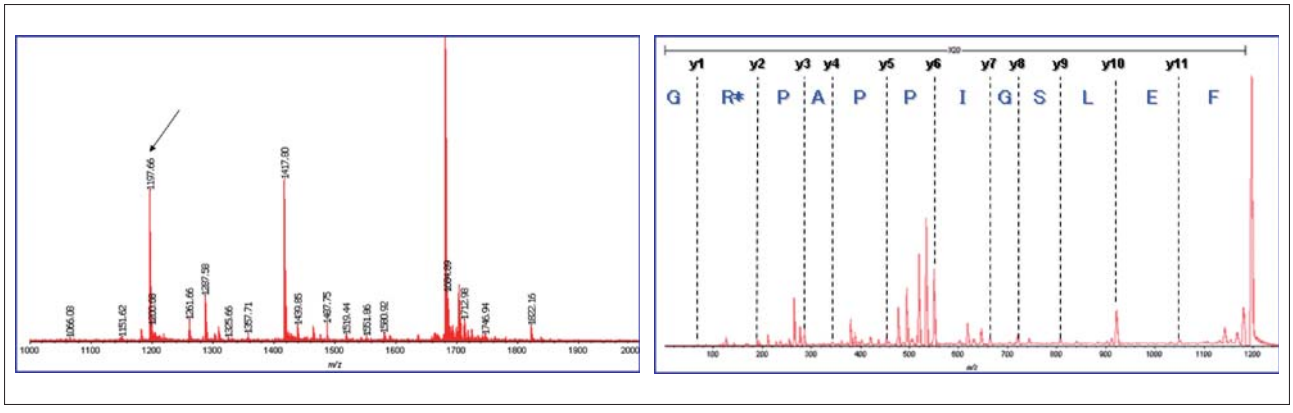


Fig. 9.1.3 Mass Spectra of Hsp70-1
(A) Spectrum of the Tryptic Digest of Hsp70-1
(B) MS/MS Spectrum of *m/z* 1197.66 (FELSGIPPAPR*G: R*, Carbonylated Arginine)

9.1 Analysis of Carbonylated Proteins (2) - MALDI-TOF MS

■Explanation

After extracting proteins from the CA1 area of a monkey hippocampus prior to, and 3, 5 and 7 days following transient cerebral ischemia, the proteins subjected to oxidative stress were labeled using 2,4-dinitrophenylhydrazine (DNPH), and separated by two-dimensional electrophoresis. In addition, Western blotting was performed using anti-DNP antibodies, and the carbonylated proteins were detected (Fig. 9.1.4 (A)). In addition, we checked for variations in all proteins using 2D-DIGE (Fig. 9.1.4 (B)). The results confirmed remarkable changes in 6 spots, and that these changes were due to carbonylated proteins. PMF analysis conducted on these 6 spots confirmed that carbonylation had affected 4 types of proteins (Table 9.1.1). In addition, after performing In-gel digestion of the C2 spot, we conducted MS/MS automatic measurement using the LC-MALDI system. The results confirmed that the 469th arginine was carbonylated. The Axima Performance system for two-dimensional electrophoresis used here is an extremely effective tool for posttranslational modification analysis, as demonstrated in these results of analysis of carbonylated proteins as one type of oxidative stress to proteins.

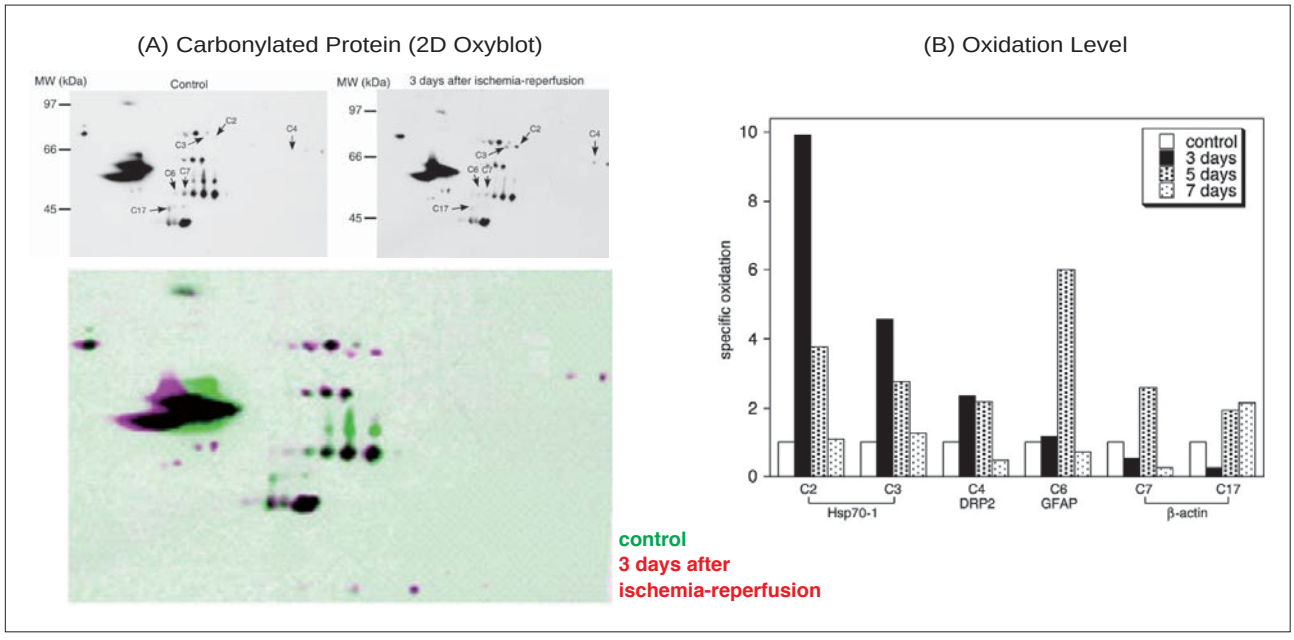


Fig. 9.1.4 (A) 2D Oxyblot (Control and Day 3 Post-Ischemic CA1 and Image Analysis (Using Progenesis PG200))
(B) Specific Oxidation Levels of Identified Proteins at 3, 5, and 7 Days After Ischemia-Reperfusion Insult

Table 9.1.1 Identification of Carbonylated Proteins

Spot	Protein Name	% Coverage	Theoretical Molecular Mass (Da)/pI
C2	Heat shock-70 kDa protein 1 (Hsp70-1)	22.6	70053/5.5
C3	Hsp70-1	29.0	70053/5.5
C4	Dihydropyrimidinase-like 2 isoform 2	28.2	73583/5.9
C6	Glial fibrillary acidic protein	37.0	47412/5.2
C7	β-Actin	39.7	41737/5.3
C17	β-Actin	32.3	41737/5.3

[References]

S. Oikawa et al. / Free Radical Biology & Medicine 46 (2009) 1472_1477

* The data presented here was acquired through joint research with Associate Professor Shinji Oikawa of the Department of Medicine, Mie University.

9.2 Phosphopeptide Enrichment Technique Using TiO_2 (1) - MALDI-TOF MS

■Explanation

Phosphorylation of proteins is one type of post-translational modification (PTM) which is important in the control of biological functions. Recently, mass spectrometry is being applied to the analysis of phosphorylation sites. However, due to the low ratio of phosphorylation as well as the marked decrease in ionization efficiency when phosphorylation is present, it is often difficult to conduct analysis of mixtures in their original state. Over the past several years, great achievements have been realized in phosphorylation research due to research into specific phosphopeptide enrichment techniques using IMAC (Immobilized Metal Affinity Chromatography) and titanium dioxide (TiO_2). Here we describe phosphorylation analysis using a combination of TiO_2-based phosphopeptide enrichment and MALDI-MS/MS (seamless PSD). Fig. 9.2.1 shows the typical enrichment protocol using TiO_2. The principle underlying the affinity of TiO_2 to the phosphate group is illustrated in Fig. 9.2.2. The phosphopeptides can be enriched by washing them with an alkaline solvent after the phosphopeptides in the mixture are trapped with TiO_2. However, since TiO_2 also shows some affinity for acidic amino acids, a process is required that will exclude adsorption to non-phosphopeptides that contain acidic amino acids. For that, a high concentration acid and high concentration organic solvent (acetonitrile) are required. Fig. 9.2.3 shows the effectiveness of TiO_2 enrichment. The phosphopeptides were enriched with specificity, and even the phosphopeptide (*m/z* 1660) that could not be detected using desalting alone was observed.

Step	Solution
Conditioning	80 % ACN, 2 - 2.5 % TFA
Sample preparation	80 % ACN, 2 - 2.5 % TFA
Adsorption	Above“ Sample preparation solution”
Washing	80 % ACN, 2 - 2.5 % TFA
Elution	NH4OH (> pH 10), ACN
Desalting	Ordinary desalting by ZipTip (Millipore), etc.

Fig. 9.2.1 Enrichment Protocol Using TiO_2

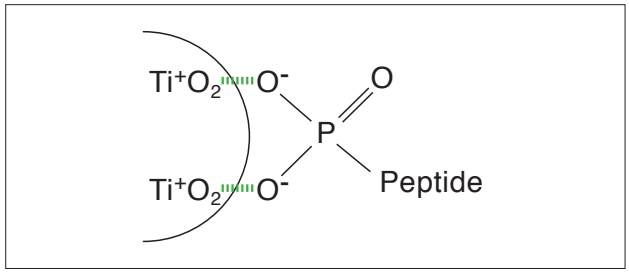


Fig. 9.2.2 TiO_2 Affinity for Phosphate Group

■Analytical Conditions

Instrument : AXIMA *Performance*
Matrix : 2, 5-DHB (Dihydroxybenzoic Acid)
10 mg/mL (50 % Acetonitrile, 0.1 % TFA)
Sample : Tryptic Digest Mix
(BSA, *α*-Casein, *β*-Casein, Ovalbumin)

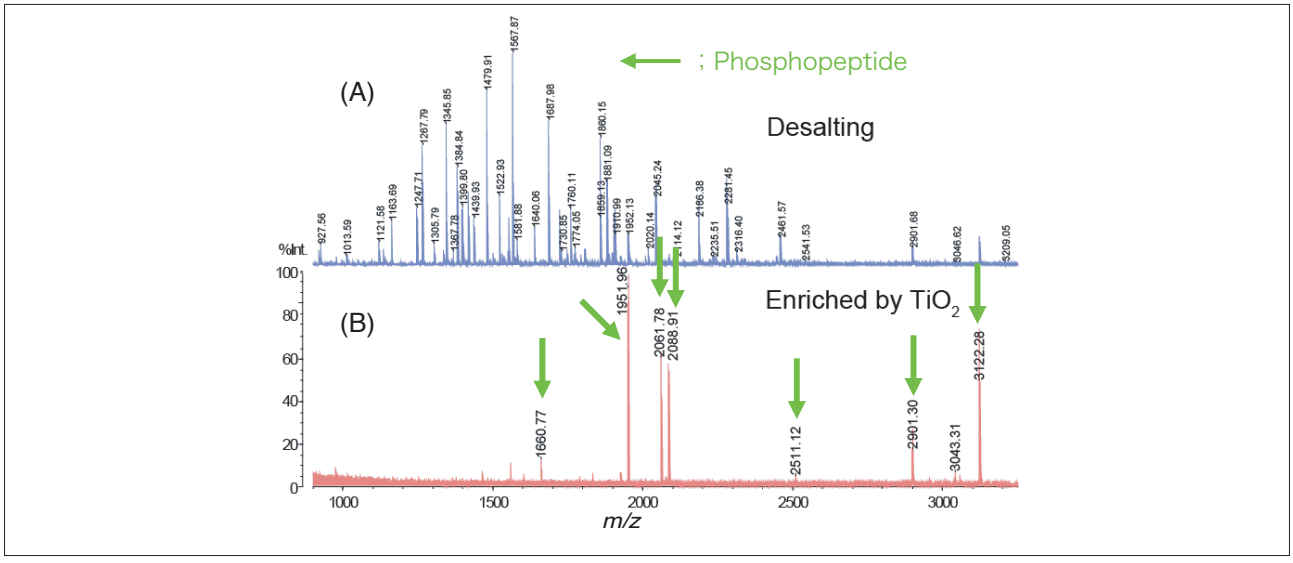


Fig. 9.2.3 Example of Phosphopeptide Enrichment Using TiO_2 (A) Desalted by C18 ZipTip (B) Enriched by TiO_2

9.2 Phosphopeptide Enrichment Technique Using TiO_2 (2) - MALDI-TOF MS

Fig. 9.2.4 shows the results of electrophoretic analysis of a whole cell lysate with abundant phosphoproteins. MS/MS (sPSD, or seamless Post Source Decay) measurement of 17 spots was conducted, and some of the results are shown in Fig. 9.2.5. The proteins of all the spots were identified, and the phosphorylation sites were specified. Particularly noteworthy is that sequence analysis including phosphorylation sites was possible by sPSD, as shown in Spot 11. Thus, enrichment using TiO_2 in combination with sPSD can be considered to be an effective method for conducting phosphorylation research.

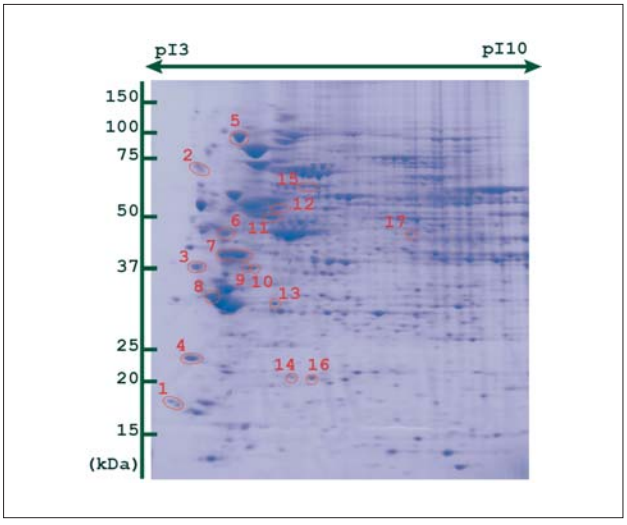


Fig. 9.2.4 Two-Dimensional Electrophoretic Profile of A431 Whole Cell Lysate (CBB-stained)

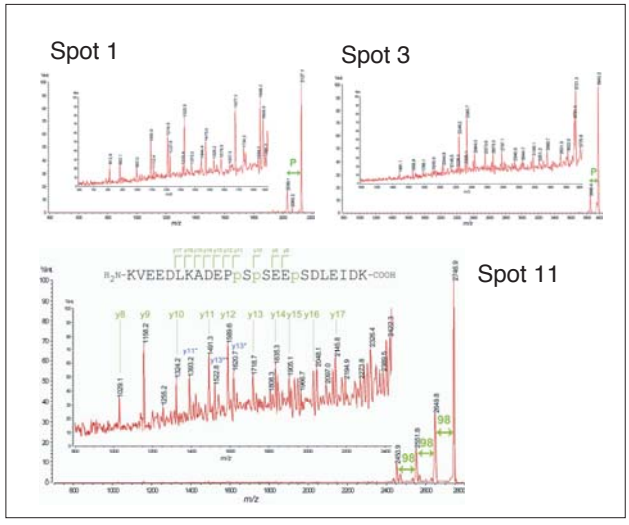


Fig. 9.2.5 sPSD Spectra of Phosphopeptides Extracted from Spots 1, 3 and 11

Table 9.2.1 Identified Phosphoproteins and Phosphorylation Sites

Spot No.	protein description	score	mass	sequence
1	60S acidic ribosomal protein P1	45	11665	K.KEEpSEEpADDDMGFGLFD.-
2	IQ domain-containing protein E	31	77696	R.VPpSPIAQApTGpSPVQEEAIVIIQpSALR.A
3	Nascent polypeptide-associated complex subunit	56	23384	K.VOGEAVSNIQENTQTPTVQEEpSEEEEVDETGVEVK.D
4	Prostaglandin E aynthase 3 - Homo sapiens	55	18982	K.DWEDDpSDEDMSNFDR.F
5	Endoplasmin precursor - Homo sapiens	17	92753	K.VEEEPEEEPEETAEDTTEDTEQDEDEEoxyMDVGpTDEEEETAK.E
6	CaM kinase-like vesicle-associated protein	26	54695	R.ATPATEESpTVPTTQSSAoxyMLATK.A
7	Hepatoma-derived growth factor	56	26902	K.GNAEGpSpSDEEGKLVIDEPAK.E
8	Elongation factor 1-beta	135	24935	K.DDDDIDLFGpSDDEEESEEAK.R K.DDDDIDLFGpSDDEEESEEAKR.L
9	Elongation factor 1-delta	44	31236	K.KPATPAEDDEDDDIDLFGpSDNEEEDKEAAQLR.E
10	Elongation factor 1-delta	73	31236	K.KPATPAEDDEDDDIDLFGpSDNEEEDKEAAQLR.E
11	Hsc70-interacting protein	47	41502	K.KVEEDLKADEPpSpSEEpSDLEIDK.E
12	Probable phospholipid-transporting ATPase IK	33	149626	R.STRAGPEPpSPAPPGPGDpTGDSDVTQEGSGPAGIRGGETVIR.A
13	Proteasome subunit alpha type-3	34	28661	K.ESLKEEDEpSDDDNoxyM.-
14	(Stathmin)			
15	Ras GTPase-activating protein-binding protein 1	108	52221	K.SpSSPAPADIAQTVQEDLR.T
16	Stathmin	73	17302	K.ESVPEFPLpSPPK.K R.ASGQAFELILpSPR.S
17	Septin-2	34	41715	K.IYHLPDAEpSDEDEDFKEQTR.L

[References]
Nature Methods., 4 (3), 231 (2007)

9.3 Glycopeptide Analysis (1) - MALDI-TOF MS

■Explanation

Recently, along with the expansion of protein analysis, the importance of posttranslational modification analysis has also come to be recognized. In particular, not only is sugar chain modification important for understanding protein functions, it is also receiving a great deal of attention as a disease marker candidate. The AXIMA *Resonance* is a mass spectrometer that combines Shimadzu's unique quadrupole ion trap with MALDI. The ion trap is a powerful tool which is suitable for analysis of glycoproteins, complex structures containing sugar chains (glycans) attached to polypeptides through posttranslational modification, because of its ability to conduct MS^n measurement (MS/MS, MS^3, MS^4). The existing AXIMA *Resonance* is receiving high acclaim from researchers in this field. Typically, a glycoprotein is analyzed by enzymatically digesting the protein portion until the fragments form glycopeptides, which are then analyzed. Previously, when the AXIMA-QIT was used to conduct this glycopeptide measurement, obtaining sufficient sensitivity proved difficult at measurement of MS^3 or greater. The AXIMA *Resonance* is an instrument that can perform MS^n at very high sensitivity, making possible analysis that has been problematic up to now. Here we introduce as an example the measurement of a glycopeptide derived from an antibody. Fig. 9.3.1 shows the results of measurement of a typical N-type glycopeptide derived from an antibody using the AXIMA *Resonance*. At greater than *m/z* 2500, a series of glycopeptide signals corresponding to the sugar chain variety is observed. Among these, *m/z* 2926.3 was selected as a precursor for MS/MS analysis. Mainly, sugar chain fragmentation products are observed in the MS/MS spectrum of Fig. 9.3.2, making it useful for conducting analysis of the sugar chain. However, since information related to the peptide portion cannot be obtained at this stage, we conducted MS^3 measurement using *m/z* 1157.5 observed in the MS/MS spectrum.

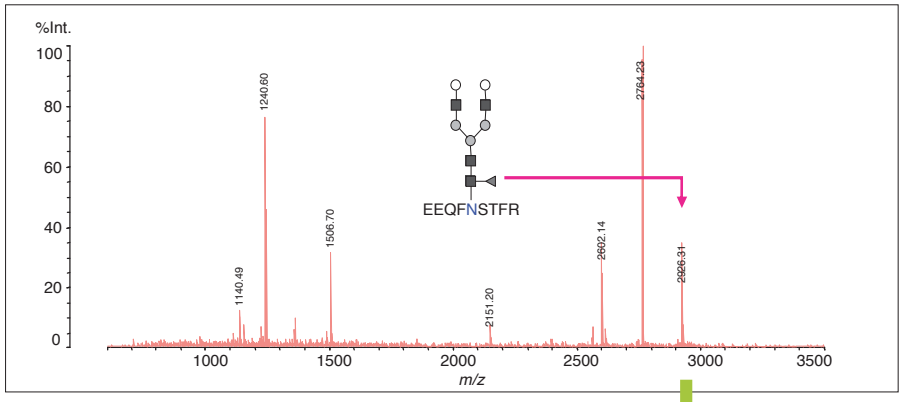


Fig. 9.3.1 MS Spectrum of a Glycopeptide derived from IgG

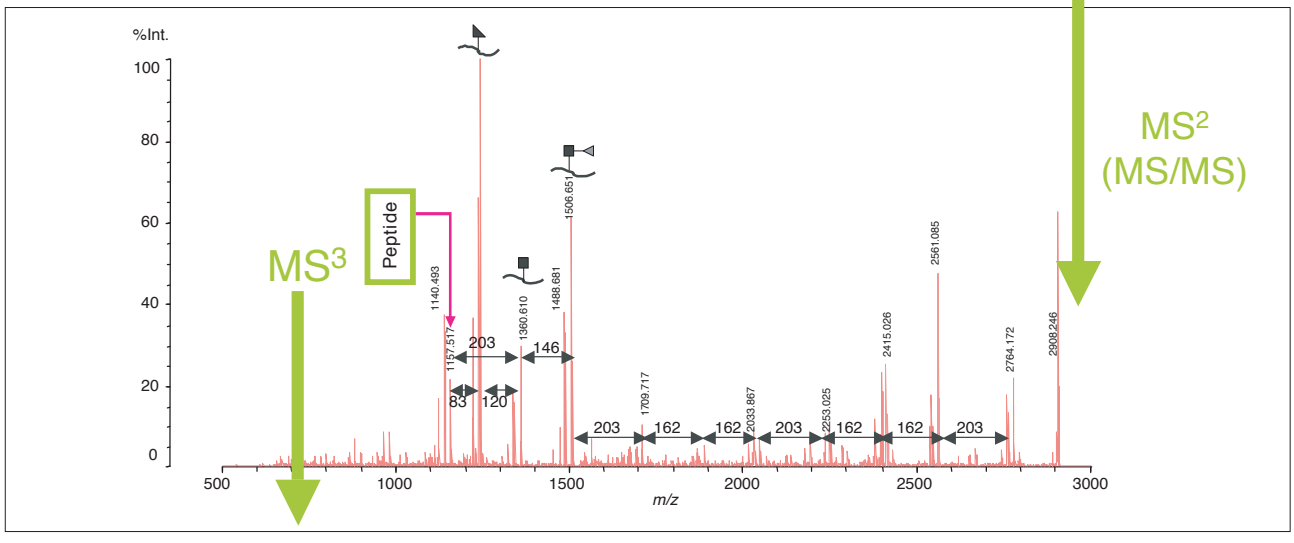


Fig. 9.3.2 MS/MS Spectrum from *m/z* 2926.3 in Fig. 9.3.1

9.3 Glycopeptide Analysis (2) - MALDI-TOF MS

An excellent MS^3 spectrum was obtained from *m/z* 1157.5 of MS/MS, as shown in Fig. 9.3.3. The fragmentation ion that supports a known peptide sequence was detected in this MS^3 spectrum. Thus, MS^n measurement using the AXIMA *Resonance* is believed to be effective not only for sequence analysis of glycopeptides sugar chains, but for peptide sequence analysis as well.

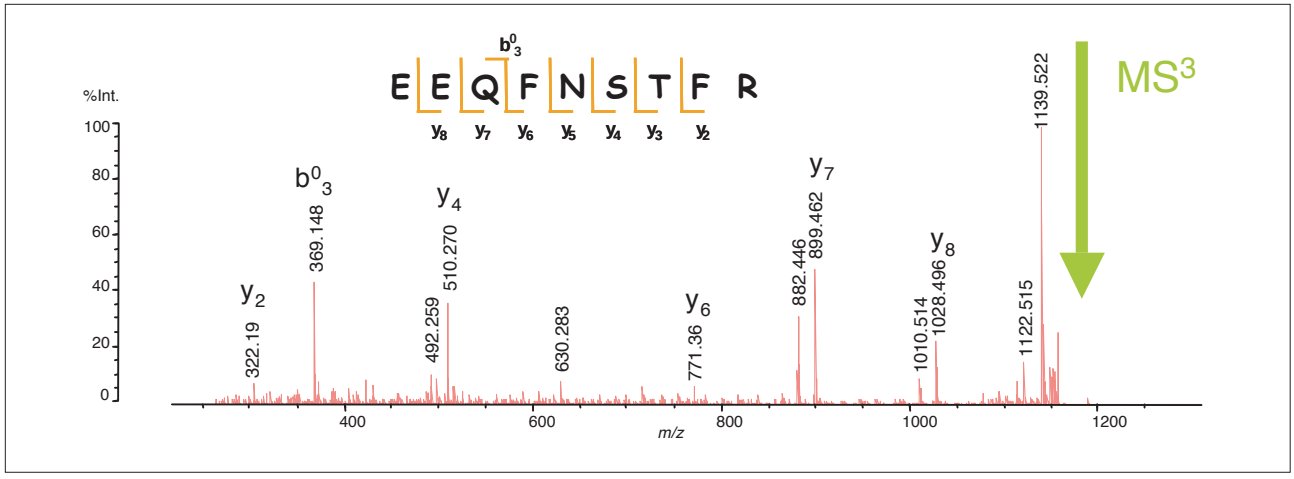


Fig. 9.3.3 MS^3 Spectrum from *m/z* 1157.5 in Fig. 9.3.2 and the Amino Acid Sequence of the Glycopeptide

9.4 Analysis of O-linked Glycopeptide (1) - MALDI-TOF MS

■Explanation

Along with the growing use and advances in proteomics research, it has become clear that many proteins, when subjected to a type of modification, become biologically active molecules. This protein modification is generally referred to as posttranslational modification, and many types of modification have been identified, including phosphorylation, methylation, etc. Among these, sugar chain modifications are receiving attention as new biomarkers for a variety of diseases such as cancer. However, when these proteins which have undergone sugar chain modification are analyzed using mass spectrometry, their detection sensitivity is extremely low compared to that of glycopeptides, with the result that they are currently difficult to analyze. The AXIMA *Resonance*™ has a detection sensitivity of 500 amol when conducting MS using peptides, and furthermore, since it is equipped with a mechanism for conducting multiple-stage mass spectrometry, it can be expected to play a powerful role in analysis of complex posttranslational modifications involving sugar chains.

Here we introduce an evaluation of ion detection sensitivity in analysis of an actual sample consisting of an O-linked glycopeptides, in addition to analysis of the glycosylation site. For the sample, we used the O-linked glycopeptides (MW: 1517.55) which binds the Core 3 structure (GlcNAcβ1-3GalNAcα1-) with threonine, the fifth amino acid in the partial sequence "AHGVTSAPDTR" of the MUC1 mucin protein (Fig. 9.4.1). Stepwise dilutions of this sample were deposited on a MALDI target plate, and after matrix solution (DHB: 2,5-dihydroxybenzoic acid) was spotted on each sample dilution, the spots were dried and MS analysis was conducted (Fig. 9.4.2). In addition to the typically used stainless steel MALDI plate, we also investigated ion detection using the µFocus MALDI plate*, equipped with 600 µm diameter measurement wells (Fig. 9.4.3). Due to the surface treatment applied to µFocus MALDI plates*, sample solution can be confined to a small area within the measurement wells, allowing ultra-small quantities of sample to be measured efficiently in this very useful plate. The results of these measurements confirm that detection of glycopeptide to the 1 fmol level is possible using the AXIMA *Resonance*™ with the selection of an appropriate MALDI plate. In addition, by not limiting analysis to simple MS and MS/MS, but extending analysis to MS^n ($n \geq 3$), it is possible to identify the site where the glycan binds to the peptide. Here, the glycopeptide molecular ion obtained in MS analysis was used as the precursor ion in conducting MS/MS analysis, making it possible to learn the composition of the glycan linked to the peptide (Fig. 9.4.4). Further, the ion of the peptide part obtained in MS/MS analysis was used in MS^3 analysis (MS^3- ②), and the ion with one sugar linked to the peptide was used as the precursor ion in MS^3 analysis (MS^3- ①). By conducting comparative analysis of these, it was possible to identify the site where the glycan binds to the target peptide (Fig. 9.4.5). As described above, by conducting MS/MS spectral analysis of the sample selected here, and then comparing the spectra obtained in MS^3 analysis, we were able to confirm that the Core 3 structure suggesting a disaccharide structure is linked to threonine, the fifth amino acid in the peptide sequence. The AXIMA *Resonance*™, with its high sensitivity, high resolution as well as its ability to conduct highly accurate multiple-stage mass spectrometric analysis, is extremely useful for this type of analysis.

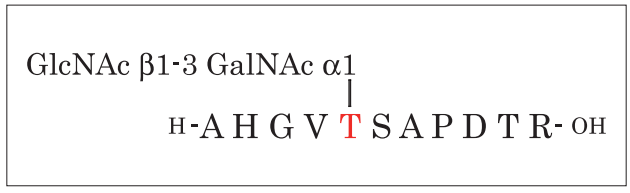


Fig. 9.4.1 O-linked Glycopeptide Sample

■Analytical Conditions

Conditions 1) for Standard Stainless MALDI Plate

Matrix	: 10 mg/mL DHB (2, 5-Dihydroxybenzoic Acid) in 50 % Acetonitrile, 0.05 % TFA (0.5 µL)
Laser Power	: 77-90
Laser Shots	: 2 Shots/Profile
Accumulation Profile	: 100 Profiles

Conditions 2) for µFocus MALDI Plate*

Matrix	: 2.5 mg/mL DHB (2, 5-Dihydroxybenzoic Acid) in 50 % Acetonitrile CN, 0.05 % TFA (0.5 µL)
Laser Power	: 85-104
Laser Shots	: 2 Shots/Profile
Accumulation Profile	: 100 Profiles

9.4 Analysis of O-linked Glycopeptide (2) - MALDI-TOF MS

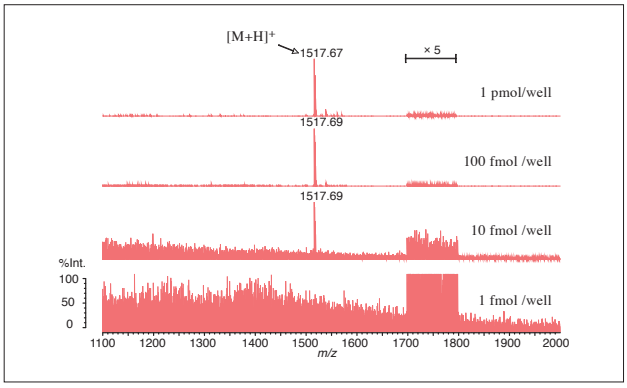


Fig. 9.4.2 Ion Detection of O-linked Glycopeptide on Standard Stainless MALDI Plate

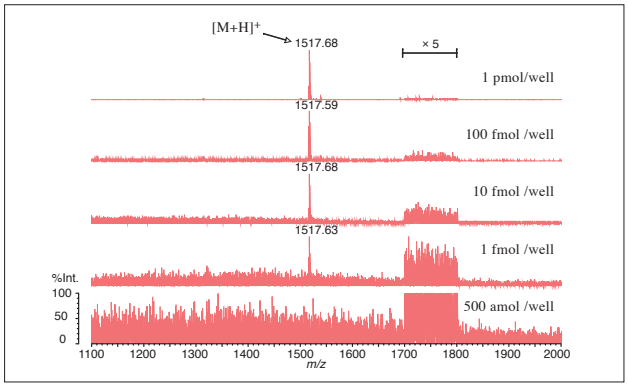


Fig. 9.2.3 Ion Detection of O-linked Glycopeptide on µFocus MALDI Plate*

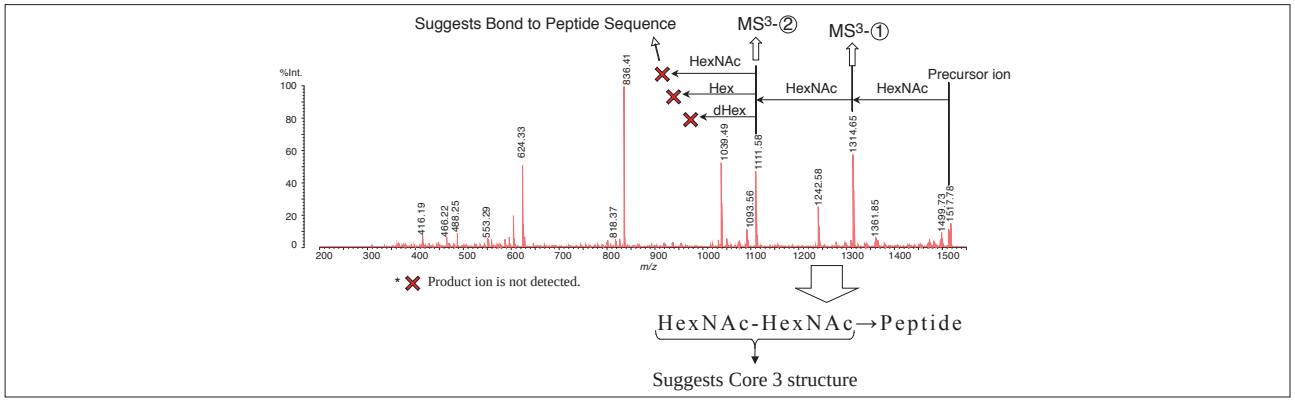


Fig. 9.4.4 Confirmation of The Glycan Sequence by Analysis of MS/MS Spectrum

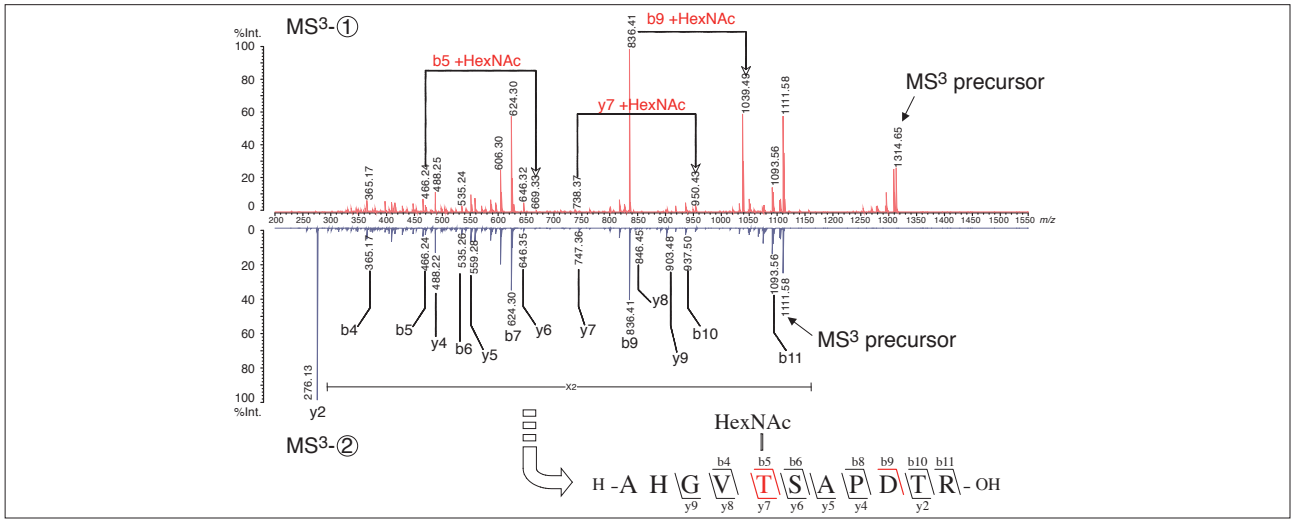


Fig. 9.4.5 Confirmation of Peptide Sequence and Determination of Glycosylation Site by Comparative Analysis of MS3 Spectra

[Acknowledgment]
We wish to offer our appreciation to Dr. Ito of the Advanced Industrial Science and Technology (AIST) Research Center for Medical Glycoscience for kindly providing the sample used in this analysis.

* µFocus MALDI plates are a product of Hudson Surface Technology, Inc.

9.5 A Simple and Highly Successful C-terminal Sequence Analysis of Proteins (1) - MALDI-TOF MS

■Explanation

Protein identification via Peptide Mass Fingerprinting (PMF) is conducted by enzymatically digesting the protein and analyzing the resulting digest using mass spectrometry. A database search is then applied to the list of peaks obtained from this analysis. However, assignment of the N and C terminal sequences is not always easily accomplished with a database search because 1) the protein N and C terminals are often changed due to processing and post-translational modification, and 2) a portion of the protein sequence may not be detectable by the mass spectrometer due to the ion suppression effect. The protein N-terminal amino acid sequence can be determined using a protein sequencer (PPSQ-31A/33A) or a protein N-terminal sequencing kit (ORFinder-NBTM). However, in the case of the C terminal, there has been a need for a technique importance of protein terminal amino acid sequence analysis is becoming more important than ever. Here we introduce an example of mass spectrometric analysis of a sample consisting of selectively collected protein C-termini, demonstrating a newly developed, successful method of amino acid sequencing.

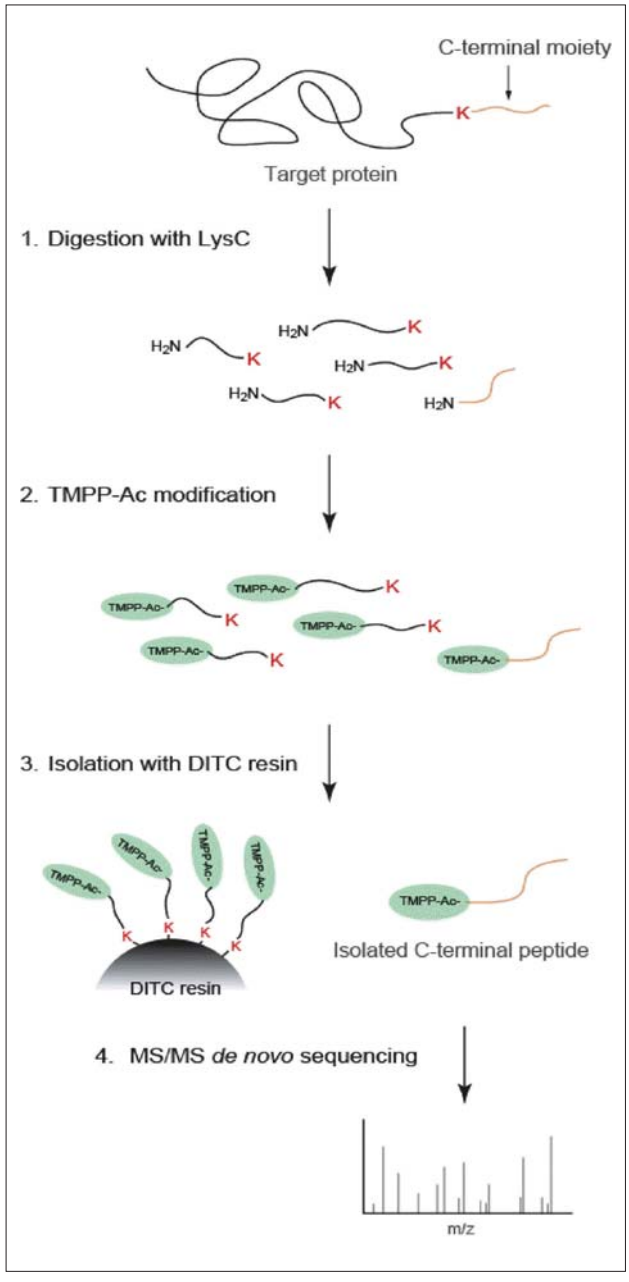


Fig. 9.5.1 Schematic Overview of C-terminal Sequence Analysis of Proteins by Mass Spectrometry

9.5 A Simple and Highly Successful C-terminal Sequence Analysis of Proteins (2) - MALDI-TOF MS

The operational flow of a protein C-terminal amino acid sequence analysis is shown in Fig. 9.5.1. In step 1, when the target protein is digested using lysyl endopeptidase (LysC), all of the digest peptide carboxy-terminal amino acids, except for those derived from the protein C-terminal, are converted to lysine (except when the protein C-terminal amino acid is lysine). In step 2, the LysC digest is reacted with (succinimidyloxycarbonylmethyl) tris (2,4,6-trimethoxyphenyl) phosphonium bromide (TMPP-Ac-OSu) so that the α amino groups are selectively TMPPAc-modified. In step 3, TMPP-Ac-modified the LysC digest is added to p-phenylenediisothiocyanate-resin (DITC resin or glass), so that all of the peptides that have lysine as an ε amino group side chain - in the sequence are trapped by the DITC resin.-Protein-C terminal-derived digest peptides without a free amino group are not trapped by the DITC resin. In step 4, when isolated protein C-terminal peptides are measured by MS/MS, only fragment ions containing the strongly positively charged TMPP are observed in the MS/MS spectrum. Assignment of the protein Cterminal amino acid sequence is possible by comparing the mass difference between these fragment ions and the terminal amino acid sequence predicted from the genetic sequence. Fig. 9.5.2 shows a MALDI-MS spectrum obtained following TMPP-Ac modification of a LysC digest of the recombinant protein complex PfuRPA consisting of 3 types of subunits. Each of the subunits, RPA14, RPA32, and RPA42 derived from the digest peptides, are observed in the MS spectrum, but the C-terminal peptides associated with RPA14 and RPA32 can hardly be seen. Next, Fig. 9.5.3 shows an MS spectrum following isolation of each of the subunit C-terminal peptides by reaction of the PfuRPA TMPP-Ac modified LysC digest with DITC resin. All the peptides derived from the internal sequence are trapped by the DITC resin, and only the peaks originating from each of the subunit C-terminal peptides are detected and observed in the MS spectrum. MS/MS measurement was conducted on all 3 of these peaks derived from the C-terminal peptides, and by comparing the terminal amino acid sequence predicted from the genetic sequence with the mass differences between the detected fragmentation ions, we were able to assign the C-terminal sequence for each subunit. Shown here as an example are the results of MS/MS measurement of the *m/z* 2504 peak using the highenergy CID method, a feature of the AXIMA *Performance* (Fig. 9.5.4).

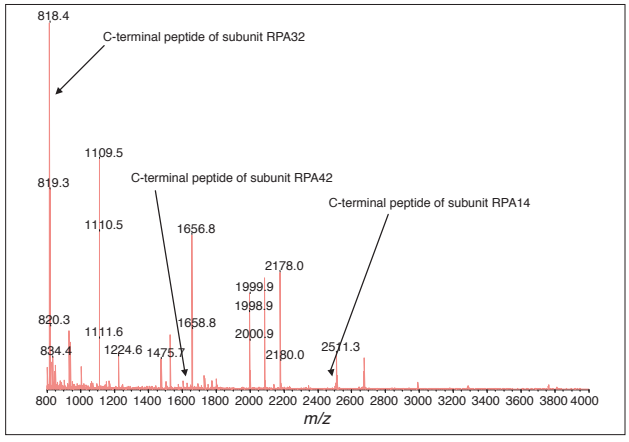


Fig. 9.5.2 MALDI-TOF Mass Spectrum after TMPP Modification of LysC Digest from PfuRPA Protein Complex

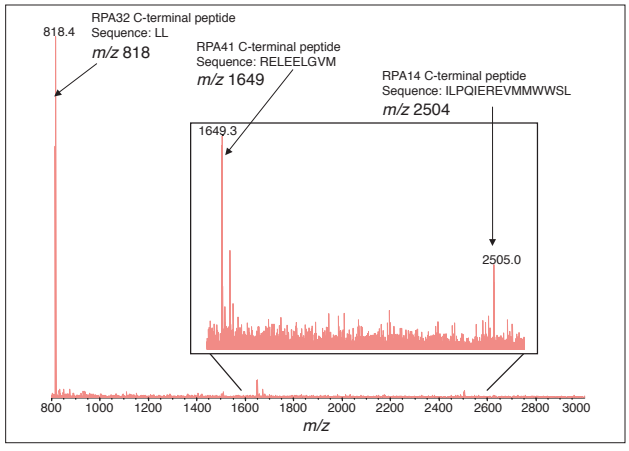


Fig. 9.5.3 MALD-TOF Mass Spectrum after Isolation of Three C-terminal Peptides using DITC Resin

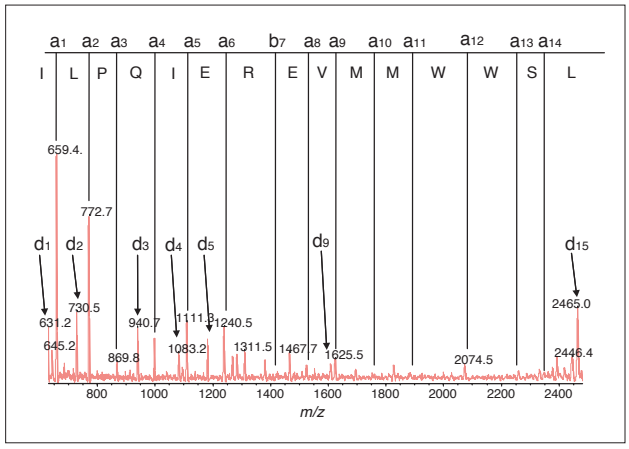


Fig. 9.5.4 MS/MS Spectrum of Isolated C-terminal Peptide from RPA14

[Reference]
Kuyama, H., Shima, K., *et al*., Proteomics 2008, 8, 1539-1550.

9.6 Differentiating βAsp Residue by PSD in a Curved Field Reflectron (1) - MALDI-TOF MS

■Explanation

Aspartic acid in proteins is known to form a 5-membered ring when it undergoes isomerization (isoaspartate; isoAsp or βAsp) due to ultraviolet irradiation or aging, etc. [1] As this isomerization means the formation of a bond between the C=O group of an aspartic acid side chain and the NH group of a neighboring residue, it is thought that this imparts instability to the main chain of the protein, eventually leading to modification of the protein structure and cohesion between proteins. In fact, it has been reported that α crystallin including isoaspartate exists in the crystalline lens of cataract patients. The detection and quantitation of isoaspartate is mainly conducted by protein sequencing and HPLC, but due to the difficulty in separating the isomers and the very small amounts present, analysis is generally difficult. MALDI-TOF MS is an effective method of analyzing trace level analytes, but because the masses of isomer residues are identical, isoaspartate detection analysis is not possible using simple molecular weight measurement. Here, we report the results of detection of the characteristic fragment ion of βAsp and its differentiation from ordinary Asp using TOF post source decay (PSD) analysis with Shimadzu's original Curved Field Reflectron technology[5].

Fig. 9.6.1 shows the structures of the Asp isomers. The characteristic feature of βAsp is the reversed main and side chain structure. Fig. 9.6.2 shows the conditions that were used for PSD measurement by MALDI-TOF MS. For the sample, an isomerized synthetic peptide from an Asp site in an α crystallin partial amino acid array was used (Fig. 9.6.2). It is known that Asp included in this partial sequence is susceptible to isomerization due to aging, etc. The PSD spectra of the synthetic T6 peptide are shown on the following page. The spectra of these different isomerized Asp included in the array are extremely similar, but it is clear that the intensities of fragmentation ions y7 and y8 before and after the Asp residue are extremely different. In addition, the characteristic y8-46 from the peptide including βAsp was detected notwithstanding its weak intensity. PSD measurement can be conducted very easily by selecting the precursor and setting the laser power to a value higher than that used in typical MS. Thus, it was shown that βAsp, which has been so difficult to distinguish by conventional methods, can easily be identified using PSD.

* This report represents part of the results obtained in joint research with Dr. Fujii of the Kyoto University Research Reactor Institute.

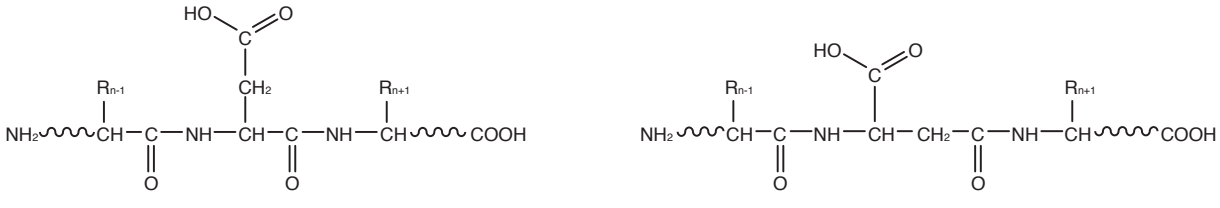


Fig. 9.6.1 Chemical Structures of α- and βAsp Residues

[Sample] T6 peptide
(Partial array of human α crystallin)
Concentration : 1 mg/mL
Sequence : TVLDSGISEVR (1175.6)

4th Asp residue was replaced with following isomers.
Lα: TVL (αD) SGISEVR
Lβ: TVL (βD) SGISEVR

[Measurement]
Instrument : AXIMA *Performance*™
Instrument conditions : Reflectron/positive
Matrix : α-CHCA 5 mg/mL 50 % Acetonitrile (0.1 % TFA)
Mass calibration : The following external standardswere used for mass calibration.
Angiotensin Ⅱ : *m/z* 1046.54
ACTH18-39 : *m/z* 2465.20

Fig. 9.6.2 Synthetic Peptides and Experimental Conditions

9.6 Differentiating βAsp Residue by PSD in a Curved Field Reflectron (2) - MALDI-TOF MS

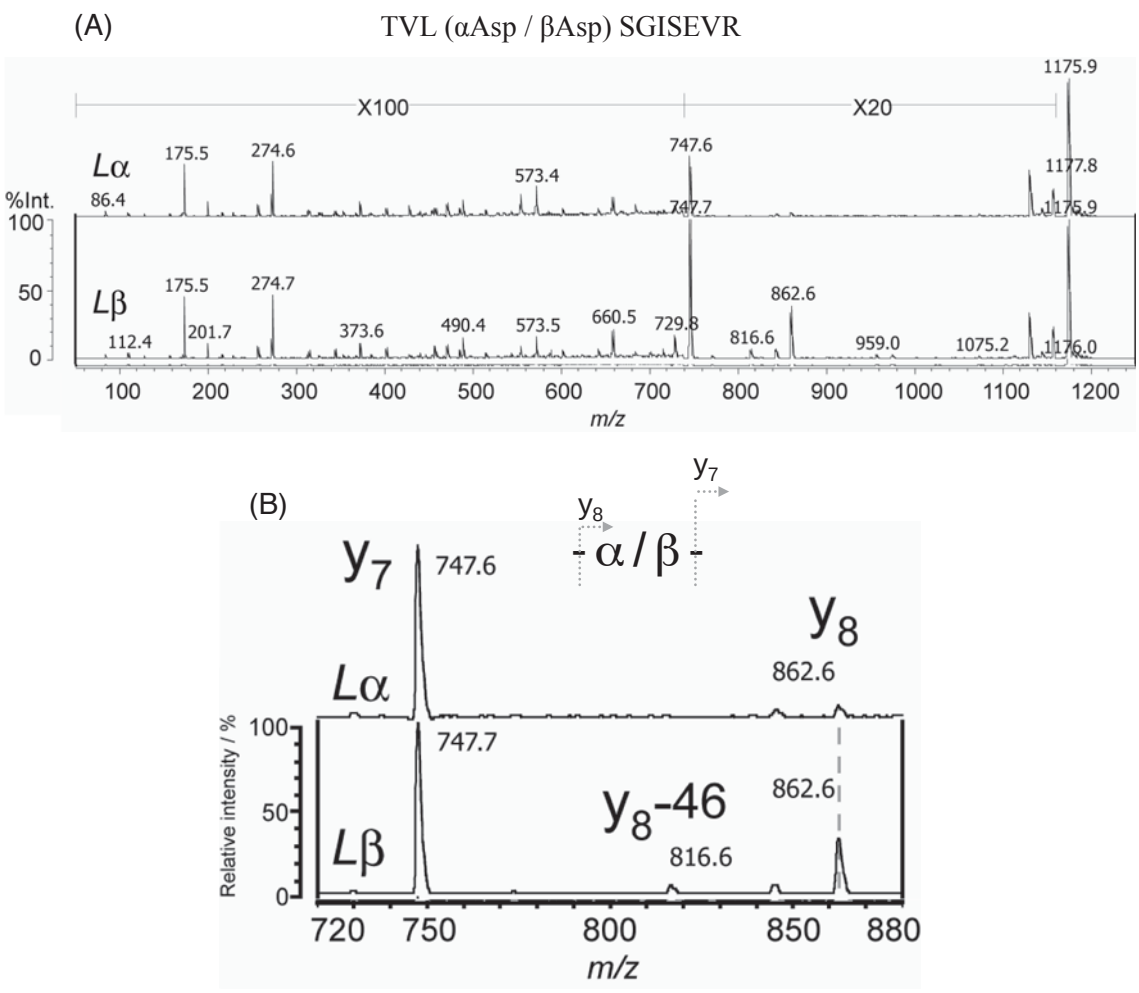


Fig. 9.6.3 PSD Spectra of T6 Peptides: (A), and Enlarged View: (B)

[References]
1) Biochem Biophys Res Commun., 294, 1047-1051 (2002)
2) Rapid Commun. Mass Spectrom., 14, 2092-2102 (2000)
3) J Am. Soc. Mass Spectrom., 18, 48-56 (2007)
4) Anal. Chem., 79, 2714-2724 (2007)
5) Anal. Chem., 82, 6384-6394 (2010)

9.7 RNA Sequence Analysis Using the Acid-Hydrolysis Method (1) - MALDI-TOF MS

■Explanation

Oligonucleotides with various types of sequences,with antisense effect as well as RNA interference effects, have been synthesized in the course of nucleic acid drug development. Although quality control of synthetic oligonucleotides is essential, no standard method has been established for base sequencing of oligonucleotides with relatively few bases, in the order of 20-30 bases. Thus, there is need for a simple, yet highly reliable sequence analysis method. Here we investigated RNA sequencing analysis using the acid-hydrolysis method. We conducted investigation of acid-hydrolysis of a 21-base synthetic siRNA and 2'-O-methylated siRNA. A mixed solution of a low molecular weight matrix (3-hydroxypicolininc acid: 3HPA) and acid (trifluoroacetic acid: TFA) was added to the sample solution, and the mass spectrum was acquired by MALDI-TOF MS. As a result, we were able to identify the entire 19-mer sequence except for 2 bases at the 3'-terminal (Fig. 9.7.1), and were also able to verify that this is also effective for the 2'-O-methylation modified RNA (Fig. 9.7.2).

■Analytical Conditions

Instrument : AXIMA *Assurance*
Measurement Conditions : Positive/Linear Mode
Sample : siRNA 21 mer 5'-UAU CAC UUG AUC UCG UAC AdTdT-3' (SIGMA)
Matrix : 50 mg/mL 3 HPA in 2.5 % TFA aq. + 10 mg/mL Diammonium Hydrogen Citrate

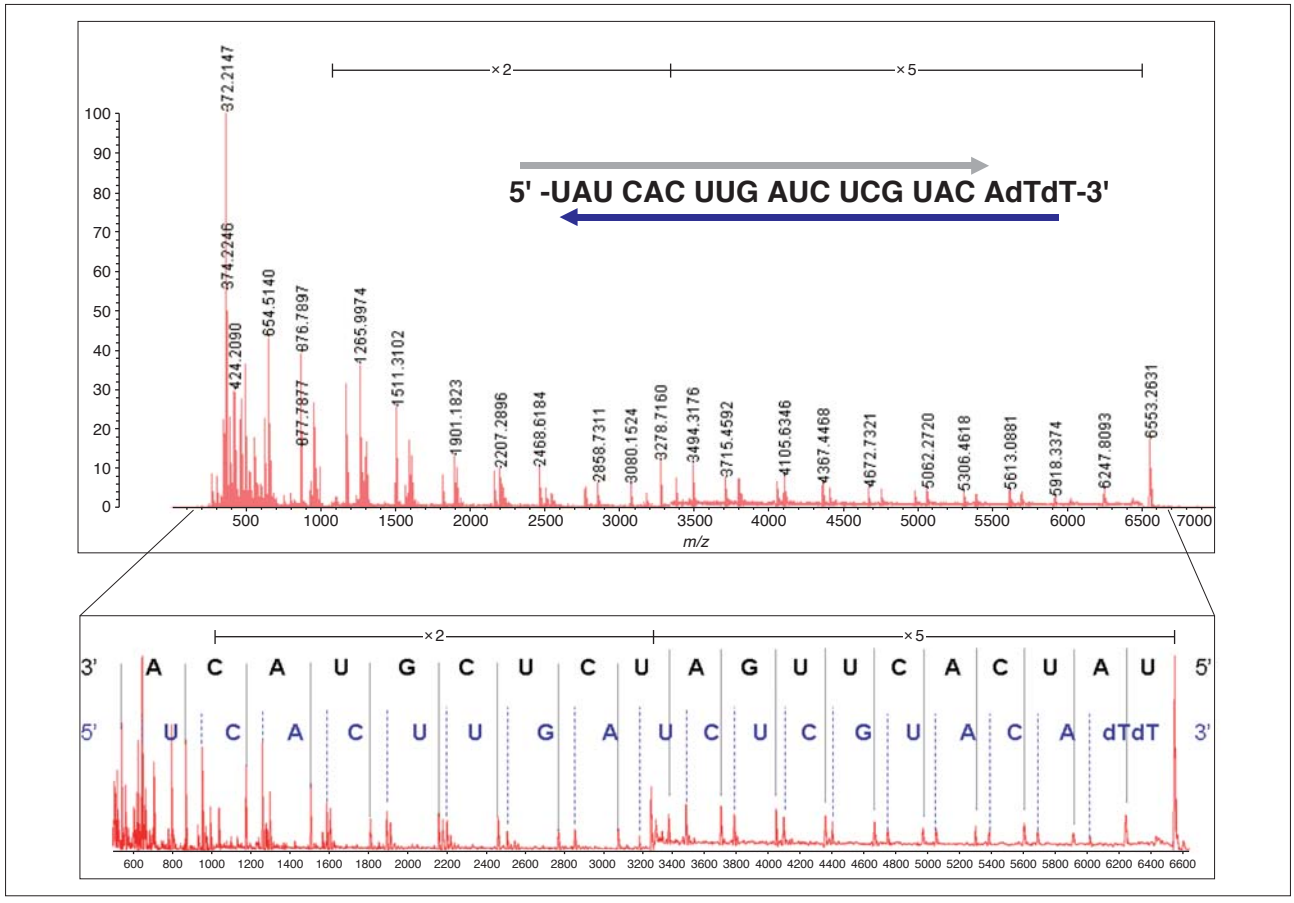


Fig. 9.7.1 Mass Spectra of siRNA (21-mer) after Acid Hydrolysis

9.7 RNA Sequence Analysis Using the Acid-Hydrolysis Method (2) - MALDI-TOF MS

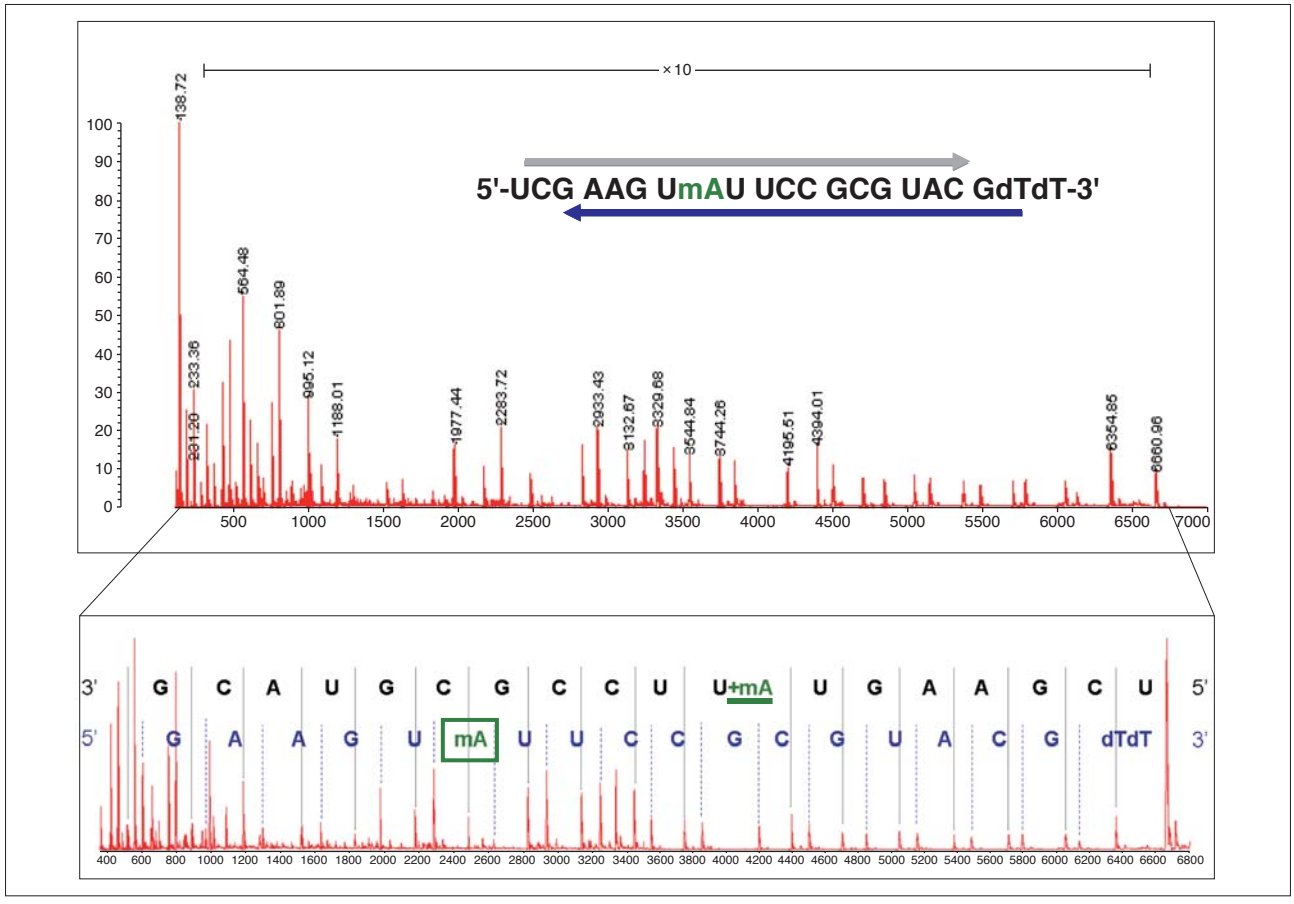


Fig. 9.7.2 Mass Spectra of siRNA (mA: Containing 2'-O-methyl adenosine) after Acid Hydrolysis

Ladder-shaped peaks due to acid hydrolysis were detected as shown in both Fig. 9.7.1 and Fig. 9.7.2, and RNA base sequencing was possible by reading the mass differences between peaks. Highly accurate base sequence information was obtained because the sequence ladder was detected one base at a time from both the 3'-terminal and 5'-terminal. In addition, the optimum concentration of TFA was investigated for determining the best analysis conditions (Fig. 9.7.3). As a result, the best spectrum information was obtained using a final concentration of 2.5 %.

The combination of MALDI-TOF MS and use of the acid-hydrolysis was confirmed to be a powerful technique providing fast and easy base sequencing of RNA sequences of approximately 20 bases. The AXIMA *Assurance* was used for measurement here. The same measurement can be performed with the AXIMA *Confidence* and AXIMA *Performance*.

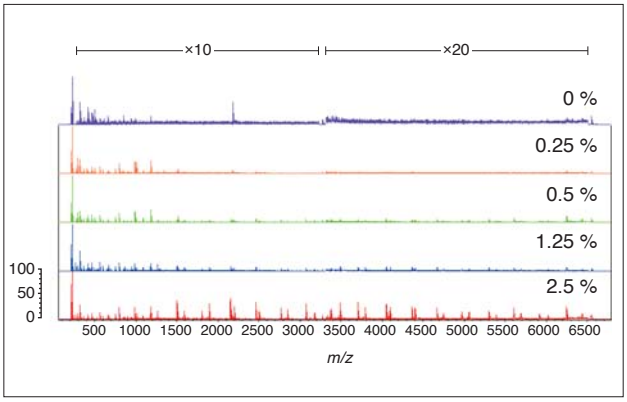


Fig. 9.7.3 Verification of Best TFA Concentration

[Reference]
Anal. Chem., 2009, 81, 3173-3179.

9.8 MALDI Mass Spectrometric Imaging for Peptides/Protein (1) - MALDI-TOF MS

■Explanation

MALDI imaging refers to a technique in which mass spectrometric analysis is conducted directly on a biological tissue sample. The distribution of biomolecules (low-molecular weight metabolites, lipids, peptides and proteins, etc.) on the tissue are mapped as a two-dimensional image based on measurement site location information and mass spectral information. This makes it possible to visually grasp the localization of biomolecules of interest. The application of the MALDI imaging technique has previously been reported for biomolecules in various tissues, and there are numerous recent manuscripts citing spatial distribution of disease-specific biomarker candidate compounds. Thus, the MALDI imaging is an effective technique for understanding the spatial distribution of molecules, and not only is there expectation for this technique with respect to the search for disease-specific biomarkers, but for its application in drug kinetics as well.

Here we present an example of MALDI imaging of peptides and proteins in which we used a crosssection of rat kidney tissue as the sample. First, the matrix was coated on a frozen tissue section of rat kidney which was placed on an electrically conductive glass slide. Generally, when conducting MALDI imaging, the matrix must be coated uniformly on the tissue sample to ensure highly reproducible mass spectral data acquisition. One of these coating techniques uses a spotter instrument to deposit micro volumes of matrix solution. Here we used a chemical inkjet printer (CHIP-1000), a micro volume dispensing instrument, to conduct repeat deposition of 300 pL of matrix solution (5 mg/mL sinapinic acid) at 200 µm intervals from spot center-to-center over the surface of the tissue section. Fig. 9.8.1 shows an image of the kidney tissue section and the other with the matrix deposited on the tissue. Next, after drying the matrix-applied sample in a desiccator, the AXIMA *Confidence* was used to conduct linear-mode mass spectrometric analysis (positive mode) on all of the matrix spots. Fig. 9.8.2 shows the mass spectrum results obtained from the measurement.

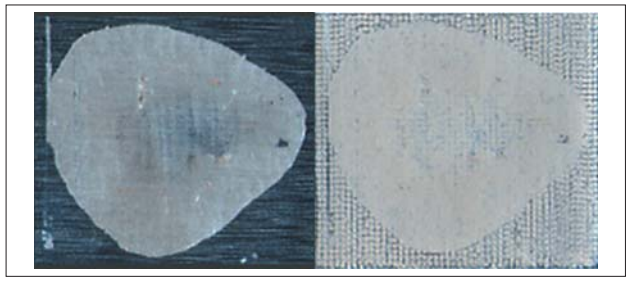


Fig. 9.8.1 Kidney Tissue Section without/with Matrix Deposition

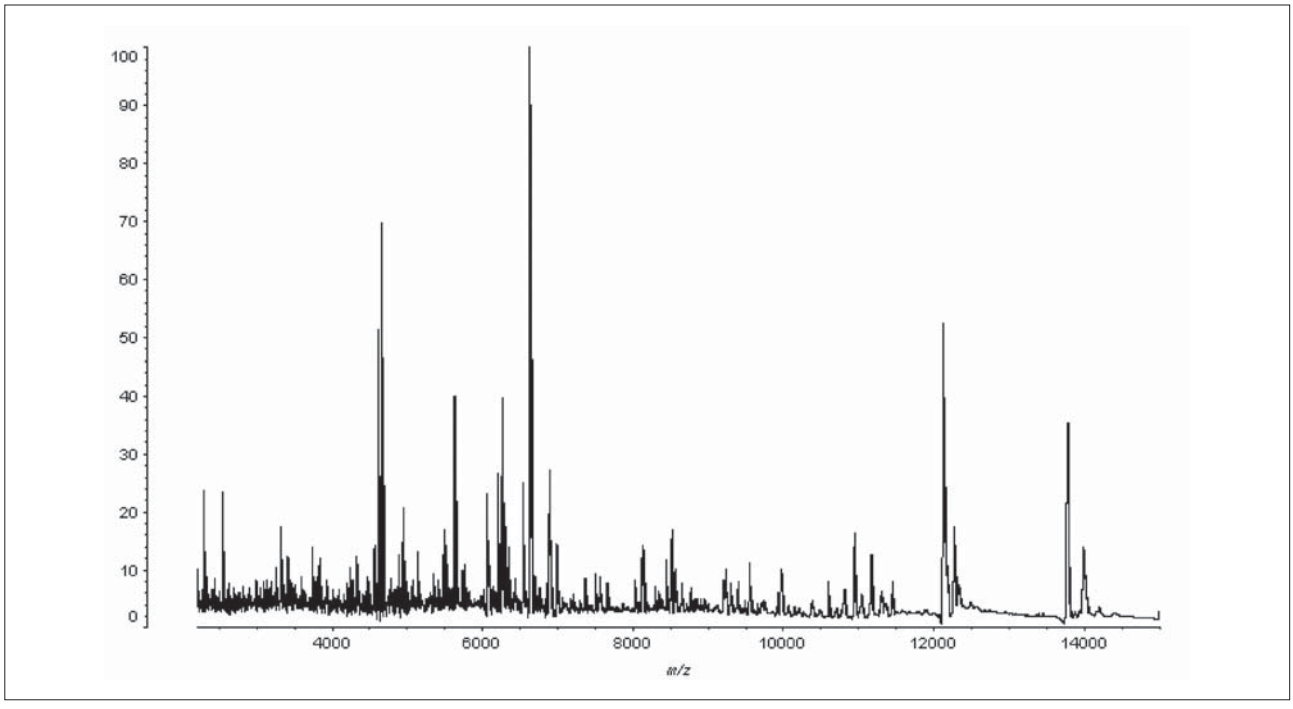


Fig. 9.8.2 Mass Spectrum of Rat Kidney Tissue Cross-Section

9.8 MALDI Mass Spectrometric Imaging for Peptides/Protein (2) - MALDI-TOF MS

From the results of Fig. 9.8.2, several MS peaks presumed to be peptides and proteins were detected directly from the tissue. For some of the MS peaks, we created an MS image based on the peak intensity and matrix spot location coordinates using the BioMap software (http://www.maldi-msi.org/) (Fig. 9.8.3). The results indicated that the spatial distribution of compounds that corresponded to each of the mass values agreed with the characteristic structures of the kidney cortex and medulla, and localization of the various biomolecules was confirmed (spatial resolution 200 μm). In addition, using the BioMap software, we created overlay images of only those MS images that displayed characteristic distributions (Fig. 9.8.4). It is clear from the overlay image results that the distributions of these peptides and proteins correspond to characteristic structure of the kidney. These results confirm the usefulness of the MALDI Imaging technique utilizing the chemical inkjet printer and the MALDI-MS (AXIMA Series) in investigating biomolecule distributions in biological tissue sections.

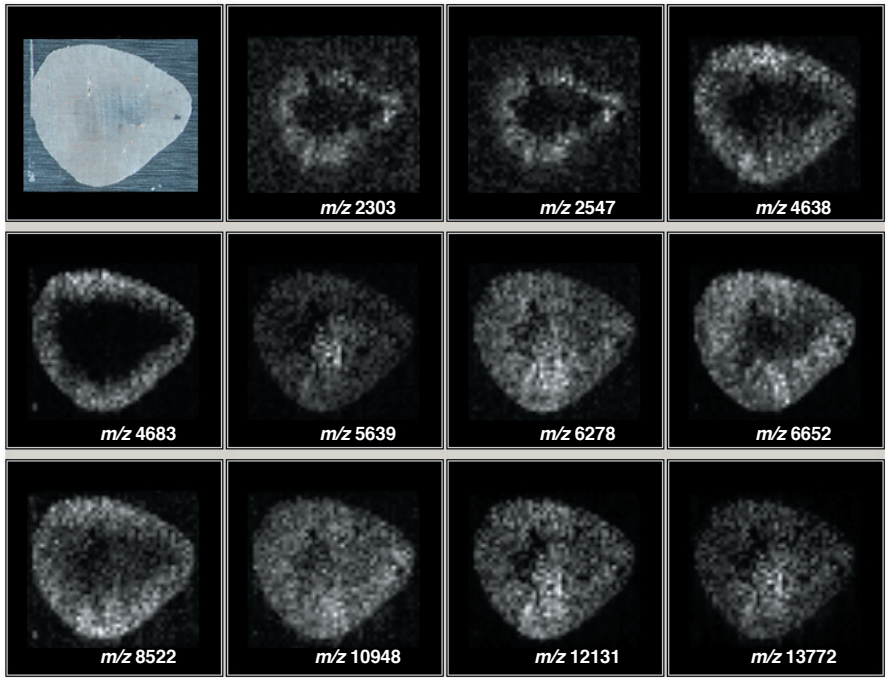
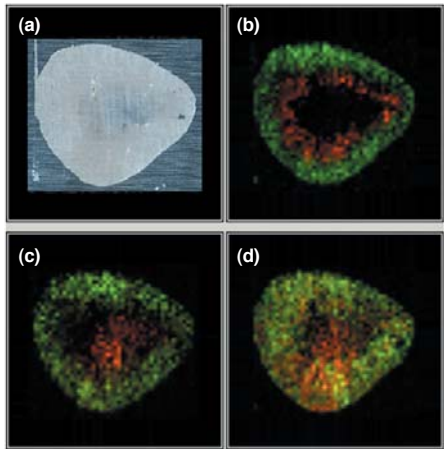


Fig. 9.8.3 MS Images of Rat Kidney Tissue Cross-Section



(a) Kidney tissue section
(b) Overlaid MS image (green: *m/z* 4683, red: *m/z* 2547)
(c) Overlaid MS image (green: *m/z* 4638, red: *m/z* 5639)
(d) Overlaid MS image (green: *m/z* 6652, red: *m/z* 6278)

Fig. 9.8.4 Overlaid MS Images

9.9 MALDI Mass Spectrometric Imaging for Tryptic Digest Peptides (1) - MALDI-TOF MS

■Explanation

MALDI imaging, a type of mass spectrometry using the MALDI technique, can display the distribution of biomolecules such as peptides and proteins without having to conduct such operations as the extraction and labeling of the biomolecules. Up to now, biomolecular MS imaging for a variety of tissue specimens has been reported, and recently there have been published reports showing the distribution of disease-specific protein biomarker candidates. However, proteins that have been detected by conducting mass spectrometry directly on a tissue section are extremely difficult to identify using the mass information alone. The typical method for identifying proteins is to conduct PMF (peptide mass fingerprinting) utilizing "tryptic digest peptides" of the target protein, together with in-silico generated database searches. Since multiple proteins are present in a tissue section, PMF cannot be used for identification of these proteins. This makes it necessary to conduct the MS/MS ion search for the tryptic digested peptides. By utilizing a spotter instrument to apply a coat of enzyme solution on the tissue section, it becomes possible to limit the analysis to micro regions of the tissue. This also allows the use of smaller amounts of expensive enzyme solution as compared to the spray method of coating. The use of spray techniques, including air brushing, can also lead to peptide diffusion on the tissue, complicating analyses. Here we present an example of protein identification conducted at a micro region of a rat liver tissue section, and an example of MALDI imaging of a tryptic digest site on a rat brain tissue section.

First, trypsin solution (40 μg/mL, 5 mM NH_4HCO_3) was deposited on a micro region of the liver tissue section using a chemical inkjet printer (CHIP-1000). Repeat deposition of 1.0 nL of trypsin solution (10 nL/spot) was conducted at 500 μm intervals on the tissue section. After allowing the enzymatic reaction to continue for 2 hours at 37 ºC in an external incubator, matrix solution (5 mg/mL DHB, 50 % methanol, 0.1 % trifluoroacetic acid) was dispensed onto the tissue. Fig. 9.9.1 shows an image of the liver tissue section with the deposited matrix. Next, after drying the tissue section in a desiccator, we conducted mass spectrometry using the AXIMA-QIT. Then, MS/MS analysis was conducted for the two peaks *m/z* 1572.78 and 1817.01 which exhibited high signal intensity in the initial mass spectrum. The obtained MS/MS spectra and the respective database search results are shown in Fig. 9.9.2.

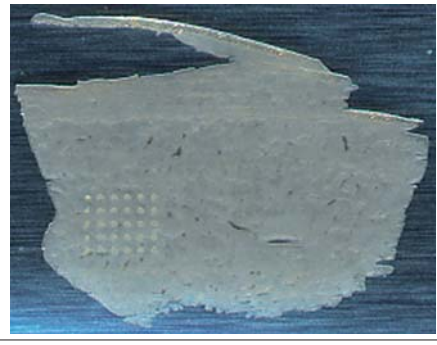


Fig. 9.9.1 Liver Tissue Section with Deposited Matrix

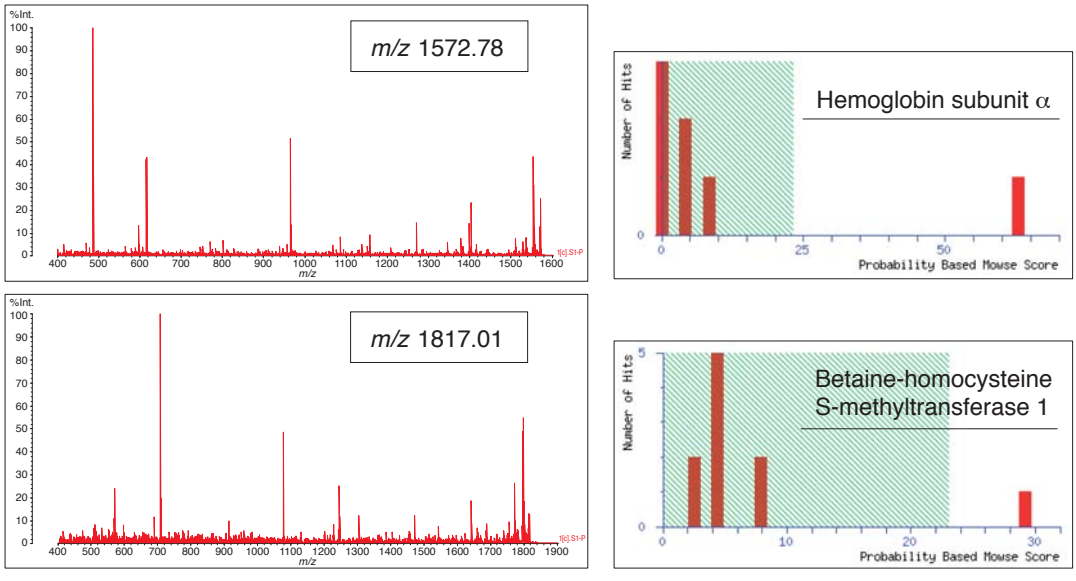


Fig. 9.9.2 MS/MS Spectra of Tryptic Digest Peptides and Database Search Results

9.9 MALDI Mass Spectrometric Imaging for Tryptic Digest Peptides (2) - MALDI-TOF MS

The AXIMA-QIT, an ion trap mass spectrometer, allows MS/MS analysis with high mass accuracy, as shown in Fig. 9.9.2. When used in conjunction with the chemical inkjet printer which can dispense micro volumes of enzyme solution, direct protein identification within a micro region of a tissue section becomes possible. Next, with the rat brain tissue section as the sample, MALDI imaging of the tryptic digest was conducted using the chemical inkjet printer and the AXIMA *Performance*. Trypsin solution (40 µg/mL) was deposited at 250 µm intervals at a micro volume of 300 pL per deposition, and following completion of the enzymatic reaction, 300 pL each of 10 mg/mL CHCA (50 % acetonitrile, 0.1 % trifluoroacetic acid) was dispensed (9 nL/spot). Analysis was conducted using the AXIMA *Performance*, and then MS images were generated based on the obtained mass spectrum positional information and the molecular ion intensity ratios. The BioMap software (http://www.maldi-msi.org/) was used to create the MS images. Fig. 9.9.3 shows the MS images for *m/z* 726.46 and *m/z* 1198.66, respectively. Each of the digested peptides shows a characteristic distribution, as can be seen in Fig. 9.9.3. In addition, MS/MS analysis of the molecular ions confirmed that they are derived from the Myelin basic protein (*m/z* 726.46) and Actin (*m/z* 1198.66), respectively (Fig. 9.9.4). Regarding the distribution of Myelin basic protein, all of the citations are reported, and the results here correlate with the reported distribution of protein in brain tissue. Thus, we have confirmed that on-tissue protein identification using the chemical inkjet printer in conjunction with the AXIMA Series, and MALDI imaging of a tryptic digest are useful for the examination of protein information obtained directly from biological tissue sections.

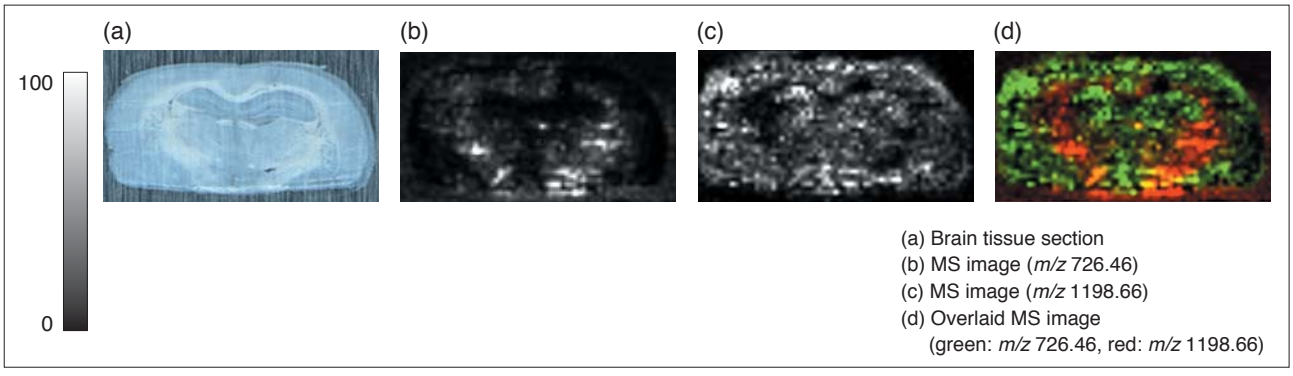


Fig. 9.9.3 MS Images of Rat Brain Tissue

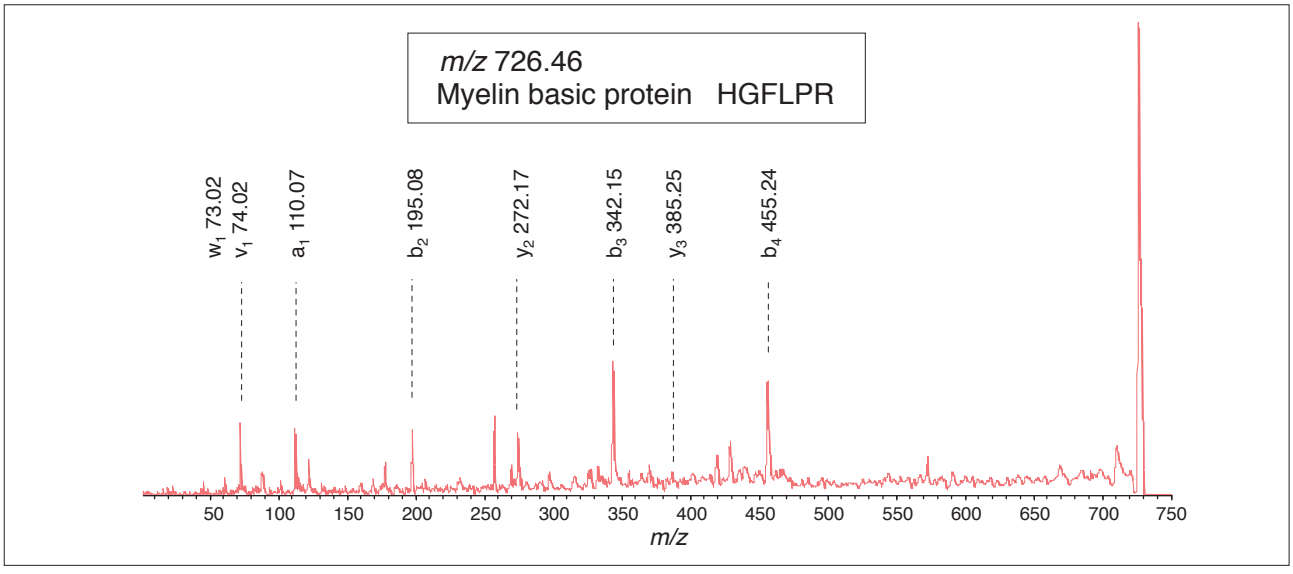


Fig. 9.9.4 MS/MS Spectrum of Tryptic Digest Peptides (Myelin basic protein)

* The BioMap copyright belongs to Novartis Institutes.

9.10 Analysis of Methylgloxal-Modified Heat Shock Protein 27 (1) - MALDI-TOF MS

■Explanation

Methylglyoxal (MG), a highly-reactive carbonyl intermediate product in the glyoxalase system and a product of a nonenzymatic glycation (Maillard reaction), is known to form target proteins and stable adducts (Advanced Glycation End-products; AGEs). It has been suggested that a specific modification due to MG plays a role in diabetic complications, and research is ongoing into its use as a useful biomarker candidate for diagnosis in the pre-disease development stage of diabetes. Here, we introduce the results of analysis of MG modification of the heat shock protein Hsp27 in response to various stresses (acidification, chemical substances, etc.) using the Prominence nano-AccuSpot-AXIMA® *Performance* LC-MALDI system. It is notable that several new modifications were found during this experiment.

The formation process of argpyrimidine, one of the products formed as a result of MG modification of arginine, is shown in Fig. 9.10.1. Various other MG modifications shown in Fig. 9.10.2 were considered besides argpyrimidine, and these were also analyzed. MG-modified Hsp27 was analyzed by LC-MALDI following enzymatic digestion with trypsin or trypsin + V8 protease. The results confirmed the formation of 5-hydro-5-methylimidazolone and carboxyethyllysine, etc. at multiple positions, as well as the formation of argpyrimidine, demonstrating the chaperone activity of Arg-188 (Table 9.10.1, Fig. 9.10.3). Argpyrimidine is an antigenic determinant of the anti-MG modification protein antibody, and we were able to identify its formation in MG modification of Hsp27 for first time ever.

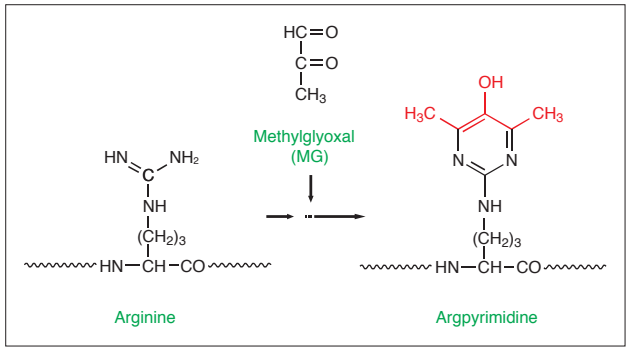


Fig. 9.10.1 Formation Process of Argpyrimidine

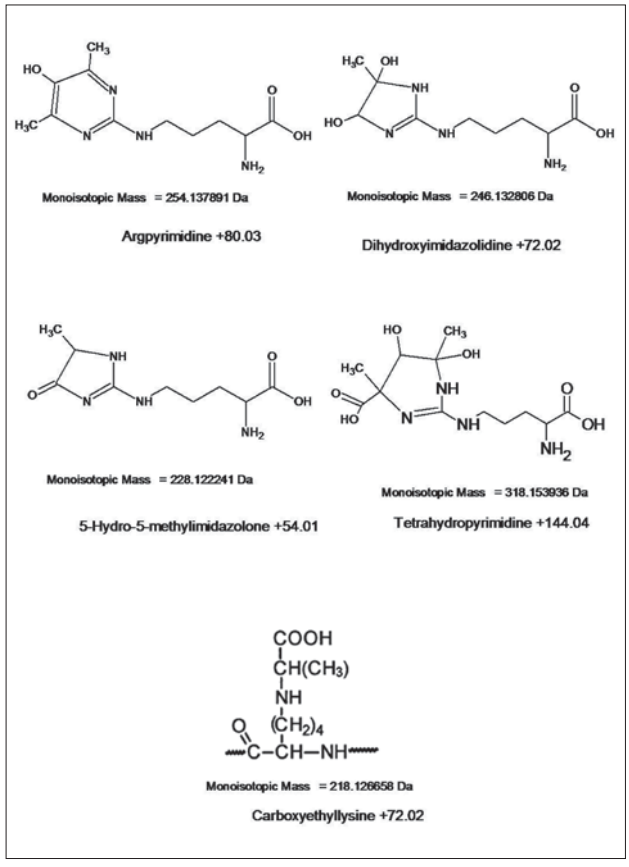


Fig. 9.10.2 Modifications by Methylglyoxal

9.10 Analysis of Methylgloxal-Modified Heat Shock Protein 27 (2) - MALDI-TOF MS

As much as 93 % of the entire MG-modified Hsp27 sequence was assigned using the LC-MALDI (Fig. 9.10.4). Most of the unassigned portion consisted of the difficult-to-assign N and C terminals. Excluding these N and C terminals, the sequence coverage rate was 96 %.

The above results demonstrate that detailed analysis of a target protein is possible using an LC-MALDI system. This system which, unlike an LC/MS/MS, allows multiple analyses of samples loaded on a sample plate, is an effective tool for conducting in-depth analysis of proteins.

Table 9.10.1 Peptides Identified by LC-MALDI Analysis for Enzymatic Digests of MG-Modified Hsp27

Theoretical Mass	Start	End	Sequence	Modifications	Enzyme
1041.25	5	12	RVPFSLLR	5-Hydro-5-methylimidazolone (R)	Trypsin
1556.76	28	40	LFDQAFGLPRLPE	5-Hydro-5-methylimidazolone (R)	Trypsin+V8
2738.01	41	64	EWSQWLGGSSWPGYVRPLPPAAIE	5-Hydro-5-methylimidazolone (R)	Trypsin+V8
1570.75	65	79	SPAVAAPAYSRALSR	5-Hydro-5-methylimidazolone (R)	Trypsin+V8
2412.61	65	87	SPAVAAPAYSRALSRQLSSGVSE	2x 5-Hydro-5-methylimidazolone (R)	Trypsin+V8
1318.44	88	96	IRHTADRWR	2x 5-Hydro-5-methylimidazolone (R)	Trypsin+V8
2180.42	95	112	WRVSLDVNHFAPDELTVK	5-Hydro-5-methylimidazolone (R)	Trypsin
1769.91	113	127	TKDGVVEITGKHEER	Carboxyethyllysine (K)	Trypsin
2856.02	113	136	TKDGVVEITGKHEERQDEHGYISR	Carboxyethyllysine (K)	Trypsin
1540.63	115	127	DGVVEITGKHEER	Carboxyethyllysine (K)	Trypsin
2626.75	115	136	DGVVEITGKHEERQDEHGYISR	Carboxyethyllysine (K)	Trypsin
2680.79	115	136	DGVVEITGKHEERQDEHGYISR	5-Hydro-5-methylimidazolone (R); Carboxyethyllysine (K)	Trypsin
1709.73	124	136	HEERQDEHGYISR	5-Hydro-5-methylimidazolone (R)	Trypsin
2274.39	124	140	HEERQDEHGYISRCFTR	5-Hydro-5-methylimidazolone (R)	Trypsin
1722.84	128	140	QDEHGYISRCFTR	5-Hydro-5-methylimidazolone (R)	Trypsin
1350.50	131	140	HGYISRCFTR	5-Hydro-5-methylimidazolone (R)	Trypsin+V8
1376.54	131	140	HGYISRCFTR	Argpyrimidine (R)	Trypsin+V8
1532.72	131	141	HGYISRCFTRK	2x 5-Hydro-5-methylimidazolone (R)	Trypsin+V8
2883.17	172	198	LATQSNEITIPVTFESRAQLGGPEAAK	5-Hydro-5-methylimidazolone (R)	Trypsin
2883.17	172	198	LATQSNEITIPVTFESRAQLGGPEAAK	5-Hydro-5-methylimidazolone (R)	Trypsin
1238.35	187	198	SRAQLGGPEAAK	5-Hydro-5-methylimidazolone (R)	Trypsin+V8
1256.37	187	198	SRAQLGGPEAAK	Dihydroxyimidazolidine (R)	Trypsin+V8
1264.39	187	198	SRAQLGGPEAAK	Argpyrimidine (R)	Trypsin+V8

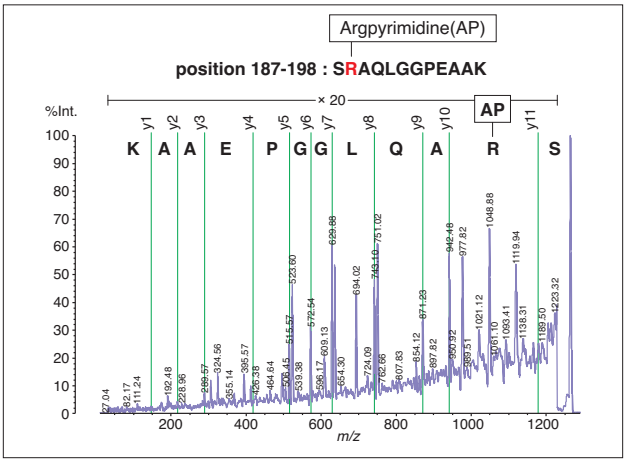


Fig. 9.10.3 MS/MS Spectrum of Argpyrimidine Adduction of MG-Modified Peptide

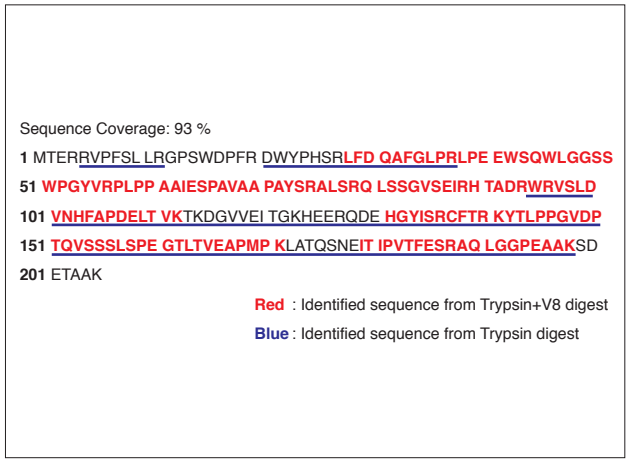


Fig. 9.10.4 Sequence Coverage of MG-Modified Hsp27 by LC-MALDI Analysis

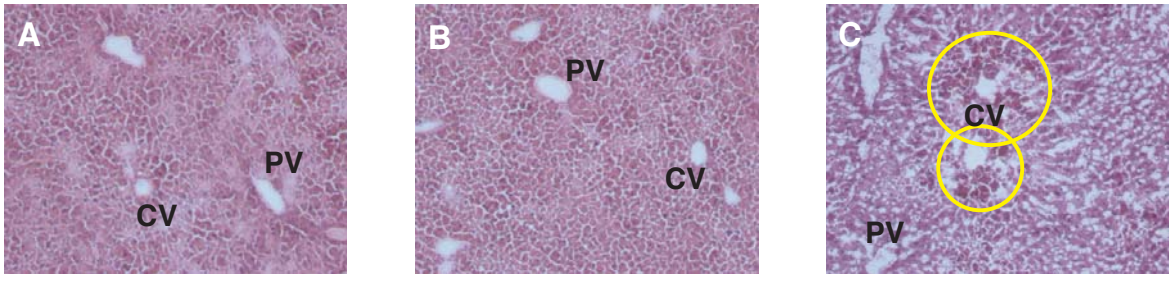
[Reference]
Oya, T. et al. J. Biol. Chem. 1999; 274 : 18492

[Acknowledgment]
This document was prepared based on the collaborative research of Tomoko Oya, Associate Professor of Biofunctional Analytical Medicine Course, School of Medicine, Kyoto Prefectural University of Medicine, Yuji Naito, Associate Professor and Toshikazu Yoshikawa, Professor of Molecular Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine.

9.11 Analysis of Changes in Amount of Lipids in Murine Hepatopathy Model due to Administration of Carbon Tetrachloride (1) - MALDI-TOF MS

■Explanation

Recently, the use of the MALDI technique to analyze target biomolecules (low-molecular-weight metabolites, lipids, peptides, proteins, etc.) directly from biological tissue sections is attracting increasing attention for its potentially strong utility in disease-related biomarker discovery. Generally, tissue sections are frozen during preparation, and then the matrix solution used in the MALDI technique is applied uniformly on the tissue section using a spotter (CHIP-1000) or other such applicator. After deposition of the matrix, analysis is conducted on the tissue section by MALDI-TOF MS to detect the *m/z* values of target biomolecules. Then, variations in MS peaks between the site of pathology and the normal site are compared in the search for disease biomarkers. Here we introduce an example of on-tissue direct detection of phospholipid changes using MALDI-TOF MS. The sample consisted of a mouse model hepatopathological tissue section in which injury was induced by administration of carbon tetrachloride, and on-tissue direct MS analysis was conducted at time intervals following administration of the carbon tetrachloride. Fig. 9.11.1 shows the H&E-stained hepatic tissue sections of 5-week-old ICR mice (♂) in which hepatopathy was induced by interperitoneal administration of carbon tetrachloride (1.0 mL/kg). The sections were taken at dissection conducted at 15 minutes and 48 hours following administration of CCl_4, respectively. Necrosis and cellular infiltration are recognizable at the periphery of a central vein in the liver after 48 hours, as shown in Fig. 9.11.1. Next, 500 pL of matrix solution consisting of 5 mg/mL α-cyano-4-hydroxycinnamic acid (60 % acetonitrile, 0.1 % trifluoroacetic acid) was deposited 25 times at 300 µm intervals on each of the hepatic tissue sections using a chemical printer (CHIP-1000). Fig. 9.11.2 shows each of the hepatic tissue section images and the deposited matrix.



A: reference murine hepatic tissue, B: 15 min post-administration, C: 48 hr post-administration, CV: central vein, PV: portal vein
A～C: X100, Yellow-encircled areas: cellular infiltration

Fig. 9.11.1 H&E Staining of Murine Liver with Hepatopathy Induced by Administration of Carbon Tetrachloride (CCl_4)

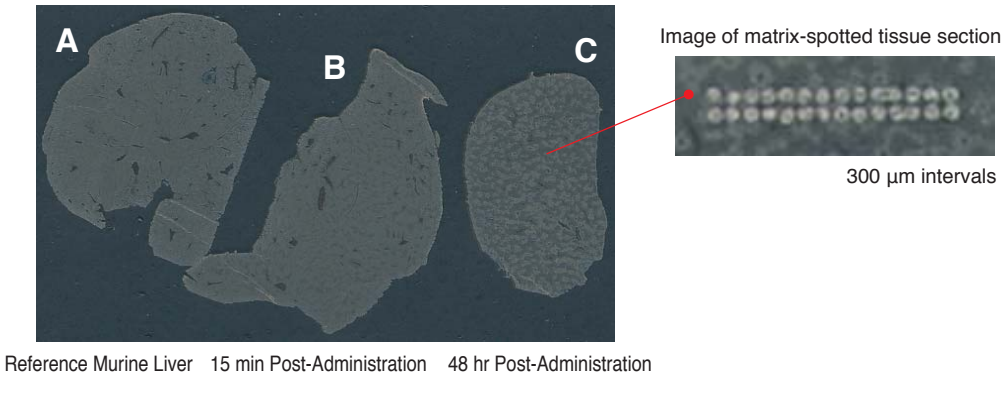
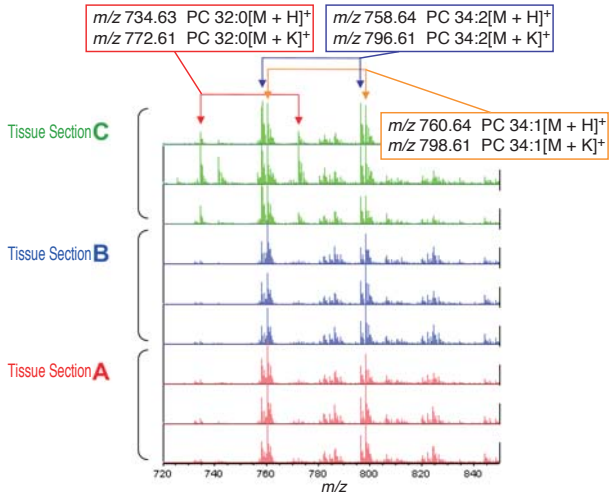


Fig. 9.11.2 Matrix Deposition onto Tissue Sections of Murine Liver

9.11 Analysis of Changes in Amount of Lipids in Murine Hepatopathy Model due to Administration of Carbon Tetrachloride (2) - MALDI-TOF MS

Fig. 9.11.3 shows typical MS spectra obtained from ontissue direct MALDI-MS analysis of the respective tissue sections following administration of carbon tetrachloride, in addition to those obtained from the reference liver tissue section. In the MS spectra of the 48-hr-elapsed tissue section results of Fig. 9.11.3, six distinctive MS peaks were confirmed to show increase and decrease. From the *m/z* values of these six MS peaks and the results of MS/MS analysis, they were determined to be the protonated molecular ions and potassium adducts of PC 32:0, PC 34:2 and PC 34:1 of phosphatidyl choline (PC).



Red: tissue section A (reference murine liver),
Blue: tissue section B (15 min post-administration),
Green: tissue section C (48 hr post-administration)

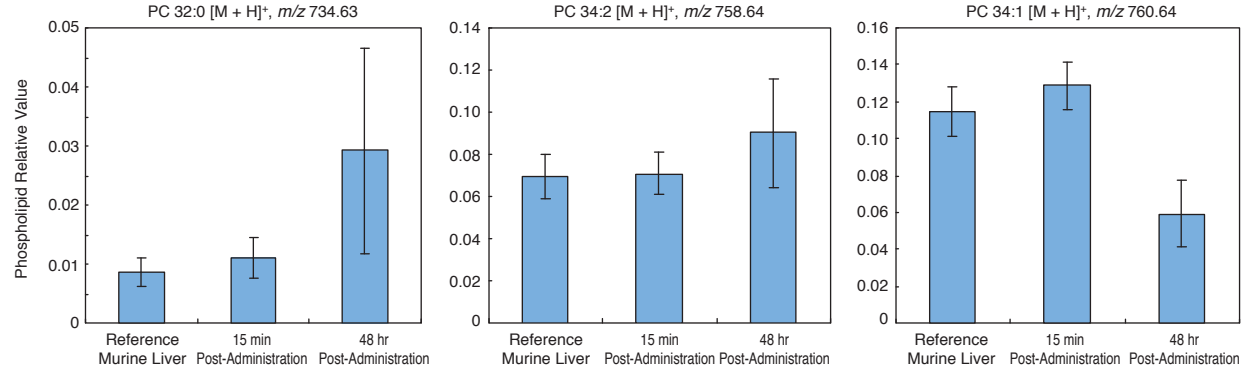
Fig. 9.11.3 MS Spectra of Respective Tissue Sections

Next, in order to conduct more in-depth analysis of the three types of PC related to the confirmed increases and decreases, ten points each of the respective tissue sections were measured, and relative comparison of the respective phospholipids $[M+H]^+$ was performed based on the obtained MS spectra. Assuming that the Relative Value of Phospholipids = Area of MS Peak of Interest/Sum of all MS Peak Areas, the relative value of phospholipids in the respective tissue sections can be plotted as shown in Fig. 9.11.4.

As indicated in Fig. 9.11.4, PC 32:0 $[M+H]^+$ = 734.63 is the highest value at 48 hours following the administration of carbon tetrachloride ($P = 0.0051$ according to t test). In addition, PC 34:2 $[M+H]^+$ decreased greatly after the elapse of 48 hours, however PC 34:1 $[M+H]^+$ with one less double bond decreased greatly after the elapse of 48 hours ($P = 0.036$, $P < 0.0001$ according to respective t tests). Furthermore, the same trend was confirmed for the respective phospholipid potassium ion adducts.

It is generally known that after the acute phase of liver cell necrosis in the carbon tetrachloride-induced liverinjured mouse model, the course eventually shifts to hepatic cell division and liver regeneration. Such ontissue direct mass analysis allowed direct observation of the distinctive changes in phospholipids in the liver regeneration phase. These results demonstrate that the on-tissue MALDI-MS analytical technique incorporating the chemical printer and MALDI-TOF MS is an effective approach to in-vivo metabolite change analysis and in-depth disease biomarker search.

This article is prepared based on collaborative study with Dr. Masaya Ikegawa , Kyoto Prefectural University of Medicine.



*The results of analysis by t test of changes in phospholipids in hepatic tissues after elapse of 48 hours from administering CCl_4 with respect to the reference mouse, indicate a significant difference $P<0.01$ for *m/z* 734.63 and *m/z* 760.64, and $P<0.05$ for *m/z* 758.64 (error bars indicate standard deviation).

Fig. 9.11.4 Changes in Amount of Phospholipids due to Administration of Carbon Tetrachloride (CCl_4)

9.12 Analysis of Spatiotemporal Changes in Energy Metabolism in a Murine Middle-Cerebral Artery Occlusion Model (1) - MALDI-TOF MS

■Explanation

Brain tissue always requires a large amount of glucose and oxygen to maintain sufficient function. For instance, blood flow volume to the brain decreases rapidly if occlusion of a blood vessel occurs due to a thrombus, instantly causing a lack of glucose. As a result, depletion of ATP (adenosine triphosphate) is caused in the ischemic region affected by the occlusion. Thus, brain function activity is severely compromised very quickly when cerebral ischemia occurs. For this reason, distinguishing the ischemic region itself from the surrounding normal tissue where compensatory neuronal recovery can be expected is critical. Therefore appropriate treatment becomes very important for cerebral ischemic damage due to cerebral vasculature infarctions. The damaged area ranges from the actual occlusion to partially damaged tissue to "normal" tissue. Therefore accurate and detailed analysis and assessment of normal versus damaged tissue of such zonal micro regions is necessary, and may impact future clinical diagnostics for such injury. Here, using the Shimadzu MALDI imaging system comprised of the CHIP-1000 and AXIMA *Performance*® to elucidate the distribution of regiospecific energy metabolism-related substances, we introduce our latest reported research results in which the ischemic region and its periphery (ischemic penumbra) are clearly distinguished. As a brain ischemia model, we used fresh frozen sections prepared from brain tissue obtained from C57BL/6J mice (male, 22 to 26 g) in which the middle-cerebral artery was occluded for 10 minutes and 60 minutes. A tissue blood flowmeter was used to confirm that the blood flow decreased by about 20% of the pre-occlusion blood flow due to occlusion. In addition, aside from the brain ischemic tissue section, brain tissue sections from a mouse in which occlusion was not conducted were used as a control, and MALDI imaging analysis of the various tissue sections was conducted. Fig. 9.12.1 shows a control (normal) tissue section and ischemic brain tissue sections (occlusion for 10 minutes and 60 minutes), respectively, stained with HE (Hematoxylin-Eosin). In the ischemic brain tissue sections (10 and 60 minutes), the ischemic regions are those within the areas delineated by the dotted lines, although these ischemic areas could not be observed in the HE-stained images.

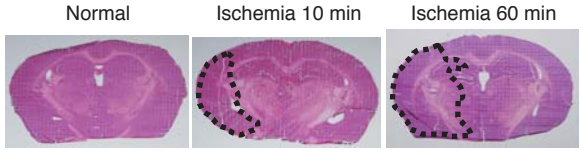


Fig. 9.12.1 HE-Stained Images of Murine Normal and Ischemic Brain Tissue Sections with Cerebral Artery Occlusion for 10 and 60 Minutes

■Analytical Conditions

Condition for printing of matrix solution

Matrix	: 4 mg/mL 9-Aminoacridine in 70 % Methanol
Volume	: 300 pL × 20 cycles
Print Pitch	: 200 µm

Condition for on-tissue MS measurement

Tuning Mode	: Linear/Negative Mode
Laser Shots	: 200 Shots/Point
Random Movement	: 60 µm Width

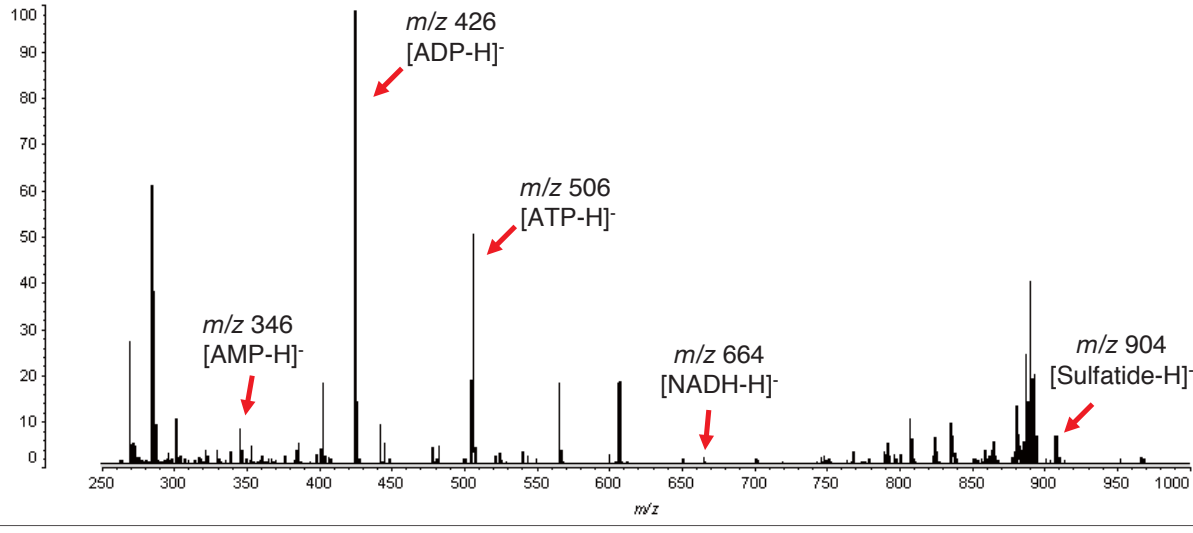


Fig. 9.12.2 Mass Spectrum of Normal Brain Tissue Section

9.12 Analysis of Spatiotemporal Changes in Energy Metabolism in a Murine Middle-Cerebral Artery Occlusion Model (2) - MALDI-TOF MS

Next, using the CHIP-1000, 300 pL spots of matrix solution (9-aminoacridine, 70 % Methanol) were applied over the affected region (spot intervals: 200 µm) of each of the tissue sections. After the matrix was applied, the tissue sections were allowed to dry thoroughly, after which they were subjected to mass spectrometry using the AXIMA *Performance*®. Fig. 9.12.2 shows the mass spectrum of the control (normal) section obtained by mass spectrometry. In the mass spectrum of Fig. 9.12.2, peaks generated from several different metabolites are observed. Metabolites associated with energy metabolism that play important roles in the body are included among these metabolite peaks. When MALDI imaging was conducted on these metabolites, their respective distribution within the brain were found to vary widely (Fig. 9.12.3).

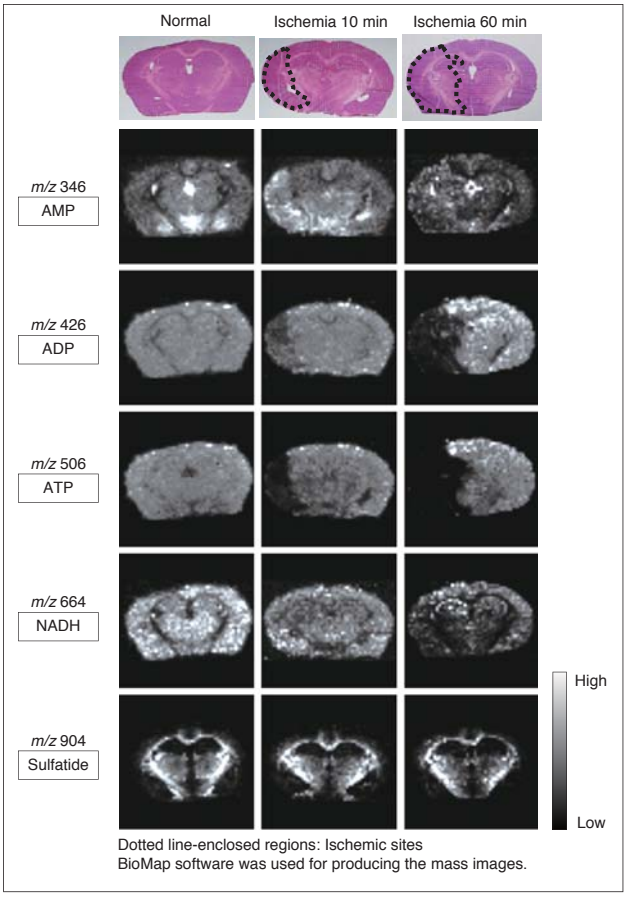


Fig. 9.12.3 Distribution of Various Metabolites in Murine Brain Tissue Sections

With respect to the MALDI imaging results in the ischemic brain, a greatly reduced presence of the energy metabolites such as ADP (adenosine diphosphate) and ATP (adenosine triphosphate) was noticed at the ischemic site. Furthermore, this tendency was more noticeable when comparing the durations of occlusion, in which differences in the presence of ADP and ATP were more pronounced at 60 minutes of sustained ischemia than with the 10 minute treatment. In contrast to this, the mass images of AMP (adenosine monophosphate) and NADH (nicotinamide adenine dinucleotide phosphate) suggest that the levels of these metabolites rise at the ischemic site particularly with the 10-minute treatment. Since sulfatides, which are not energy metabolites, show the same distribution regardless of the tissue section, substances like the energy metabolites ADP and ATP become depleted in the ischemic site, suggesting that those energy metabolites are present at greatly different levels with respect to normal tissue regions. By conducting MALDI imaging on a brain ischemia model mouse, we were able to clearly distinguish between the ischemic site and its periphery (ischemic penumbra). Moreover, the regiospecific or zonal changes in characteristic energy metabolites at the ischemic site and the ischemic penumbra were visualized for the first time as distributions in the functioning state *in-vivo* in this particular animal model. Substances associated with the energy metabolism play important roles in living organisms, and examination of their precise anatomical distribution provides very useful information for clarifying complex biological functions in both normal and pathological states. MALDI tissue imaging using CHIP-1000 for regiospecific printing of MALDI matrix on various types of tissue sections is very effective for studying various metabolite changes not limited to distribution of energy metabolites, but a wide variety of MALDI tissue imaging applications potentially impacting clinical diagnosis, treatment and prognosis. Expectations are promising for this type of application in biomarker research and pharmacokinetic studies for determining and treatment of various disease states.

[Abbreviations]

AMP : adenosine monophosphate
ADP : adenosine diphosphate
ATP : adenosine triphosphate
NADH : nicotinamide adenine dinucleotide phosphate

[Reference]

K. Hattori *et al*. Antioxidants & Redox Signaling 13 (8) 2010, 1157-1167

* This document is based on data obtained as a result of joint research with Dr. Makoto Suematsu, Department of Biochemistry and Integrative Medical Biology, School of Medicine, Keio University.

10. Supplements

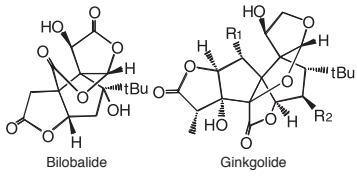
10.1 Analysis of Terpenoids in Ginkgo Biloba - LC

■Explanation

Ginkgo biloba extract contains flavonoids and terpenoids that have been reported to be effective for improving poor blood circulation in the brain as well as poor peripheral blood vessel circulation. This ginkgo biloba extract is used as a health dietary supplement in Japan and the United States. Here we present an example of analysis of terpenoids in ginkgo biloba extract using the ELSD-LT Ⅱ evaporative light scattering detector.

■Analysis of Standard Solution

Terpenoids that are known to be present in large quantities in ginkgo biloba include bilobalide, ginkgolide A, ginkgolide B and ginkgolide C (Fig. 10.1.1). Because these compounds have no chromophores, use of the evaporative light scattering detector together with reversed-phase gradient elution is an effective means of analysis. Fig. 10.1.2 shows a chromatogram obtained from analysis of a standard solution of these four terpenoids (200 mg/L each, methanol).



Compound	R_1	R_2
Ginkgolide A	H	H
Ginkgolide B	OH	H
Ginkgolide C	OH	OH

Fig. 10.1.1 Structures of 4 Terpenoids

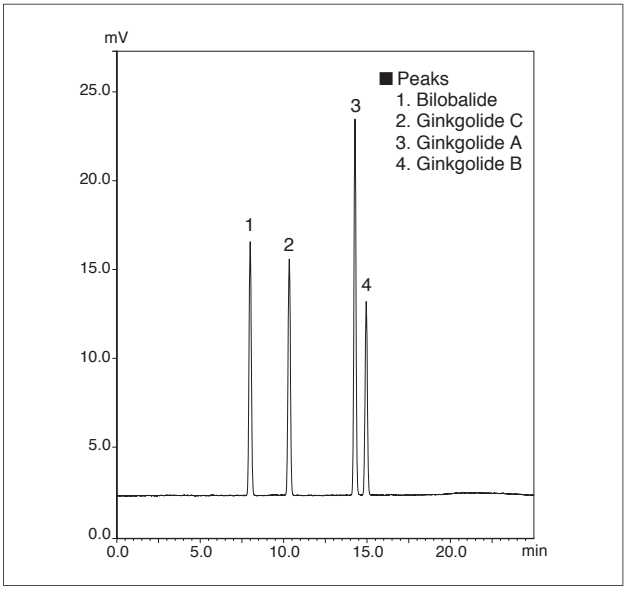


Fig. 10.1.2 Chromatogram of a Standard Mixture of 4 Terpenoids in Ginkgo Biloba (200 mg/L each, 10 µL injected)

■Analytical Conditions

Column : Shim-Pack FC-ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase : A: Water
B: Methanol
Gradient Elution Method
Time Program : B 20 % (0 min) → 45 % (16 min)
→ 80 % (16.01-20 min)
→ 20 % (20.01-30 min)
Flowrate : 1.0 mL/min
Column Temp. : 50 ºC
Injection Volume : 10 µL
Detection : Evaporative Light Scattering Detector ELSD-LT Ⅱ
Temperature : 40 ºC
Gain : 6
Nebulizer Gas : N_2
Gas Pressure : 350 kPa

* Column washing with 80 % methanol is included

■Analysis of Dietary Ginkgo Biloba Supplement

Analysis was conducted after performing sample preparation of a commercially available dietary ginkgo biloba supplement according to the procedure shown in Fig. 10.1.3. Fig. 10.1.4 shows the chromatogram.

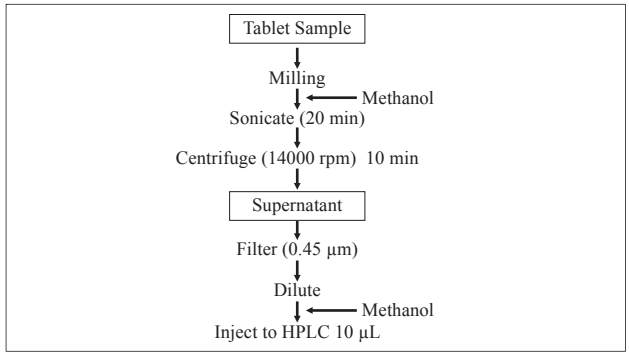


Fig. 10.1.3 Sample Preparation

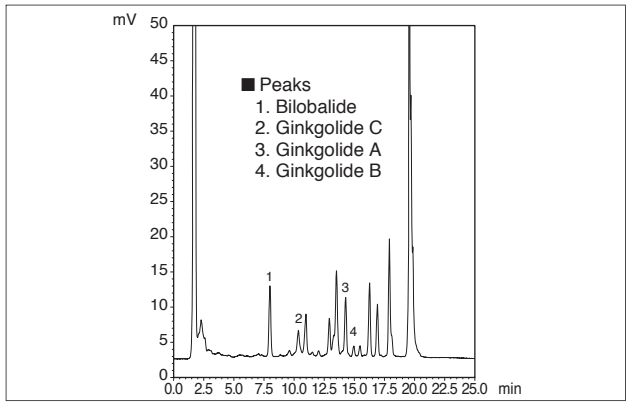


Fig. 10.1.4 Chromatogram of Dietary Ginkgo Biloba Supplement

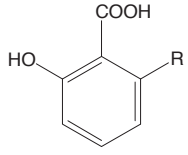
10.2 Analysis of Ginkgolic Acids in Ginkgo Biloba Extract (1) - LC

■Explanation

Ginkgo leaf extract (ginkgo biloba extract), which contains active ingredients extracted from ginkgo biloba leaves, is reported to be effective in improving cerebral and peripheral blood circulation deficiencies. It is used in Japan and the United States in the form of a dietary supplement, and in Germany, France, and other European countries as a prescription medication. However, alkylphenols which are present in the ginkgolic acids contained in ginkgo leaves are known to cause allergic reactions. For this reason, the United States Pharmacopeia (USP) has established an upper limit for ginkgolic acid content in ginkgo biloba extract. Here we introduce an example of analysis of gingkolic acids contained in ginkgo biloba leaves.

■Analysis of Standard Solution

The ginkgolic acids in ginkgo biloba leaves that were analyzed include ginkgolic acid C13:0 (hereafter, GA C13:0), GA C15:0, GA C15:1, and GA C17:1. Fig. 10.2.1 shows the structural formula of these 4 substances. Due to the high hydrophobicity of these ginkgolic acids, the Shim-pack CLC-C8 in which the silica gel is modified with an octyl group (C8) was used, and chromatography was conducted using gradient elution. For detection, the SPD-M20A photodiode array detector was used. Fig. 10.2.2 shows the spectrum of GA C17:1, and Fig. 10.2.3 shows a chromatogram of a standard mixture of 4 ginkgolic acids.



Compound		R
GA C13:0	Ginkgolic Acid C13:0	$C_{13}H_{27}$
GA C15:0	Ginkgolic Acid C15:0	$C_{15}H_{31}$
GA C15:1	Ginkgolic Acid C15:1	$C_{15}H_{29}$
GA C17:1	Ginkgolic Acid C17:1	$C_{17}H_{33}$

Fig. 10.2.1 Structures of Ginkgolic Acids

■Analytical Conditions

Column	: Shim-Pack CLC-C8 (250 mmL. × 4.6 mm I.D.)
Mobile Phase	: A: 0.01 % Phosphoric Acid (85 %) -Water B: 0.01 % Phosphoric Acid (85 %) -Acetonitrile Gradient Elution Method
Time Program	: B 80 % (0 min) → 90 % (15-18 min) → 80 % (18.01-25 min)
Flowrate	: 1.0 mL/min
Column Temp.	: 35 ℃
Injection Volume	: 20 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 311 nm

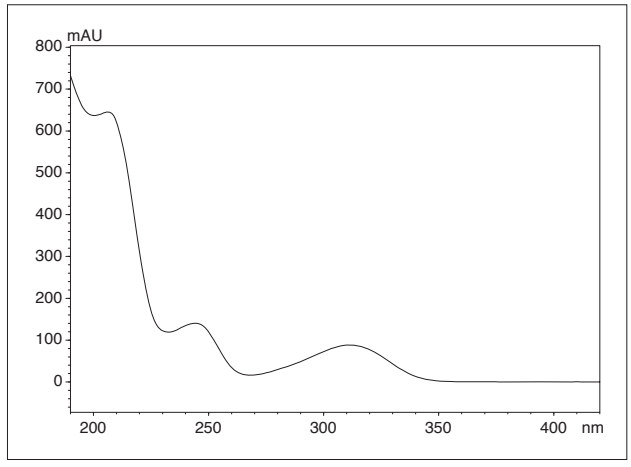


Fig. 10.2.2 Spectrum of Ginkgolic Acid C17:1

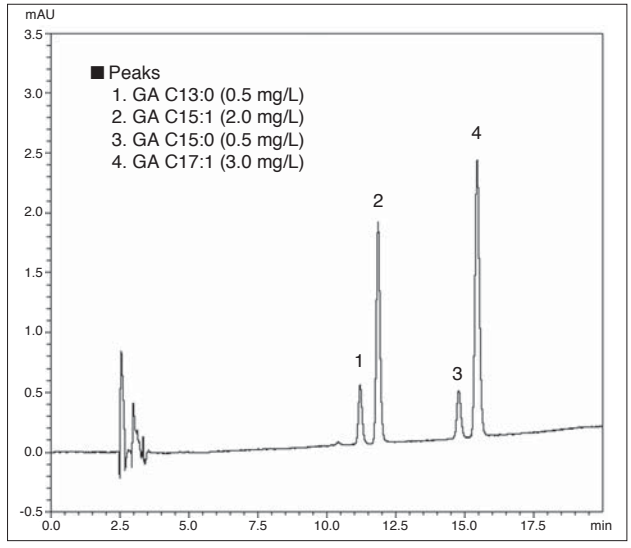


Fig. 10.2.3 Chromatogram of a Standard Mixture of 4 Ginkgolic Acids

10.2 Analysis of Ginkgolic Acids in Ginkgo Biloba Extract (2) - LC

■Analysis of Ginkgo Biloba Extract Supplement

Analysis of the ginkgo biloba extract was conducted after performing sample pretreatment of the commercial supplement containing the extract as shown in Fig. 10.2.4. Fig. 10.2.5 shows the chromatogram. Since ginkgolic acid was barely detected in this supplement, the chromatogram also shows the results of analysis of the prepared sample solution spiked with ginkgolic acid standard.

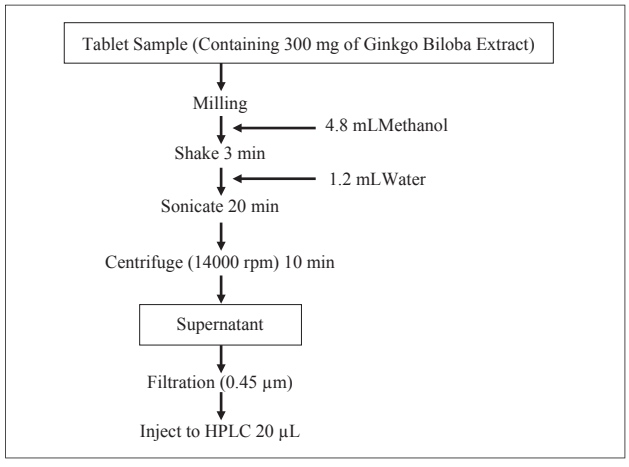


Fig. 10.2.4 Sample Preparation

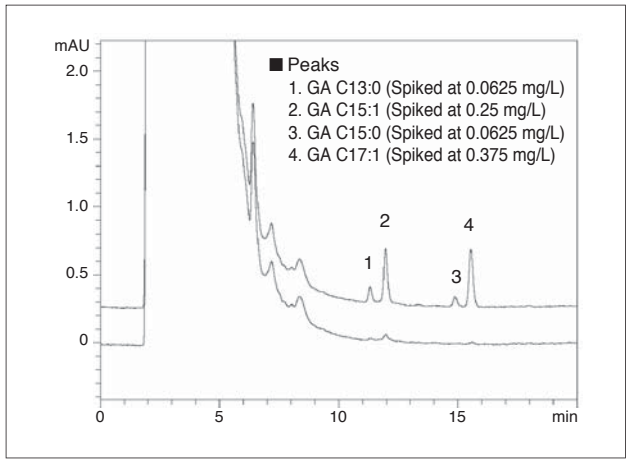


Fig. 10.2.5 Chromatogram of Ginkgo Biloba Extract Supplement (Upper: Spiked, Lower: Not Spiked)

[Reference]

United States Pharmacopeia (USP32-NF27)

10.3 High Speed Analysis of Glucosylceramide - LC

■Explanation

Glucosylceramide is a type of lipid derived from plants and is reported to possess moisturizing, anti-atopic, and other health-related actions, and is widely used in cosmetics, dietary supplements, etc.
Glucosylceramides are a type of glycosphingolipid consisting of glucose bound to ceramide, and because they display almost no optical absorption, the evaporative light-scattering detector is effective when analyzing these substances by HPLC.
Here, we introduce an example of high speed analysis of glucosylceramide in a dietary supplement using the Nexera UHPLC (Ultra High Performance Liquid Chromatography) System.

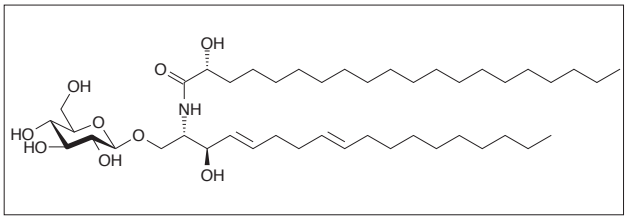


Fig. 10.3.1 Structure of Glucosylceramide from Rice (Primary Compound)

■Analysis of Dietary Supplement Containing Glucosylceramide from Rice

We conducted analysis of a commercially-available dietary supplement containing glucosylceramide derived from rice. The sample preparation procedure is shown in Fig. 10.3.2, and the obtained chromatogram is shown in Fig. 10.3.3. Detection of Glucosylceramide was easily accomplished and this method can be analyzed it only 8 min.

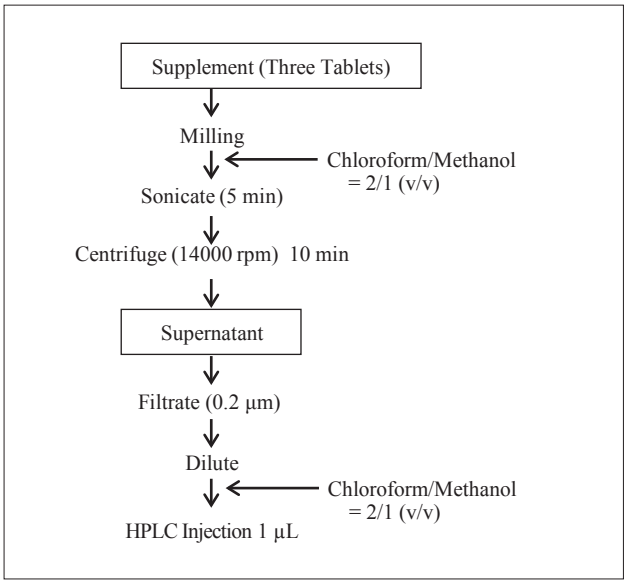


Fig. 10.3.2 Sample Preparation

■Analytical Conditions

Column	: Shim-pack XR-SIL (75 mmL. × 3.0 mm I.D.,2.2 µm)
Mobile Phase	: A: Chloroform B: Methanol/Water = 95/5 (v/v) Gradient Elution Method
Time Program	: B 1 % (0.0 min) → 25 % (3.0 min) → 90 % (4.0 min) → 1 % (5.0 min) → 1 % (8.0 min)
Flowrate	: 0.8 mL/min
Column Temp.	: 35 ºC
Injection Volume	: 1 µL
Detection	: Evaporative Light Scattering Detector ELSD-LT Ⅱ
	Temperature : 40 ºC
	Gain : 6
	Nebulizer Gas : N_2
	Gas Pressure : 350 kPa

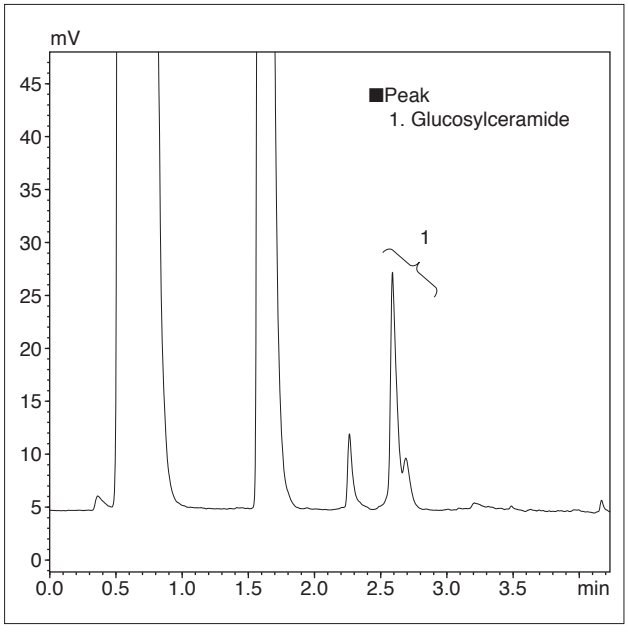


Fig. 10.3.3 Chromatogram of Dietary Supplement

[Reference]
M. Kashima, K. Nakagawa, T. Sugawara, T. Miyazawa, C. Murakami, R. Miyashita, J. Ono, F. S. Deschamps, and P. Chaminade: J. Oleo. Sci., 51,347-354 (2002)

10.4 Determination of Coenzyme Q10 in Food - LC

■Explanation

In Japan, coenzyme Q10 has traditionally been used as a pharmaceutical for improving myocardial metabolism. In accordance with revisions to the Food and Medicine Differentiation List (Pharmaceuticals and Food Safety Bureau, Ministry of Health, Labor and Welfare, Japan) in 2001, coenzyme Q10 was moved to the food section of the list. It is now the focus of attention as a food supplement. According to the Japanese Pharmacopoeia, which lists coenzyme Q10 under the pharmacological name, "Ubidecarenone", the recommended analysis method for coenzyme Q10 is the HPLC method. Here we introduce an analysis of coenzyme Q10 in commercially available food products using the Prominence Photodiode Array UV-Vis detector.

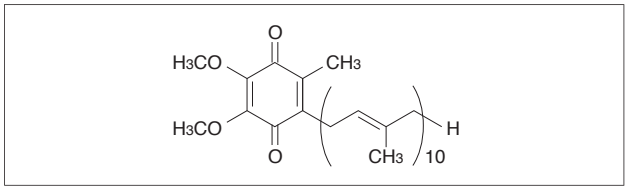


Fig. 10.4.1 Structure of Coenzyme Q10

■Analysis of Standard Solution

Fig. 10.4.1 shows the structure of coenzyme Q10. Fig. 10.4.2 shows the chromatogram obtained by injecting 5 µL of the coenzyme Q10 standard solution (5.0 mg/L, ethanol). Due to high fat solubility of coenzyme Q10, nonaqueous mobile phase is used when performing analysis using reversed phase chromatography. Coenzyme Q10 has the maximum absorption wavelength at 275 nm, consequently it is easily detected by UV detector.

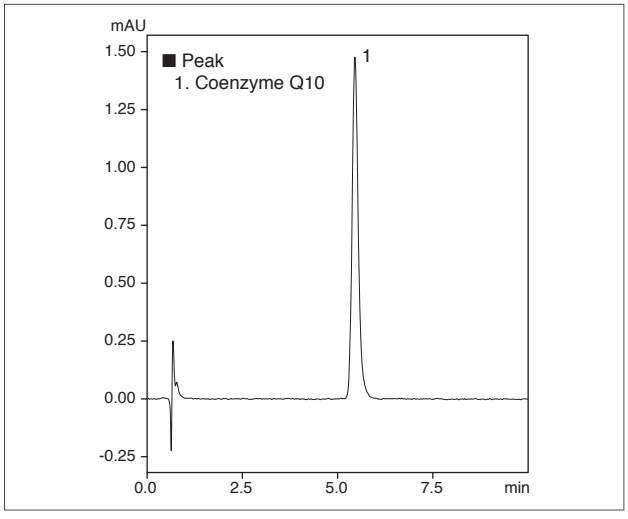


Fig. 10.4.2 Chromatogram of Coenzyme Q10 (5.0 mg/L, 5 µL injection)

■Analytical Conditions

Column	: Shim-pack FC-ODS (75 mmL. × 4.6 mm I.D.)
Mobile Phase	: Methanol / Ethanol = 13/7 (v/v)
Flowrate	: 1.5 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 5 µL
Detection	: UV-VIS Absorbance Detector SPD-20AV at 275 nm (Standard Solution) Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 275 nm (Sample Solution) Slit Width : 8 nm Cell Temp. : 40 ºC

■Analysis of Food Sample

Fig. 10.4.3 shows the resulting chromatogram, using a photodiode array detector, of a food sample (capsule) containing coenzyme Q10. The sample was dissolved* in ethanol (10 g/L) and the solution was filtered through a membrane filter (0.45 µm) before injection (5 µL). Fig. 10.4.4 shows comparison of the spectra of coenzyme Q10 in the standard solution and that of corresponding peak in the sample solution. We can see that the spectra closely match. Using a photodiode array detector makes it easy to obtain qualitative information from the UV absorption spectrum.

* A high concentration sample was used in this measurement. However, dilution by a factor of 100 is recommended for routine analysis in order to reduce load on the column.

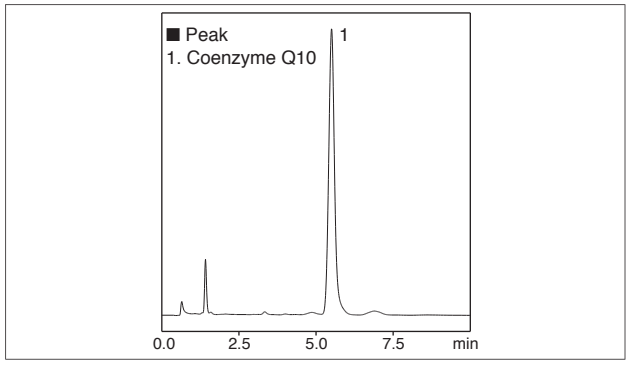


Fig. 10.4.3 Chromatogram of Food Sample (Capsule)

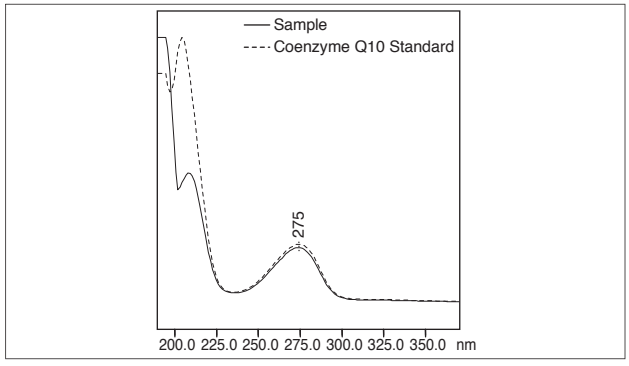


Fig. 10.4.4 UV Spectra of Coenzyme Q10

10.5 Analysis of α-Lipoic Acid in Dietary Supplement - LC

■Explanation

Lipoic acid, also referred to as thioctic acid, exists as (+)-α-lipoic acid in the natural world. In the body, it acts as a coenzyme for various enzymes, in particular, it is receiving attention for its activity as a coenzyme for enzymes that exist in mitochondria. Moreover, in recent years, it has found extensive use as a dietary supplement. Here, we introduces an example of analysis of α-lipoic acid in a food supplement product using a Photodiode Array Detector.

■Analysis of Standard Solution

Fig. 10.5.1 shows the structural formula for α-lipoic acid. In addition, Fig. 10.5.2 shows a spectrum of an a-lipoic acid standard, in which the maximum absorption peak is evident in the vicinity of 333 nm.

Fig. 10.5.3 shows the results of analysis of a 500 mg/L standard solution. After preparing a standard source solution consisting of 1000 mg/L of acetonitrile, the standard solution was prepared by diluting with water.

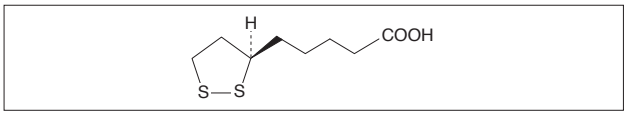


Fig. 10.5.1 Structure of α-Lipoic Acid

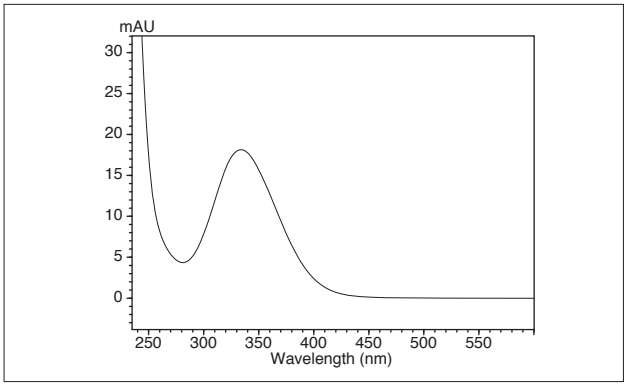


Fig. 10.5.2 Spectrum of α-Lipoic Acid

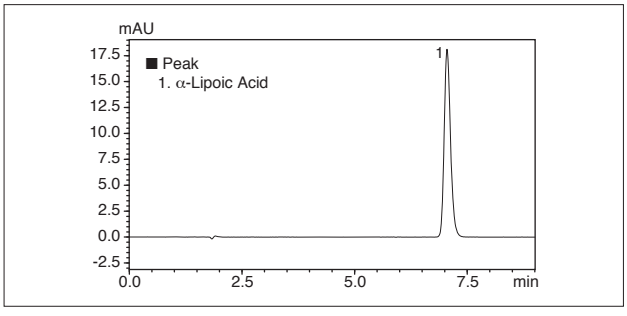


Fig. 10.5.3 Chromatogram of α-Lipoic Acid (500 mg/L, 10 µL injection)

■Analytical Conditions

Column	: Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)
Guard Column	: Shim-pack GVP-ODS (10 mmL. × 4.6 mm I.D.)
Mobile Phase	: 10 mmol/L Sodium Phosphate Buffer (pH 2.6) /Acetonitrile = 6/4 (v/v)
Flowrate	: 1.0 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 5 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 333 nm (Slit Width : 8 nm)

■Analysis of Dietary Supplement

Fig. 10.5.4 shows the chromatogram obtained from analysis of commercial capsule-type dietary supplement, in addition to the 3-dimensional plots of the result. The content of the supplement was dissolved in 10mL of acetonitrile, and after 10 minutes of ultrasonic extraction, the supernatant was filtered through a 0.45 µm membrane filter. Then, after diluting the filtrate with pure water (dilution factor: 20), 10 µL was injected.

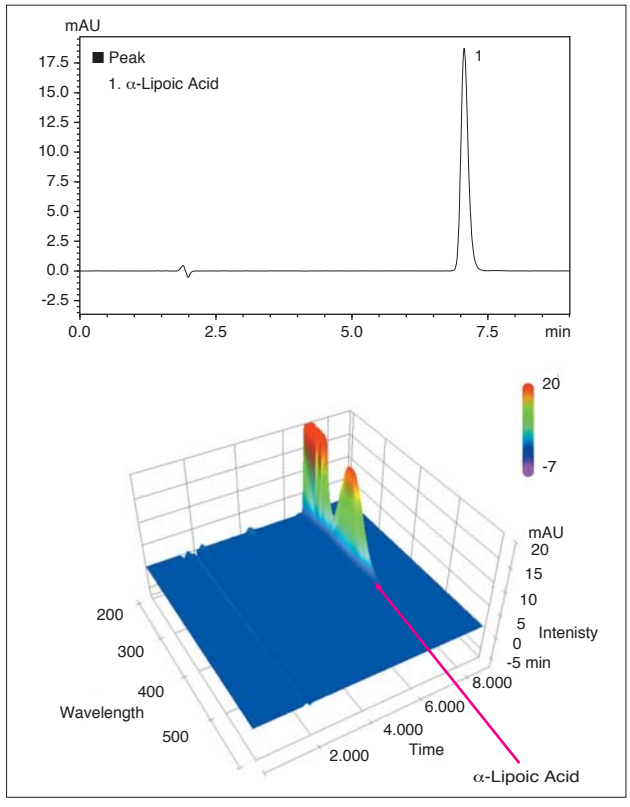


Fig. 10.5.4 Chromatogram and 3-D Plot of Dietary Supplement

10.6 High Speed Analysis of Lutein and Zeaxanthin in Dietary Supplement (1) - LC

■Explanation

Lutein and zeaxanthin are types of carotenoids, and are constituents of marigold pigment, a food additive which also occurs naturally in various foods. Recent studies have suggested that these substances may be effective in preventing cataracts and age-related macular degeneration syndrome (AMD). Here, we introduce an example of analysis of lutein and zeaxanthin in marigold supplement extract using the ultra fast LC "Prominence UFLC" with the SPD-M20A photodiode array detector.

■Analysis of Standard Solution

Fig. 10.6.1 shows the structures of lutein and zeaxanthin, which reveals that these 2 compounds are structurally similar, and in fact, structural isomers. Complete separation of such substances generally requires the use of reversed phase chromatography. In order to achieve more efficient separation through improved selectivity, we added tetrahydrofuran to the water / methanol mobile phase. In addition, use of the Shim-pack XR-ODS high speed, high resolution column (particle diameter 2.2 µm) allows the analysis time to be shortened to about 1/4 the time achieved with the conventational Shim-pack VP-ODS (particle diameter 4.6 µm), while maintaining the same resolution. In the analytical conditions, column washing procedure was included for analysis of the actual sample.

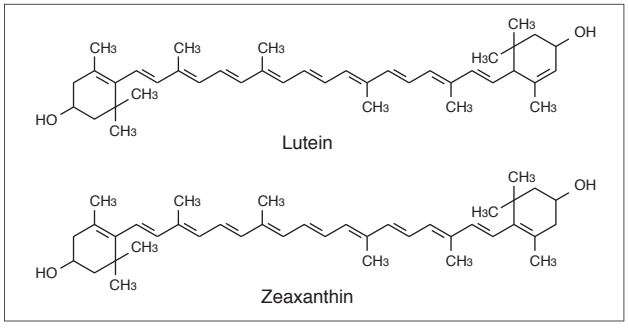


Fig. 10.6.1 Structure of Lutein and Zeaxanthin

■Analytical Conditions

Column	: Shim-pack XR-ODS (75 mmL. × 3.0 mm I.D., 2.2 µm) Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D., 4.6 µm)
Mobile Phase	: A: Methanol/Tetrahydrofuran/Water = 45/30/25 (v/v/v) B: Tetrahydrofuran Gradient Elution Method
Time Program	: (XR-ODS) B 0 % (0.00-4.50 min) → 100 % (4.51-5.50 min) → 0 % (5.51-7.50 min) (VP-ODS) B 0 % (0.00-15.50 min) → 100 % (15.51-20.00 min) → 0 % (20.01-25.00 min)
Flowrate	: 0.8 mL/min (XR-ODS), 1.0 mL/min (VP-ODS)
Column Temp.	: 50 ºC
Injection Volume	: 2 µL (XR-ODS), 5 µL (VP-ODS)
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 450 nm
Flow Cell	: Semi-micro cell (XR-ODS) Conventional cell (VP-ODS)

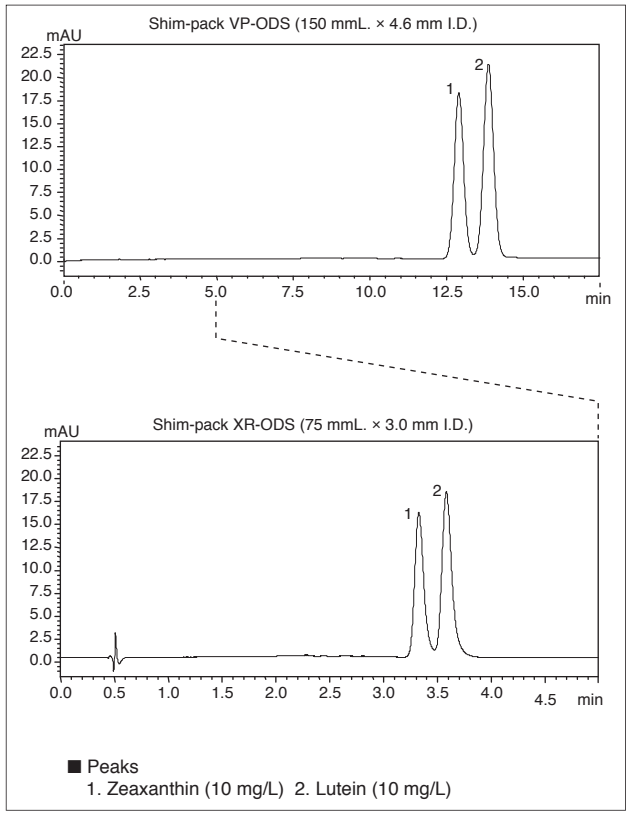


Fig. 10.6.2 Chromatograms of Lutein and Zeaxanthin Standard Solution

10.6 High Speed Analysis of Lutein and Zeaxanthin in Dietary Supplement (2) - LC

■Analysis of Dietary Supplement

Fig. 10.6.3 shows the analysis results of commercial marigold extract contained in a dietary supplement (capsule form). Fig. 10.6.4 shows the sample preparation procedure. Fig. 10.6.5 shows overlay spectra of standard and sample solutions of lutein and zeaxanthin in a dietary supplement, respectively. In addition, Fig. 10.6.6 shows a 3-D plot of the dietary supplement.

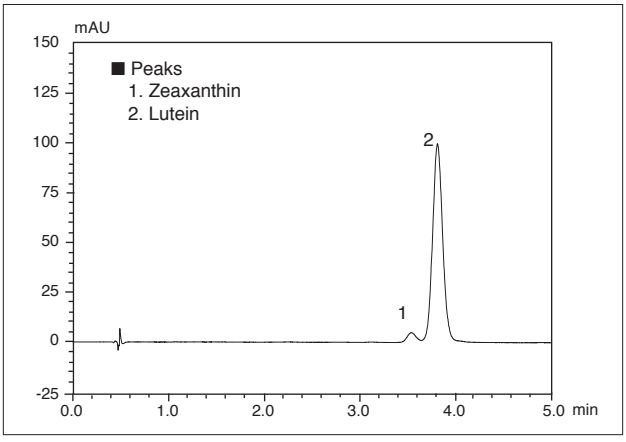


Fig. 10.6.3 Chromatogram of Dietary Supplement

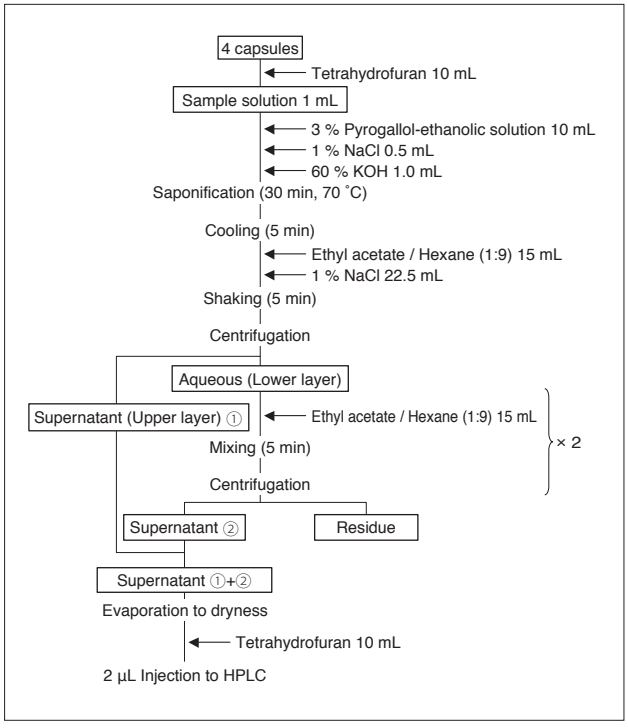


Fig. 10.6.4 Sample Preparation

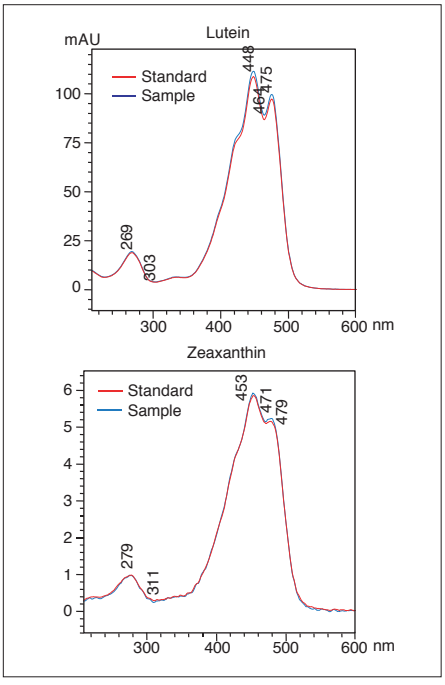


Fig. 10.6.5 Spectra of Lutein and Zeaxanthin

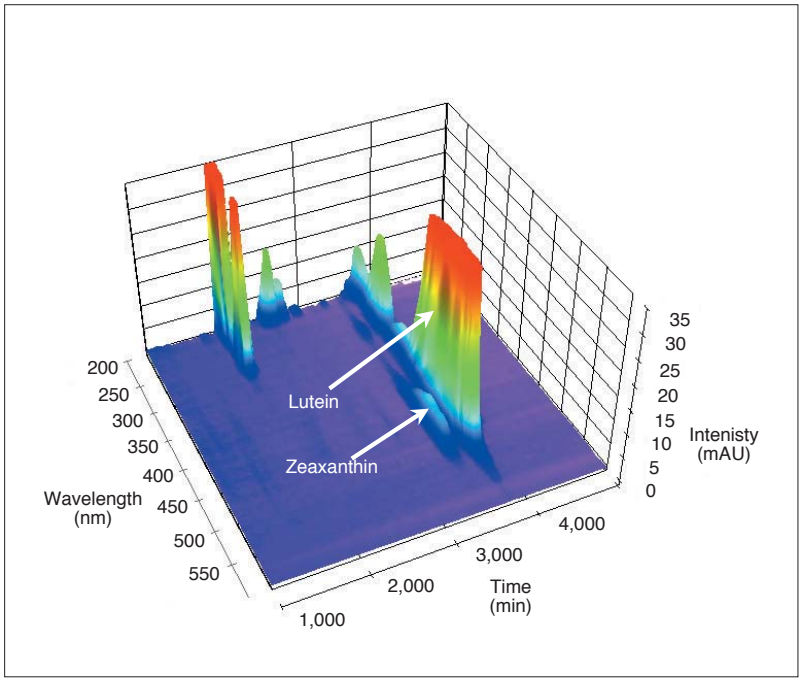


Fig. 10.6.6 3-D Plot of Dietary Supplement

[References]

1) Japan Food Research Laboratories: Revision 5 Japan Food Standard Ingredients Label Explanation of Analysis Manual
2) Egawa Sachie Baba Tsuyomi: Nagasaki Prefectural Institute for Environmental Research and Public Health, 49,100. (2003)

10.7 Measurement of Minerals in Dietary Supplements (1) - AA

■Explanation

Recently, the development and sales of a variety of dietary supplements have increased dramatically against the background of rising public interest regarding health. Here we introduce the method of analysis of minerals in dietary supplements as specified in the Pharmacopoeia of the United States (USP 32), where the supplement market now stands at about three trillion yen (33 billion dollars). As one example, in the case of tablets of oil –and water-soluble vitamins with minerals, the sample preparation and measurement methods are specified for the quantitation of the minerals Ca, Cr, Cu, Fe, K, Mg, Mn, Mo, Se, and Zn, in which flame atomic absorption spectroscopy is used for conducting the quantitation.

■Sample Preparation

The sample preparation differs for (1) Ca, Cr, Cu, Fe, K, Mg, Mn, Zn and (2) Mo, Se in the above supplement. For the elements in group (1), at least 20 tablets are crushed and a quantity corresponding to 5 tablets are transferred to a porcelain crucible. After ashing at 550 ºC in a muffle furnace, hydrochloric acid is added and the contents are heated to dissolve the residue. Adjust the final solution to 0.125 N hydrochloric acid. For the elements in group (2), at least 20 tablets are crushed, and a quantity corresponding to 1000 µg of the measurement element is weighed. This is decomposed using nitric acid and perchloric acid, and is finally brought to a fixed 2 % solution of ammonium chloride.

■Standard Concentrations of Elements

According to the USP, calibration curves are to be generated using standard solutions having the concentrations shown in Table 10.7.1, and quantitation is conducted using a calibration curve approximated by a straight line using a standard solution prepared for the concentration indicated in bold type in the Table. Examples of the target element calibration curves are shown in Fig. 10.7.1 – 10.7.10, but 0 µg/mL is not included in accordance with USP. In the case of Zn, since the high concentration of the standard solution causes curvature of the calibration curve at normal sensitivity, the angle of the burner was changed and measurement was conducted at lower sensitivity to improve the linearity.

■Measurement Conditions

The measurement wavelength, type of flame, and matrix modifier used are shown in Table 10.7.1. The N_2O-C_2H_2 flame was used for measurement of Ca and Mo, and the air-C_2H_2 flame was used for all the other elements. La was added as a matrix modifier for measurement of Ca and Mg, and ammonium chloride was added for measurement of Mo and Se.

Table 10.7.1 Measurement Conditions

Element	Wavelength	Flame	Matrix Modifier
Ca	422.7 nm	N_2O-C_2H_2	0.1 % La
Cr	357.9 nm	Air-C_2H_2	
Cu	324.7 nm	Air-C_2H_2	
Fe	248.3 nm	Air-C_2H_2	
K	766.5 nm	Air-C_2H_2	
Mg	285.2 nm	Air-C_2H_2	0.1 % La
Mn	279.5 nm	Air-C_2H_2	
Mo	313.0 nm	N_2O-C_2H_2	2 % Ammonium Chloride
Se	196.0 nm	Air-C_2H_2	2 % Ammonium Chloride
Zn	213.8 nm	Air-C_2H_2	

Table 10.7.2 Element Concentrations for Calibration Curves

Element	STD (g/mL)						
	1	2	3	4	5	6	8
Mn		0.5	0.75	**1**		1.5	2
K	0.5	**1**	1.5	2	2.5		
Zn	0.5	1	1.5	**2**	2.5		
Cu	0.5	1		**2**		3	4
Cr		**1**		2		3	4
Fe		2		4	**5**	6	8
Mg		0.2	0.3	**0.4**	0.5	0.6	
Ca		1	1.5	**2**	2.5	3	
Mo	5	**10**			25		
Se	5	**10**			25		

10.7 Measurement of Minerals in Dietary Supplements (2) - AA

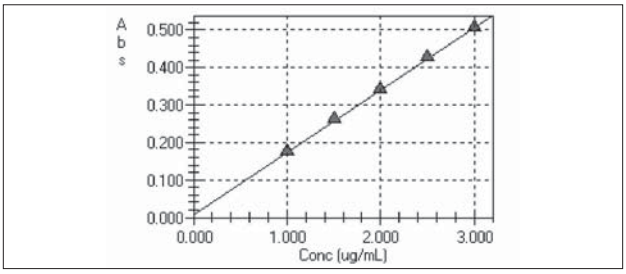


Fig. 10.7.1 Calibration Curve of Ca

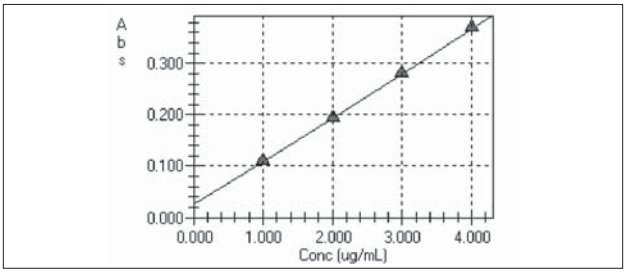


Fig. 10.7.2 Calibration Curve of Cr

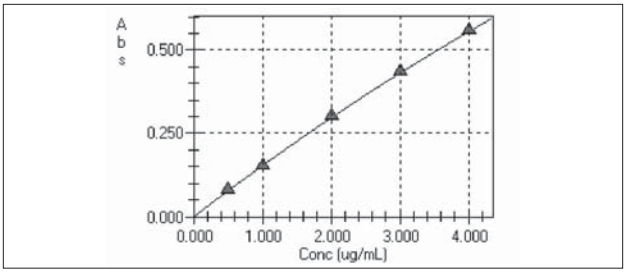


Fig. 10.7.3 Calibration Curve of Cu

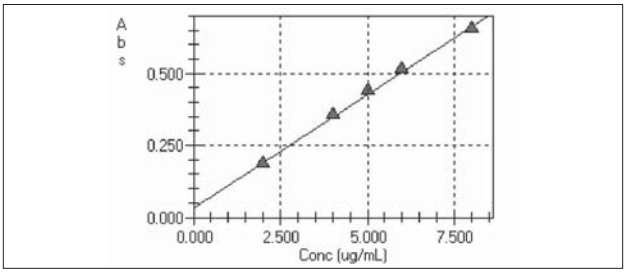


Fig. 10.7.4 Calibration Curve of Fe

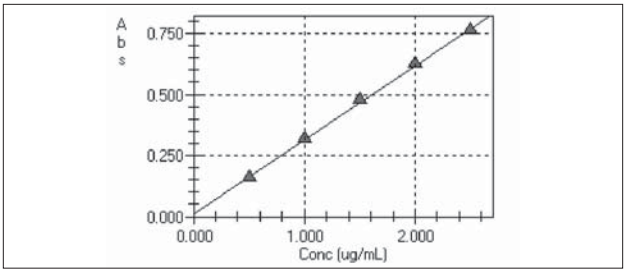


Fig. 10.7.5 Calibration Curve of K

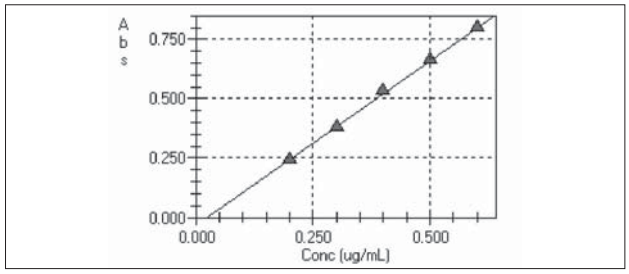


Fig. 10.7.6 Calibration Curve of Mg

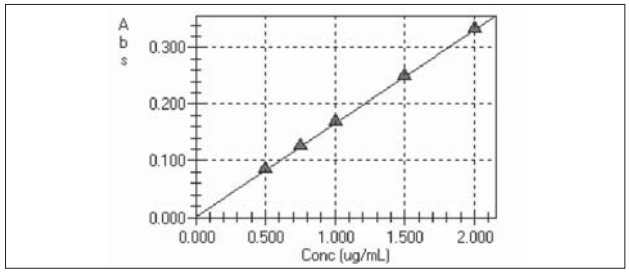


Fig. 10.7.7 Calibration Curve of Mn

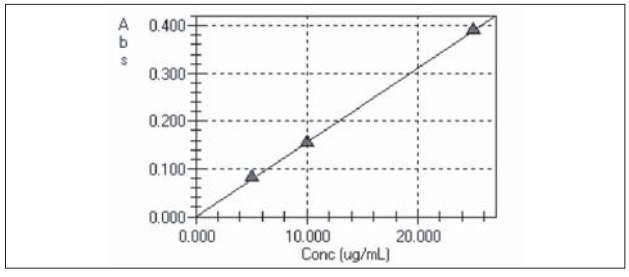


Fig. 10.7.8 Calibration Curve of Mo

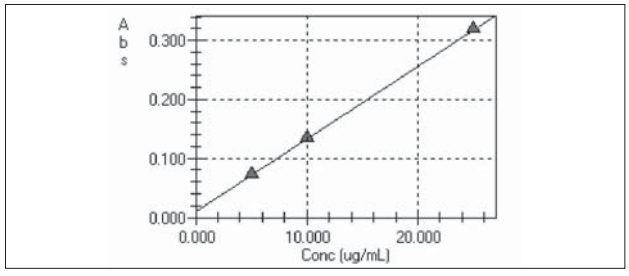


Fig. 10.7.9 Calibration Curve of Se

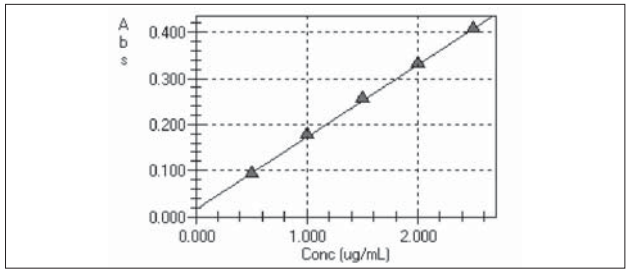


Fig. 10.7.10 Calibration Curve of Zn

10.8 Analysis of Arsenic and Lead in Dietary Supplement (1) - EDX

■Explanation

In recent years, dietary supplements have become widely available in convenience stores and supermarkets. They are defined as food products that promote and maintain health and are used to improve disease prevention and enhance immunity. They are available in various types and forms, including tablet and powdered supplements, and processed herbal products, etc. Among these are products that are subject to safety standards that address the presence and concentrations of heavy metals, etc.[1)]

Analysis of toxic heavy metals such as As and Pb is typically conducted using an emission spectrophotometer (ICP) or atomic absorption spectrophotometer (AA), however, these require time-consuming preparation procedures. For analyte quantities ranging from a few to tens of ppm, measurement can be conducted using an X-ray fluorescence spectrometer, which permits very easy sample preparation. Using an energy dispersive X-ray luorescence spectrometer, we conducted quantitative analysis of As and Pb in a dietary supplement (herbal medicine), and evaluated their lower limit of detection and quantitation, respectively.

1) Example: Japan Health and Nutrition Food Association (JHNFA)

■Standard Samples

Seven standard samples were prepared by mixing herbal powder with a standard solution used for atomic absorption analysis. The elements and standard values are shown in Table 10.8.1, and the preparation procedure is shown in Fig. 10.8.1.

Table 10.8.1 Standard Values

No.	As	Pb
(1)	50	0
(2)	30	5
(3)	20	10
(4)	10	20
(5)	5	30
(6)	0	50
(7)	0	0

Unit: ppm

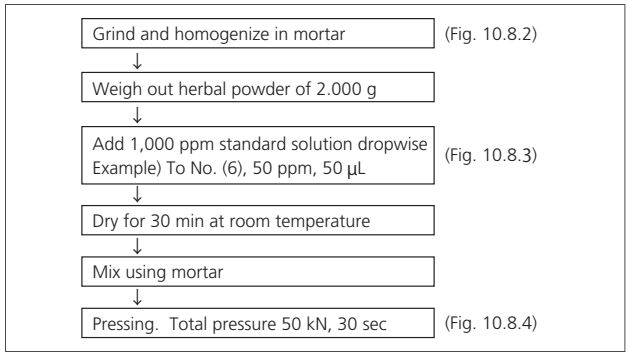


Fig. 10.8.1 Preparation Procedure

Fig. 10.8.2 Homogenization by Pulverizing

Fig. 10.8.3 Blend in Standard Solution

Fig. 10.8.4 Formed Briquette

■Calibration Curves

The calibration curves for As (Kα line) and Pb (Lβ line) are shown in Fig. 10.8.5 and Fig. 10.8.6, respectively. Correction by the dj method was conducted for As, which is overlapped by Pb.

We also generated those calibration curves with the internal standard which line is the RhKα C scattering (Compton) (figure not shown). Table 10.8.2 shows the accuracy of the respective calibration curves with and without internal standard correction. Accuracy refers to the variation of the calibration point using a numerical value indicated as 1σ.

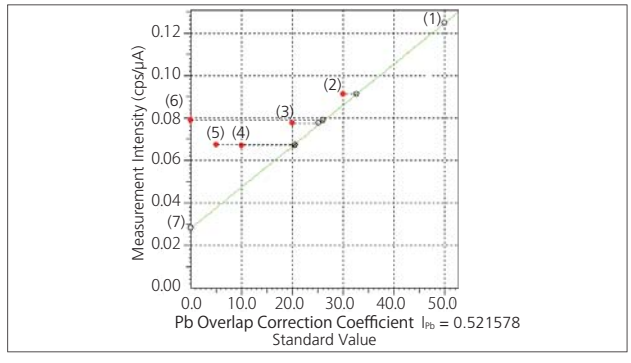


Fig. 10.8.5 Calibration Curves for As

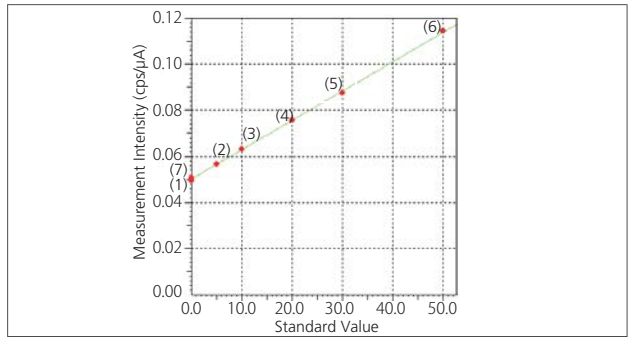


Fig. 10.8.6 Calibration Curves for Pb

10.8 Analysis of Arsenic and Lead in Dietary Supplement (2) - EDX

Table 10.8.2 Accuracy of Calibration Curve

Internal Standard Correction	Without		With	
Internal Standard Line	–		RhKαC	
Element	As	Pb	As	Pb
Analytical line	Kα	Lβ1	Kα	Lβ1
Accuracy (1 σ)	0.23	0.42	0.60	0.83

Unit: ppm

■Profile

Fig. 10.8.7 shows the profile overlap of standard sample No. (4) (As: 10 ppm, Pb: 20 ppm) and No. (7) (Blank). Since the AsKα line and PbLα line are adjacent, one or a combination of the following processing methods is selected.

A) Intensity peak separation
B) Intensity overlap correction
C) Overlap correction of the dj method on Calibration curve

Here, we applied method C) only.

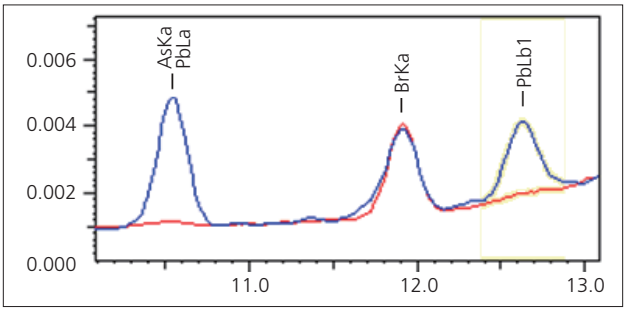


Fig. 10.8.7 Qualitative Profile Overlap of As and Pb
Blue: No. (4), Red: No. (7) (Blank)

■Lower Limits of Detection and Quantitation

Ten times repeatability test of the No.(7) Blank were conducted, and the lower limit of detection (3σ) and lower limit of quantitation (10σ) were determined. Table 10.8.3 shows the results obtained using 2 different sample preparation methods, pressing and simple compression. In the case of simple manual compression, quantitation was conducted using an internal standard calibration curve to correct for flatness and density effects. The powder preparation procedure used is shown in Fig. 10.8.8.

Table 10.8.3 Lower Limits of Detection and Quantitation for As and Pb

Preparation Method	Pressing		Powder, Sample Container	
Internal Standard Correction	Without		With	
Element	As	Pb	As	Pb
Average Value	(-0.08)	0.35	0.32	(-0.18)
Standard Deviation	0.18	0.26	0.18	0.26
Lower Limit of Detection (3σ)	0.53	0.77	0.55	0.78
Lower Limit of Quantitation (10σ)	1.8	2.6	1.8	2.6

Unit: ppm

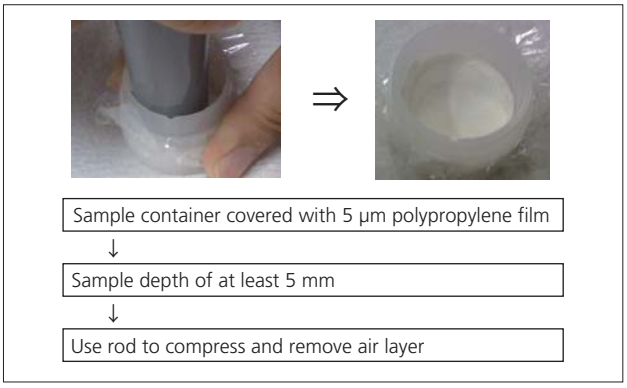


Fig. 10.8.8 Simple Powder Compression by Hand

■Conclusion

Table 10.8.2, which shows the accuracy of the calibration curves, indicates that without conducting internal standard correction, accuracy improved 2.6 times for As, and 2.0 times for Pb. The cause is thought that the fluctuation of the RhKαC is added to the fluctuations in the respective intensities of AsKα and PbLβ1.

On the other hand, both for As and Pb, Table 10.8.3 indicates that the lower limit of detection and lower limit of quantitation are the same for both formed briquette samples and manually compressed samples.

The reason for this lack of any substantial difference is thought to be due to a zero net intensity for the blank.

Therefore, since either method is valid for measuring dietary supplements, using the pressing sample preparation method without the use of an internal standard or the easy compression (powder) sample preparation method with an internal standard is suitable.

■Analytical Conditions

Instruments : EDX-720 (EDX-GP)
X-ray Tube : Rh target
Tube Voltage : 50 kV
Tube Current : (Auto sensitivity control) μA
Filter : #3 (EDX-720)
#4 (EDX-GP)
Atmosphere : Air
Measurement Diameter : 10 mmϕ
Measurement Time : 1200 sec
Dead Time : Max 40 %

11. Veterinary Drugs

11.1 Analysis of Polyether Antibiotics in Animal Feeds (1) - LC

■Explanation

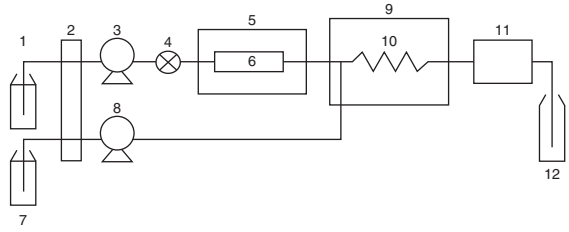
The Japanese Ministry of Agriculture, Forestry and Fisheries designates polyether antibiotics salinomycin sodium and monensin sodium as animal feed additives for enhancing the effectiveness of nutrients contained in animal feeds. These substances contained in poultry and bovine feeds were conventionally analyzed by microbiotic quantitation in accordance with the Animal Feed Analysis Standards. However, as this method requires two days for results to be obtained, faster quantitative methods were being pursued.

Given this situation, the Animal Feed Analysis Standards were partially revised as of April 10, 2002, to incorporate LC post-column derivatization method to analyze salinomycin sodium, monensin sodium, narasin and semduramicin sodium.

■Detection Method

Polyether antibiotics produce color when heated with vanillin (4-hydroxy-3-methoxybenzaldehyde) in sulfuric acid and methanol. This reaction is known as a Komarowsky reaction, and this post-column derivatization system uses the Komarowsky reaction. Polyether antibiotics narasin and semduramycin are also analyzed by the same method.

Fig. 11.1.1 shows the flow diagram for this system. A vanillin reagent is continuously added to the polyether antibiotics that were separated in the reversed-phase column, and the target substances are detected with a visible absorption detector (520 nm) after being heated at 95 °C in the reaction chamber.



1:Mobile Phase
2:Degasser
3:Pump for Mobile Phase
4:Injector
5:Column Oven
6:Column
7:Reaction Reagent
8:Pump for Reaction Reagent
9:Reaction Bath
10:Reaction Coil
11:UV-VIS Detector
12:Waste

Fig. 11.1.1 System Flow Diagram

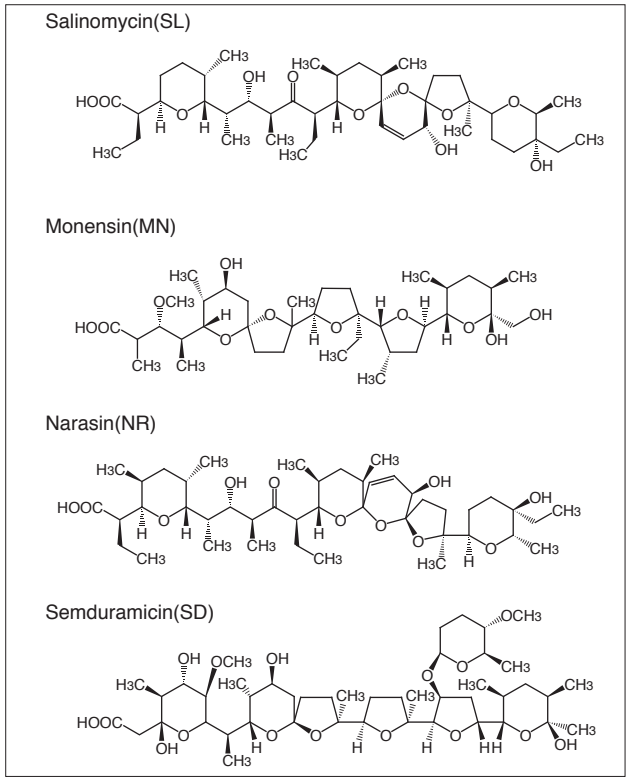


Fig. 11.1.2 Polyether Antibiotics Structures

■Analytical Conditions

<Separaition Condition>

Column	: Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase	: Water / Methanol / Acetic Acid = 60 mL / 940 mL / 1 mL (v / v / v)
Flowrate	: 0.6 mL/min
Column Temp.	: 40 ˚C

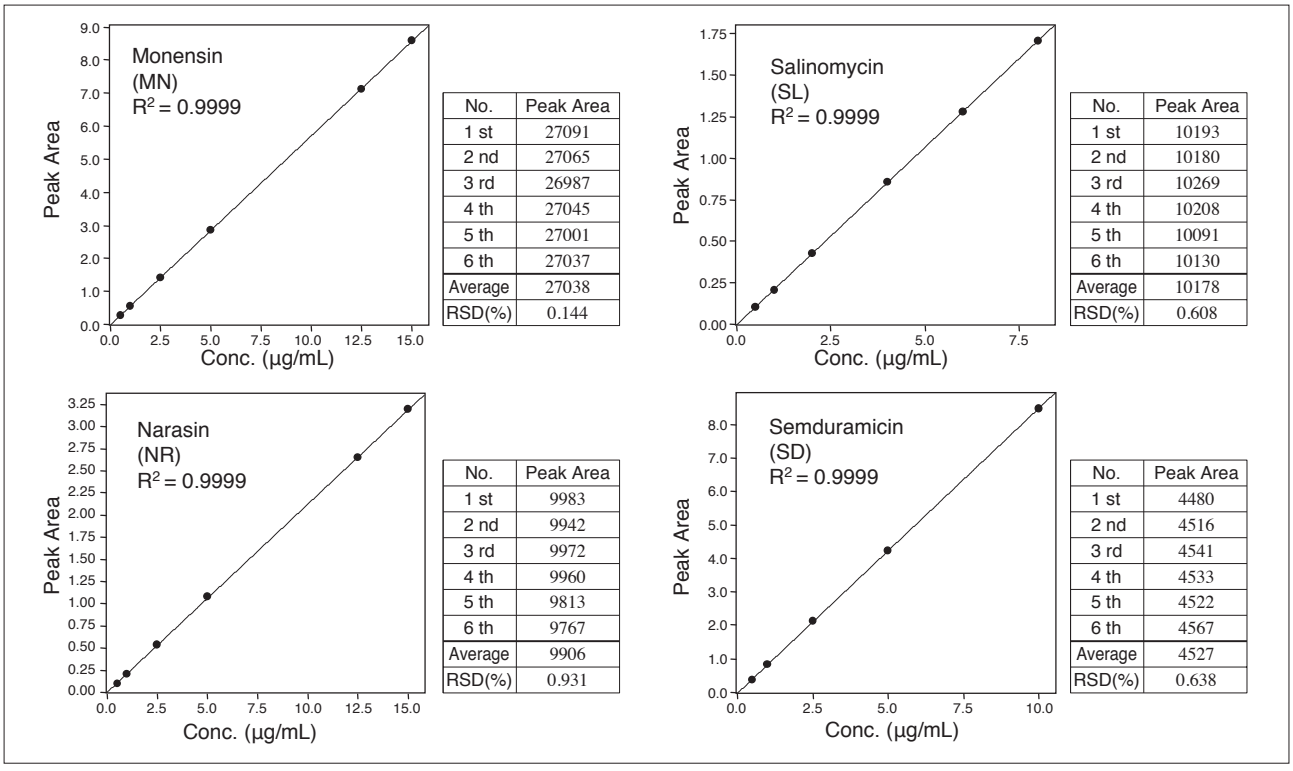
<Detection Condition>

Reaction Reagent	: Methanol / Sulfuric acid / Vanillin = 95 mL / 2 mL / 3 g (v/v/w)
Flowrate	: 0.6 mL/min
Reaction Temp.	: 95 ˚C
Reaction Coil	: 5 mL. (SL·MN·NR) or 10 mL. (SD) × 0.5 mm I.D.
Detection	: UV-VIS Absorbance Detector at 520 nm

11.1 Analysis of Polyether Antibiotics in Animal Feeds (2) - LC

■Linearity and Reproducibility

Fig.11.1.3 shows the calibration curve (horizontal axis: µg /mL (through titration)) for each compound generated within the concentration range specified in the Animal Feed Analysis Standards and the peak area reproducibility (n = 6) for each standard solution (0.5 µg/mL (through titration)).



No.	Peak Area
1 st	27091
2 nd	27065
3 rd	26987
4 th	27045
5 th	27001
6 th	27037
Average	27038
RSD(%)	0.144

No.	Peak Area
1 st	10193
2 nd	10180
3 rd	10269
4 th	10208
5 th	10091
6 th	10130
Average	10178
RSD(%)	0.608

No.	Peak Area
1 st	9983
2 nd	9942
3 rd	9972
4 th	9960
5 th	9813
6 th	9767
Average	9906
RSD(%)	0.931

No.	Peak Area
1 st	4480
2 nd	4516
3 rd	4541
4 th	4533
5 th	4522
6 th	4567
Average	4527
RSD(%)	0.638

Fig. 11.1.3 Linearity and Peak Area Reproducibility

■Analysis of Standard Solutions

Fig. 11.1.4 shows the chromatographic results obtained following a 20 µL injection of the SL, MN and NR standard solution (1 µg/mL each (through titration)). The length of the reaction coil was 5 m. Fig. 11.1.5 shows the results following a 20 µL injection of the SD standard solution (2.5 µg/mL (through titration)). In this case, a 10 m reaction coil was used.

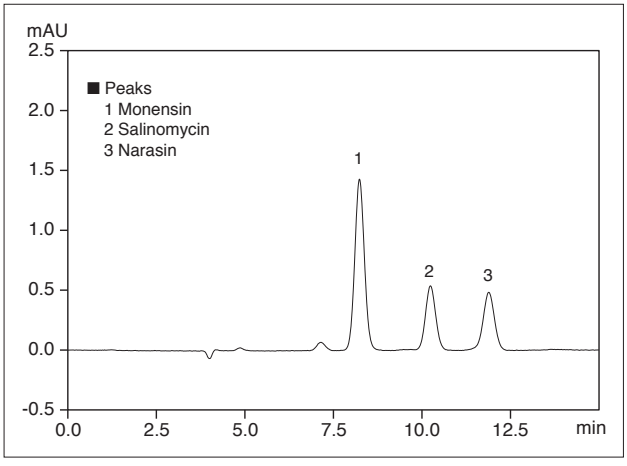


Fig. 11.1.4 Chromatogram of Standard Mixture of Monensin, Salinomycin and Narasin (1 µg/mL each, 20 µL injected)

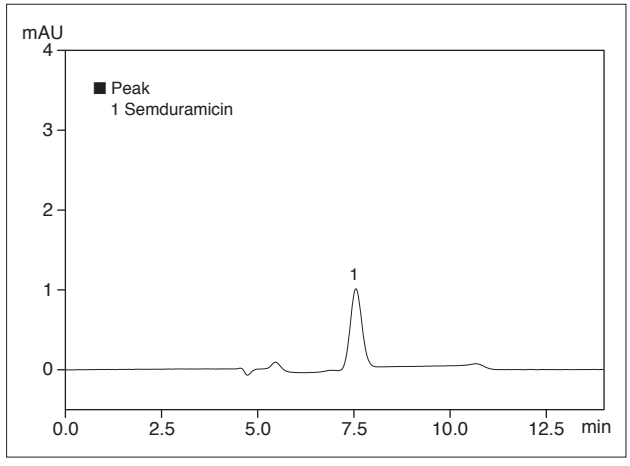


Fig. 11.1.5 Chromatogram of Semduramicin (2.5 µg/mL, 20 µL injected)

11.1 Analysis of Polyether Antibiotics in Animal Feeds (3) - LC

■Explanation

Lasalocid sodium (Fig. 11.1.6), unlike the other four compounds referred to in the previous page, does not present a colorimetric reaction with vanillin (refer to previous analysis), and cannot, therefore, be detected using post-column derivatization method. However, as it is naturally fluorescent, it can be analyzed using a fluorescence detector. Here we introduce an example of analysis of lasalocid sodium using the fluorescence detection method, as specified.

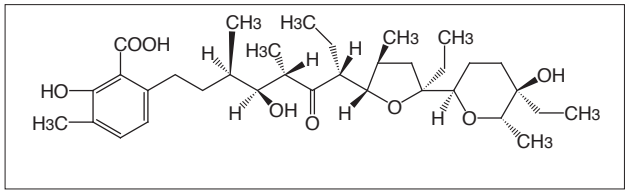


Fig. 11.1.6 Structure of Lasalocid

■Analysis of Standard Solution

Fig. 11.1.7 shows the chromatographic results following a 20 µL injection of lasalocid sodium standard solution (1 µg/mL (through titration)).

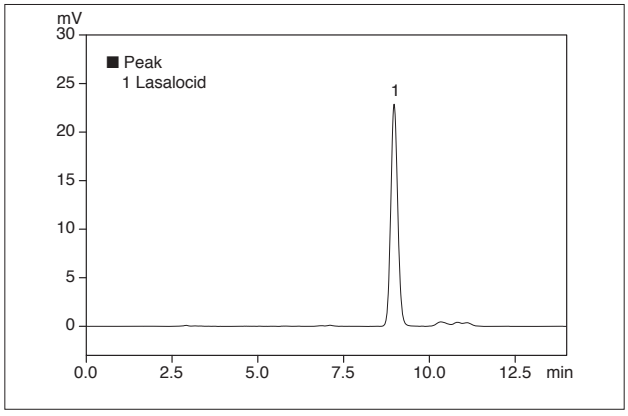


Fig. 11.1.7 Chromatogram of Lasalocid
(1 µg/mL, 20 µL injected)

■Analytical Conditions

Column : Shim-pack VP-ODS (250 mmL. × 4.6 mm I.D.)
Mobile Phase : Phosphate Buffer* / Methanol = 1/9 (v/v)
Flowrate : 1.0 mL/min
Column Temp. : 40 °C
Detection : Fluorescence Detector
(Ex : 310 nm Em : 420 nm)

* KH_2PO_4 2.72 g + water
→ Total 1000 mL
→ pH=2.9-3.0 by aq. H_3PO_4 (1+10)

■Analysis of Feed

Fig. 11.1.8 shows the pretreatment procedure according to the Animal Feed Analysis Standards. Fig. 11.1.9 shows the chromatographic results obtained from analysis of a 20 µL injection of a feed sample (concentration of 75 µg/g in feed), prepared adding 100 mL of lasalocid sodium standard solution (7.5 µg/mL (through titration), methanol solution), instead of methanol 100 mL, to 10 g of feed sample in the pretreatment procedure shown in Fig. 11.1.8. The results indicate that quantitation of lasalocid sodium is possible without interference from contaminating components.

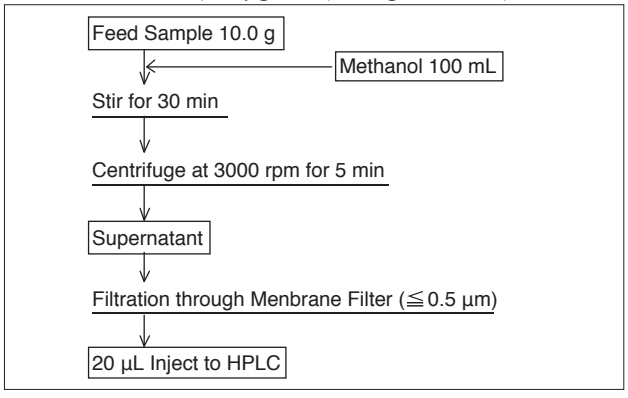


Fig. 11.1.8 Pretreatment Procedure

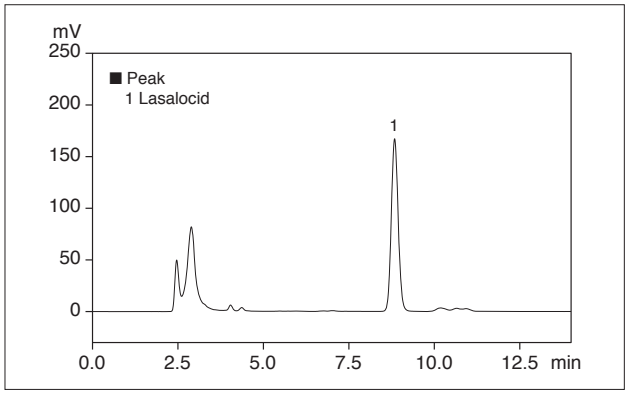


Fig. 11.1.9 Chromatogram of Feed
(Feed Spiked with 75 µg/g Lasalocid, 20 µL injected)

11.2 Analysis of Aminoglycoside Antibiotics (1) - LC/MS

■Explanation

Some of the ministerial ordinances related to standards on the constituents of milk and dairy products were revised in accordance with Notification No. 170 issued by the Ministry of Health, Labour and Welfare on 26 November 2003. New standards and test methods (LC/MS) for the residual amounts of streptomycin and dihydrostreptomycin in milk were established. In the example presented here, the analysis of these two constituents and of gentamycins, spectinomycin, and neomycin, constituents for which LC/MS was already specified as the test method, is performed.

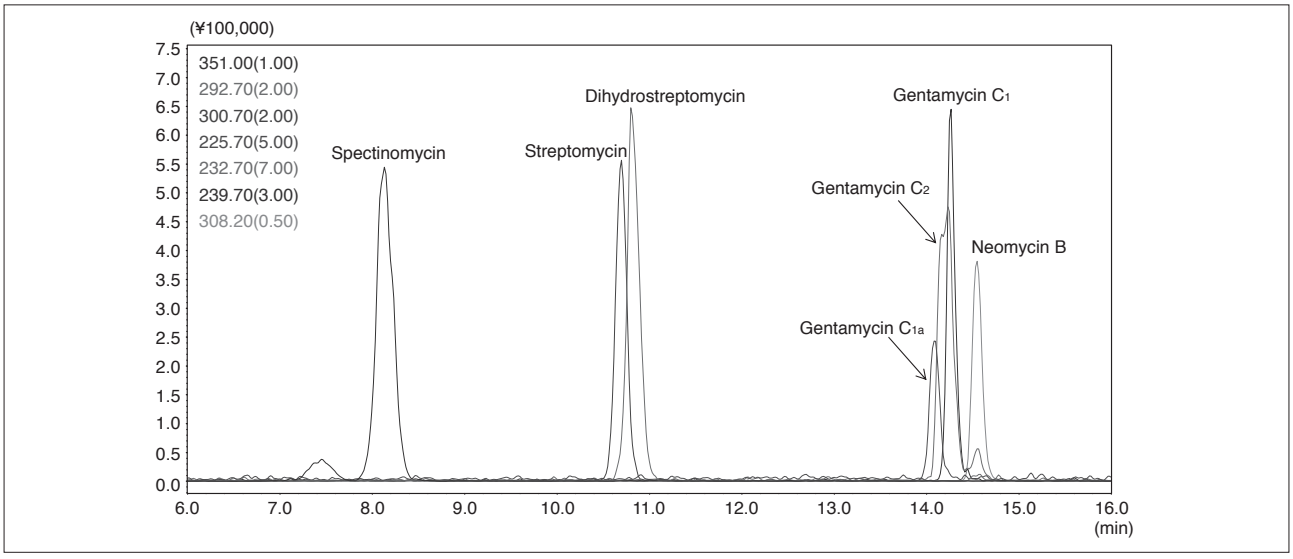


Fig. 11.2.1 ESI Mass Chromatogram for Aminoglycoside Antibiotics

■Analytical Conditions

Instrument	: Shimadzu LCMS-2010A
Column	: Shim-pack VP-ODS (150 mmL. × 2.0 mm I.D.)
Mobile Phase A	: 5 mmol/L Perfluorobutyric Acid (PFBA)-Water
Mobile Phase B	: Acetonitrile
Flowrate	: 0.4 mL/min
Gradient Program	: B 10 % (0 min) → 40 % (15-20 min)
Column Temp.	: 40 ˚C
Injection Volume	: 10 µL
Ionization Mode	: Positive ESI
Applied Voltage	: 4.5 kV
Neburizing Gas Flow	: 1.5 L/min
Drying Gas Pressure	: 0.1 MPa
CDL Temp.	: 200 ˚C
Block Temp.	: 200 ˚C
CDL Voltage	: S-Mode
SIM	: *m/z* 351.0 for Spectinomycin *m/z* 300.7 for Streptmycin *m/z* 292.7 for Dihydrostreptmycin
	m/z 225.7 for Gentamycin C1a *m/z* 232.7 for Gentamycin C2 *m/z* 239.7 for Gentamycin C1 *m/z* 308.2 for Neomycin B

11.2 Analysis of Aminoglycoside Antibiotics (2) - LC/MS

■Samples with Standard of Regulated Concentration Added and Pork Blanks

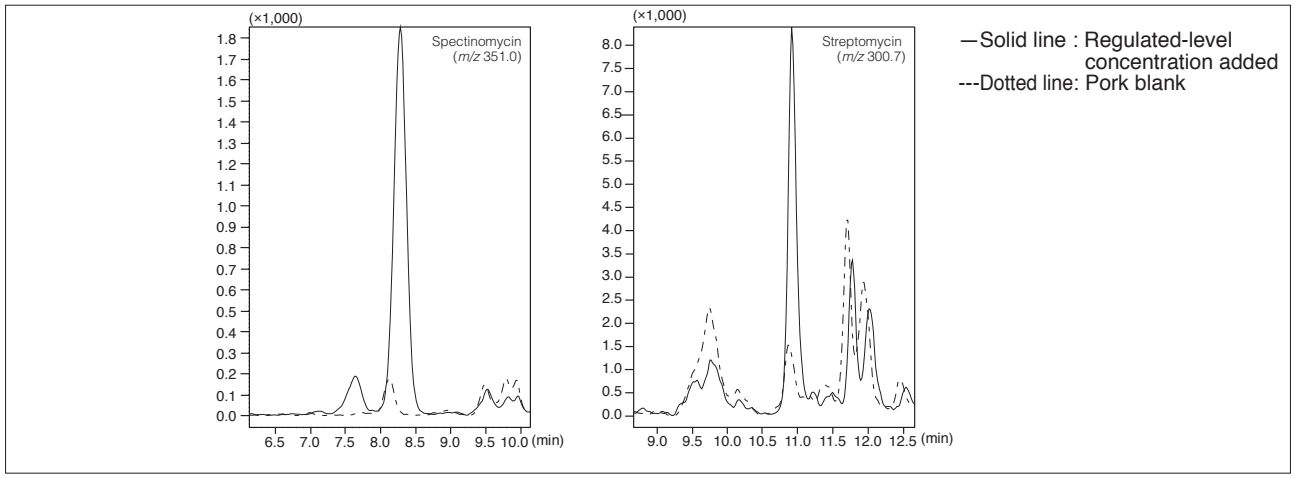


Fig. 11.2.2 Samples with Standard of Regulated-Level Concentration Added and Pork Blanks (1)

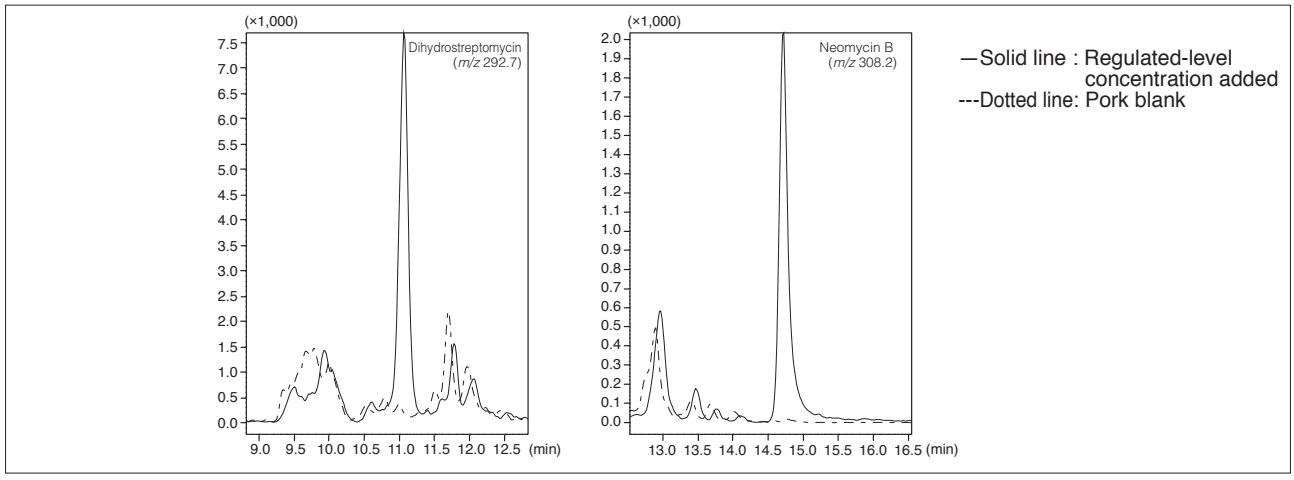


Fig. 11.2.3 Samples with Standard of Regulated-Level Concentration Added and Pork Blanks (2)

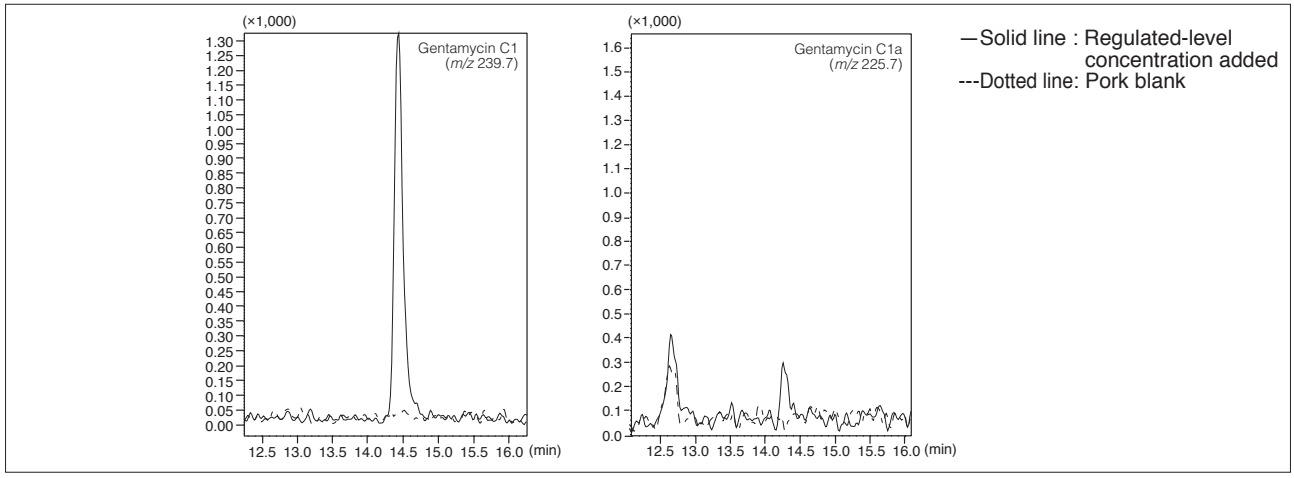


Fig. 11.2.4 Samples with Standard of Regulated-Level Concentration Added and Pork Blanks (3)

11.3 Analysis of Chloramphenicol in Honey - LC/MS

■Explanation

Chloramphenicol is a widely applied bacteriostatic antimicrobial that is useful as a broad-spectrum antibiotic. However, due to the serious side effects associated with this substance, the FAO and WHO specify that all food products be free of any residual chloramphenicol. In the Positive List System for such substances as pesticide residues that began to be enforced by Japan in 2006, certain substances such as agricultural chemicals are specified to be "Non-detectable" in food products upon testing using the specified test methods. In addition, due to frequent reports of detection of chloramphenicol not only in livestock and marine products, but in honey and royal jelly as well, it has been specified as a substance to be monitored in food imports. Here we introduce an example of analysis of chloramphenicol in honey according to the test method specified in the Japanese official notification. Chloramphenicol is ionized by electrospray ionization (ESI), and is detected as the *m/z* 321 deprotonated molecule $[M-H]^-$. Fig. 11.3.1 shows the structure of chloramphenicol.

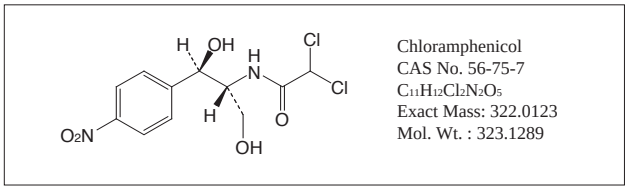


Fig. 11.3.1 Structure of Chloramphenicol

The sample preparation for chloramphenicol testing in honey is shown in the flow chart of Fig. 11.3.2. The SPEcolumn used is a divinylbenzene-*N*-vinylpyrrolidone copolymer mini-column (60 mg).

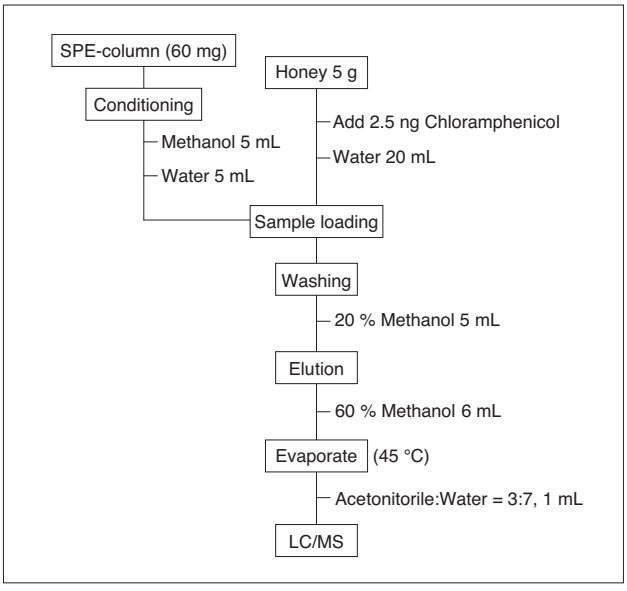


Fig. 11.3.2 Sample Preparation

■Analytical Conditions

Column	: Shim-pack VP-ODS (150 mmL. × 2.0 mm I.D.)
Mobile Phase	: 10 mmol/L Ammonium Formate–Water/Acetonitrile = 70/30
Flowrate	: 0.2 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 3 µL
Probe Voltage	: -3.5 kV (ESI-Negative Mode)
DL Temp.	: 250 ºC
BH Temp.	: 350 ºC
Nebulizing Gas Flow	: 1.5 L/min
Drying Gas Flow	: 15 L/min
SIM	: *m/z* 321 (0.5 sec)

Fig. 11.3.3 shows the overlaid mass chromatograms of a honey control sample (1), and a honey sample spiked with 2.5 ng chloramphenicol (2). The detection limit of chloramphenicol specified in the official notification test method is 0.0005 mg/kg (= 0.5 ng/g). To obtain a test sample with the specified detection limit concentration, 2.5 ng of chloramphenicol was added to 5 g of honey. In the final sample extract, the concentration of chloramphenicol is 2.5 µg/L, sufficient to allow good detection. The recovery of chloramphenicol in the spiked honey averaged 89 % (CV% =1.72, n =3).

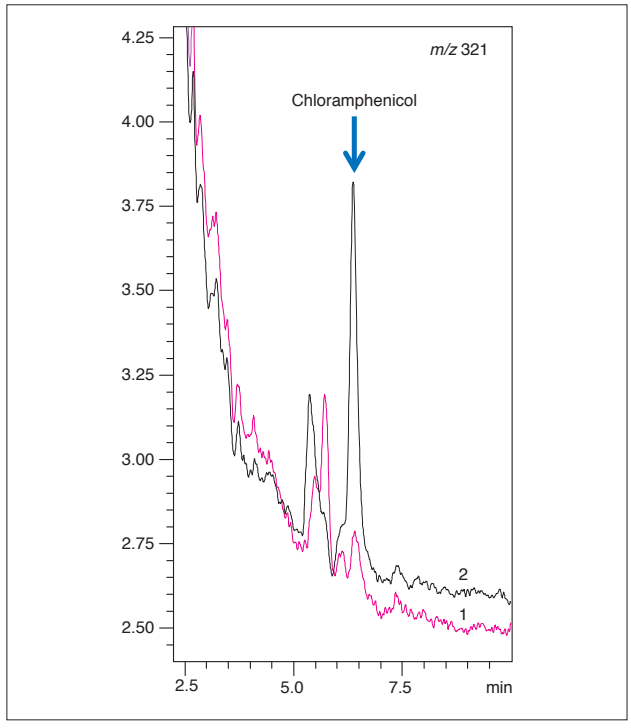


Fig. 11.3.3 Analytical Results for Chloramphenicol in Honey (1: honey control, 2: honey + chloramphenicol)

11.4 High Speed Analysis of Quinolone Antibacterial Agents - LC

■Explanation

Many types of quinolone synthetic antibacterials are widely used for the prevention and treatment of infectious diseases in domestic animals, domestic fowl, and cultured fish. Here, we introduce an example of simultaneous analysis of a mixture of synthetic quinolone antibacterials using the Nexera ultra high performance LC system with the RF-20Axs high-sensitivity fluorescence detector.

■Shortening of Analysis Time

We conducted simultaneous analysis of a mixture of 11 synthetic quinolone antibacterials using the RF-20Axs fluorescence detector. A standard mixture (1 mg/L each, prepared in 30 % methanol) was injected. Analysis was conducted using 3 different columns, one of which was a conventional column, and the other two which were high-speed analysis columns. Fig. 11.4.1 shows the chromatograms (1)-(3) obtained with the respective columns. Use of the 2.2 µm particle size Shim-pack XR-ODS allowed the analysis time to be shortened to 1/6 that of the conventional column, and use of the 1.6 µm particle size Shim-pack XR-ODS shortened the analysis time further to about 1/12 the original time.

■Analytical Conditions

Column : (1) Shim-pack VP-ODS
(150 mmL. × 4.6 mm I.D., 4.6 µm)
(2) Shim-pack XR-ODS
(75 mmL. × 3.0 mm I.D., 2.2 µm)
(3) Shim-pack XR-ODS Ⅲ
(50 mmL.×2.0 mm I.D., 1.6 µm)

Mobile Phase : A: 0.1 % Formic Acid-Water
B: 0.1 % Formic Acid-Acetonitrile

Flowrate : (1) 1.0 mL/min (2) 1.3 mL/min
(3) 0.8 mL/min

Gradient Program :

(1) B 3 % (0 min) → 15 % (18 min) → 35 % (21-27 min) → 95 % (27.01-31.8 min) → 3 % (31.81-45 min)
• Mixer: 20 µL

(2) B 3 % (0 min) → 15 % (3 min) → 35 % (3.5-4.5 min) → 95 % (4.51-5.3 min) → 3 % (5.31-7.5 min)
• Mixer: 20 µL

(3) B 3 % (0 min) → 15 % (1.5 min) → 35 % (1.75 - 2.25 min) → 95 % (2.26-2.65 min) → 3 % (2.66-3.75 min)
• Mixer: 20 µL

Column Temp. : 65 ºC

Injection Volume : (1) 2 µ L (2) 0.8 µL (3) 0.4 µL

Detection : Fluorescence Detector (RF-20Axs)
Ex: 299 nm Em: 455 nm Gain×4 (Peak 1-8)
Ex: 325 nm Em: 365 nm Gain×16 (Peak 9-11)

Cell Temp. : 20 ºC

Flow Cell : (1) Coventional Cell
(2) Semi-micro Cell
(3) Semi-micro Cell

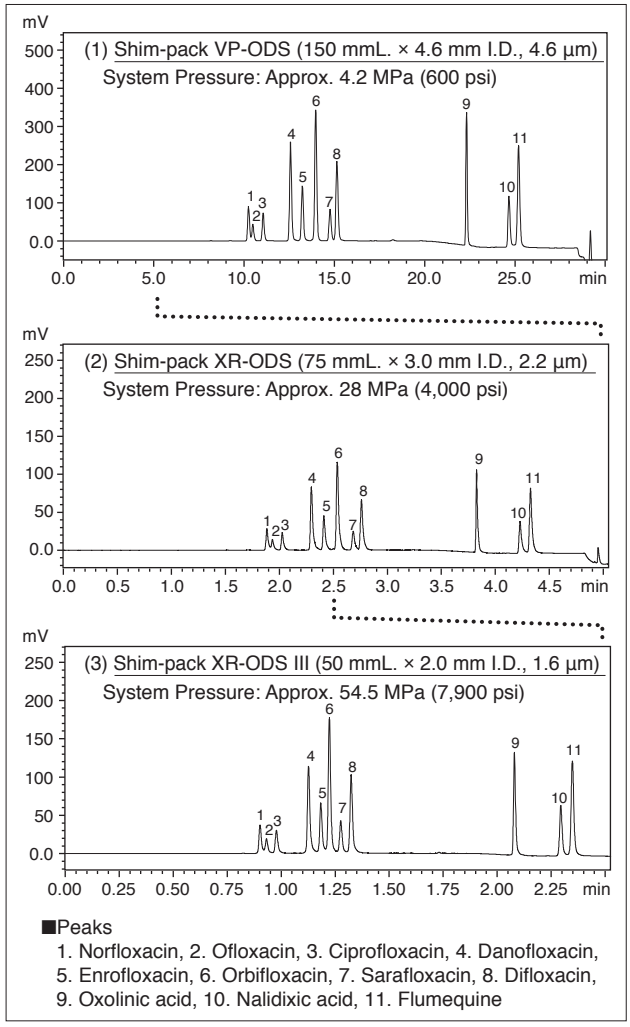


Fig. 11.4.1 Chromatograms of a Standard Mixture of 11 Quinolones (1 mg/L each)

11.5 Analysis of New Type Quinolone Antibacterial Agents in Poultry - LC/MS

■Explanation

In Japan, some of the standards for food products and additives were revised in accordance with Notification No. 369 issued by the Ministry of Health, Labour and Welfare on 26 November 2003. New standards and test methods for the residual amounts of sarafloxacin and danofloxacin in meat were established, and LC/MS is now used for confirmation tests. In the example presented here, LC/MS is used in the analysis of new type quinolone antibacterial agents (sarafloxacin and danofloxacin).

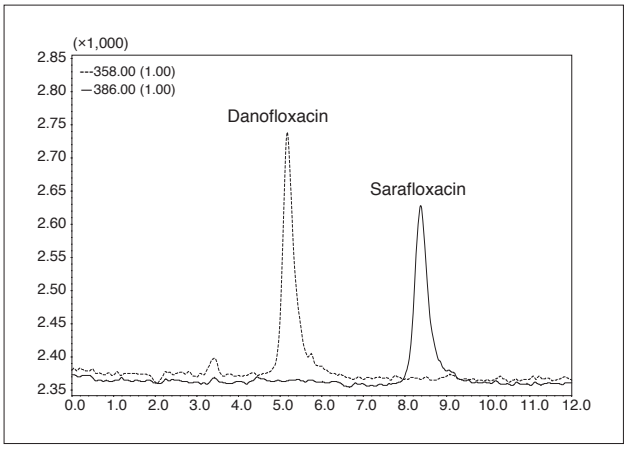


Fig. 11.5.1 Analysis of Standard Samples (50 ppb for each constituent, 50 µL injected)

■Analytical Conditions

Instrument	: LCMS-2010A
Column	: Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase	: A: 0.05 %TFA-Water B: Acetonitrile A/B = 4/1 (v/v)
Flowrate	: 0.8 mL/min
Column Temp.	: 40 ˚C
Sample Store Temp.	: 5 ˚C
Ionization Method	: ESI-Positive
Applied Voltage	: 4.5 kV
Nebulizer Gas Flow	: 1.5 L/min.
Drying Gas Pressure	: 0.2 MPa
CDL Temp.	: 200 ˚C
BH Temp.	: 200 ˚C
CDL Voltage	: S-Mode
Q-array Voltage	: S-Mode
SIM	: *m/z* 358.00 $(M+H)^+$ for Danofloxacin *m/z* 386.00 $(M+H)^+$ for Sarafloxacin

■Confirming Addition of Regulated-Level Concentration

Although unwanted peaks are obtained at retention times different to that of danofloxacin, the regulated-level concentration can be easily detected.

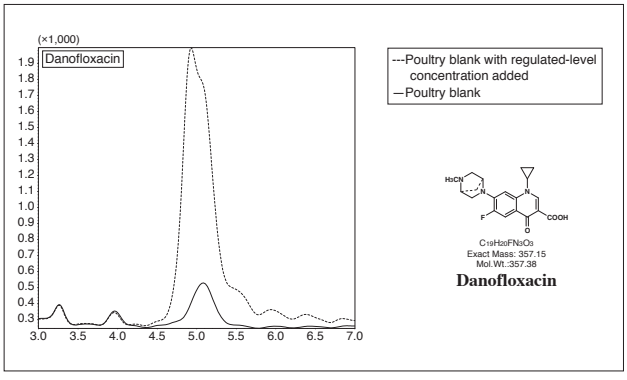


Fig. 11.5.2 Chromatogram for Addition of Regulated-Level Concentration (Danofloxacin)

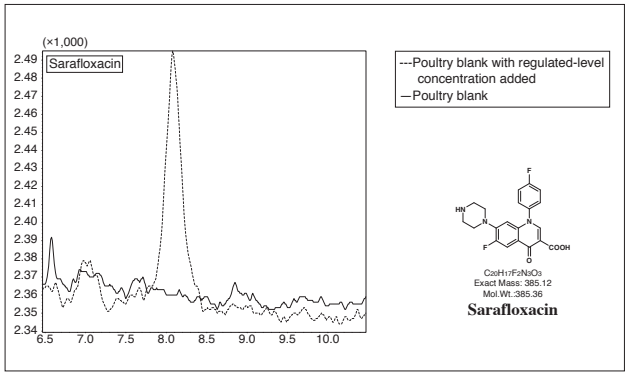


Fig. 11.5.3 Chromatogram for Addition of Regulated-level Concentration (Sarafloxacin)

11.6 Analysis of Enrofloxacin in Broiled Eels (1) - LC

■Explanation

Enrofloxacin is one kind of the new quinolones, synthetic antibiotics, and is used to prevent and treat pneumonia and E. coli bacterial diarrhea syndrome in cows and pigs. In Japan, however, it is not permitted to use this agent in farmed fish.

The enrofloxacin residue analysis method is specified in "Analysis Method for Enrofloxacin in Eel" (Japanese Ministry of Health, Labour and Welfare dated June 5, 2003 Food Control Notification No.0605002), in which the HPLC method with a fluorescence detector and the LC/MS method using electrospray ionization (ESI) are described in parallel. Introduced here are examples of analysis of commercially available broiled eel (unscorched) that conform to the HPLC and LC/MS analysis methods.

■Analysis of Standard Solution

Fig. 11.6.1 shows the structural formula for enrofloxacin. The structure common to the new quinolones synthetic antibiotics is characterized as having a carboxyl group in the 3rd position, a carbonyl group in the 4th position and fluorine in the 6th position, with the first two positions indispensable in demonstrating the anti-bacterial activity. Although the new quinolones synthetic antibiotics can also be analyzed using a UV detector, since the substance possesses natural fluorescence, HPLC high sensitivity analysis is performed using a fluorescence detector. Fig. 11.6.2 shows chromatograms obtained from analysis of the enrofloxacin standard solutions (5 µg/L, 50 µg/L, 100 µg/L), using 5 µL injections.

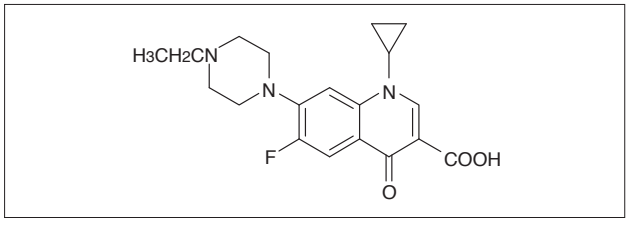


Fig. 11.6.1 Structure of Enrofloxacin

■Analytical Conditions for HPLC

Column	:	Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase	:	A: McIlvain Buffer (pH = 3.0) B: Acetonitrile A/B = 85/15 (v/v)
Flowrate	:	1.0 mL/min
Column Temp.	:	40 ºC
Injection Volume	:	5 µL
Detection	:	Fluorescence Detector RF-10AXL (Ex: 285 nm Em: 460 nm)
Cell Temp.	:	25 ºC

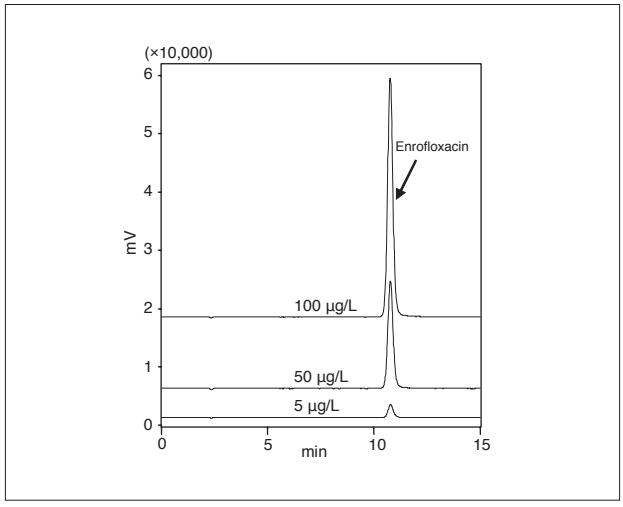


Fig. 11.6.2 Chromatograms of Enrofloxacin

■Analysis of Broiled Eel by HPLC

Fig. 11.6.3 shows the pretreatment procedure for the broiled eel described in the analysis method. Fig. 11.6.4 shows the results following injection of 5 µL of the sample prepared as shown in Fig. 11.6.3. In addition, Fig. 11.6.5 shows the chromatogram obtained from the broiled eel sample preparation spiked with 50 µg/L enrofloxacin, using the same analytical conditions.

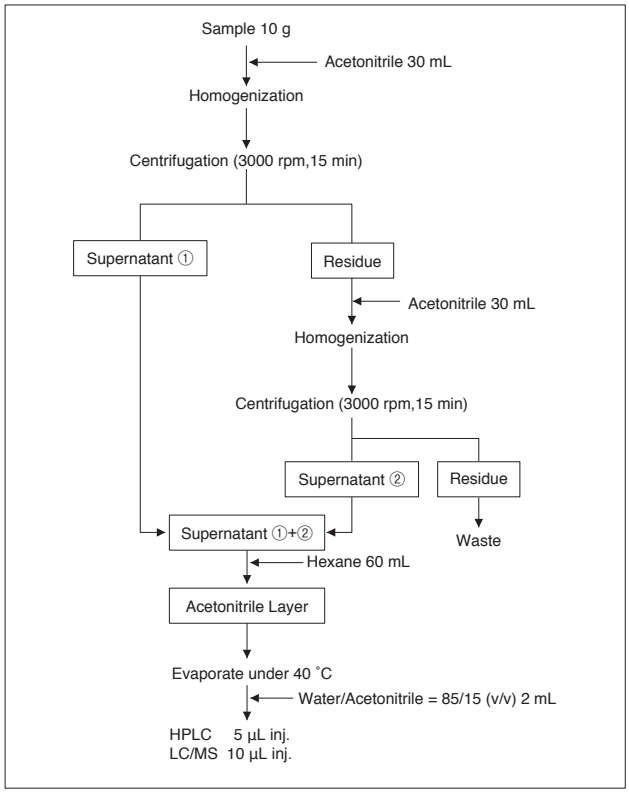


Fig. 11.6.3 Sample Preparation

11.6 Analysis of Enrofloxacin in Broiled Eels (2) - LC/MS

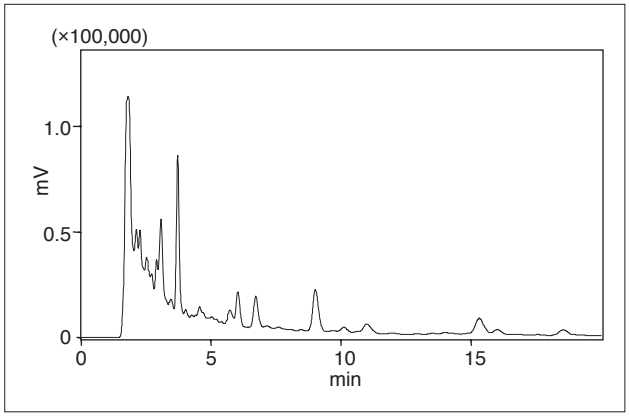


Fig. 11.6.4 Chromatogram of Broiled Eels

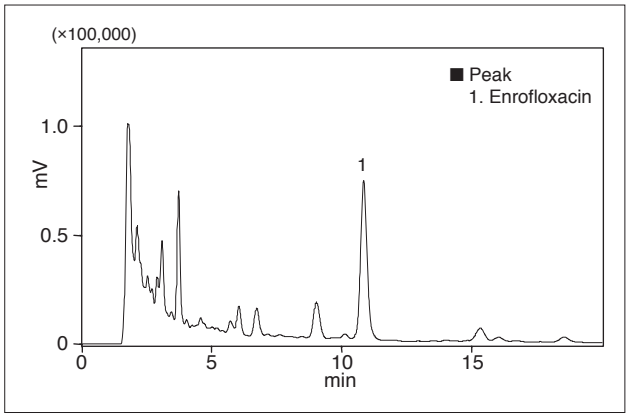


Fig. 11.6.5 Chromatogram of Broiled Eels Spiked with 50 µg/L Enrofloxacin

■Analysis of Broiled Eel by LC/MS

Fig. 11.6.6 shows a SIM chromatogram from analysis of broiled eel using LC/MS ESI in the positive mode. The broken line corresponds to the pretreated sample, and the solid line the sample spiked with 50 µg/L enrofloxacin (molecular weight 359). *m/z* 360 is a protonized molecule of enrofloxacin.

■Analytical Conditions for LC/MS

Column	: Shim-pack VP-ODS (150 mmL. × 2.0 mm I.D.)
Mobile Phase A	: 0.1 % Formic Acid-Water
Mobile Phase B	: 90 % Acetonitrile-Water containing 0.1 % Formic Acid
	Gradient Elution Method
Time Program	: B 0 % (0 min) → 100 % (20-25 min) → 0 % (25.01 - 35 min)
Flowrate	: 0.2 mL/min
Injection Volume	: 10 µL
Column Temp.	: 40 ºC
Ionization Method	: ESI-Positive
Applide Voltage	: 4.5 kV
Nebulizer Gas Flow	: 1.5 L/min
Drying Gas	: 0.15 MPa
CDL Temp.	: 200 ºC
BH Temp.	: 200 ºC
SIM	: *m/z* 360

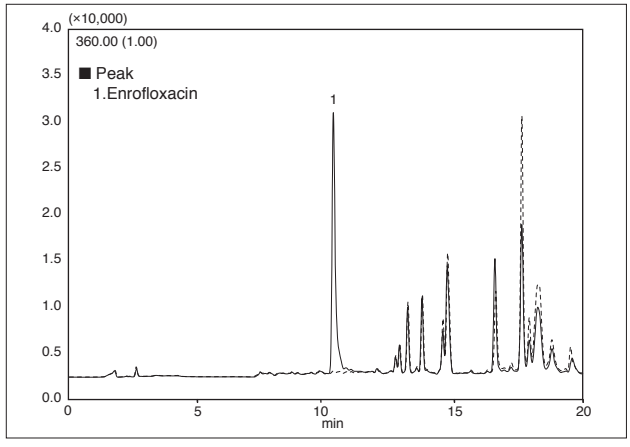


Fig. 11.6.6 SIM Chromatogram of Broiled Eels Spiked with 50 µg/L Enrofloxacin

11.7 Analysis of Carbadox and Quinoxaline-2-Carboxylic Acid in Pork - LC/MS

■Explanation

Carbadox (CDX) is a synthetic antibacterial agent used in pork which is relatively quickly metabolized to form quinoxaline-2-carboxylic acid (QCA) after passing through several intermediate metabolic stages. Here we introduce an example of simultaneous analysis of CDX and QCA. As CDX and QCA are highly polar compounds, analysis was conducted using an acidic mobile phase, and positive ion ESI method was used for ionization. Their mass spectra are shown in Fig. 11.7.1. Fig. 11.7.2 shows analyses of swine muscle spiked with CDX and QCA.

■Analytical Conditions

Column	: Phenomenex Gemini 5u C18 110A (150 mmL. × 2.0 mm I.D.)
Mobile Phase	: Water containing 0.1 % Formic Acid / Acetonitrile = 88 / 12
Flowrate	: 0.2 mL/min
Injection Volume	: 10 µL
Column Temp.	: 40 ˚C
Probe Voltage	: +4.5 kV (ESI-Positive Mode)
Nebulizing Gas Flow	: 1.5 L/min
Drying Gas Pressure	: 0.1 MPa
CDL Temp.	: 250 ˚C
BH Temp.	: 200 ˚C
CDL, Q-array Voltage	: using Default Values
Scan Range	: *m/z* 50 - 400
SIM	: *m/z* 263 (CDX), 175 (QCA)

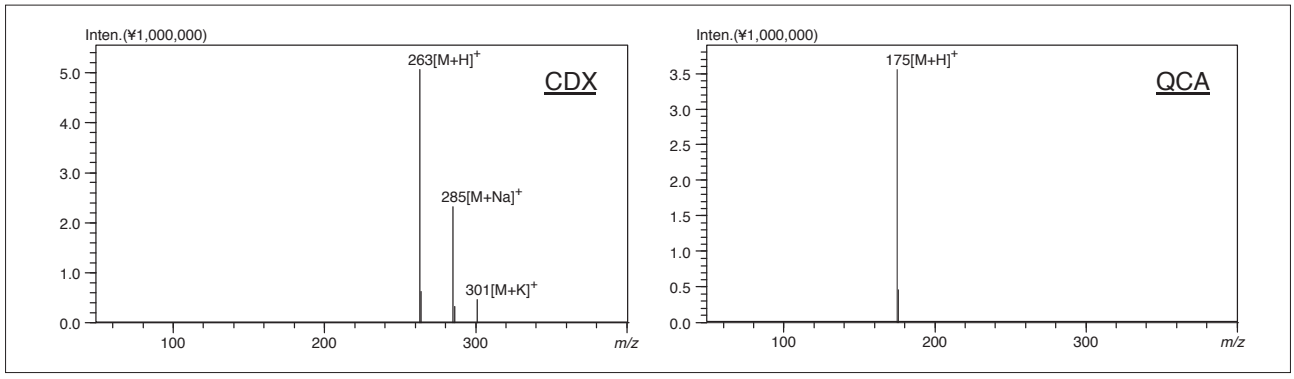


Fig. 11.7.1 Mass Spectra of Carbadox and Quinoxaline-2-carboxylic Acid

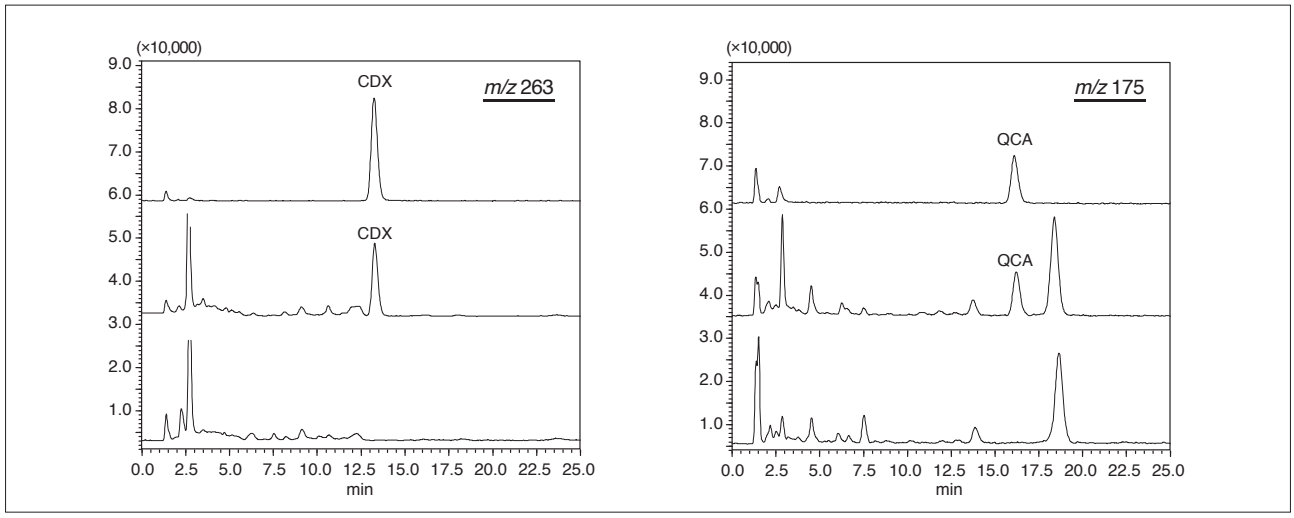


Fig. 11.7.2 SIM Chromatograms of (upper) Standard Solutions of Carbadox and Quinoxaline-2-carboxylic Acid (50 ng/mL each, equivalent to 10 ng/mL in swine muscle), (middle) Swine Muscle Extract Spiked with 10 ng/mL Each of Carbadox and Quinoxaline-2-carboxylic Acid, (lower) Swine Muscle Extract

11.8 Analysis of Tetracyclines - LC

■Explanation

Tetracycline, oxytetracycline and other tetracycline antibiotics are used as veterinary pharmaceuticals. Fig. 11.8.1 shows a chromatogram of a standard mixture of tetracycline and oxytetracycline (10 µg/L and 15 µg/L, respectively, dissolved in methanol, diluted with 1.36 % mono-potassium phosphate solution). The quantitation limit concentration determined based on the individual test method "sample solution preparation" procedure is 100 µg/L each.

■Analytical Conditions

Column	: Phenomenex Gemini-NX 5 µm C18 110 Å (150 mmL. × 4.6 mm I.D., 5 µm)
Mobile Phase	: Imidazole Buffer* / Methanol =17/3 (v/v)
	* Imidazole 68.08 g + EDTA·2Na 0.37 g +Magnesium acetate 10.72 g, pH 7.2 adjusted with acetic acid in 1000 mL water
Flowrate	: 0.8 mL/min
Column Temp.	: 30 ºC
Injection Volume	: 10 µL
Detection	: Fluorescence Detector RF-20Axs (Ex : 380 nm Em: 520 nm) Cell Temp. : 30 ºC

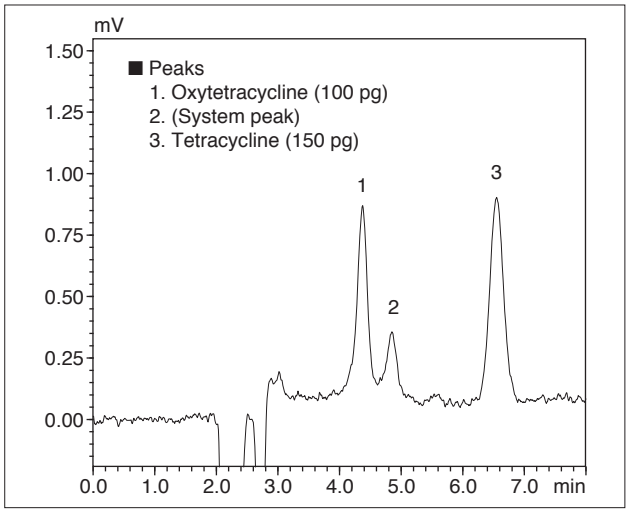


Fig. 11.8.1 Chromatogram of a Standard Mixture of Oxytetracycline (10 µg/L) and Tetracycline (15 µg/L)

■High Speed Analysis of Tetracyclines

Here, we show the example of high speed analysis of a standard mixture of 3 components of tetracyclines using the Prominence UFLC System. Fig. 11.8.2 shows a chromatogram of tetracycline, oxytetracycline and chlortetracycline.

■Sample Preparation

A 1 g/mL concentration solution of each standard sample was prepared using a mixed solution of methanol and 10 mmol/L oxalic acid aqueous solution (3:2 v/v).

■Analytical Conditions

Column	:Shim-pack XR-ODS (50 mmL. × 3.0 mm I.D.)
Mobile Phase	:A: 10 mmol/L Oxalic Acid aq. Solution
	B: 10 mmol/L Oxalic Acid aq. Solution /Acetonitrile =1/1 (v/v)
	Gradient Elution Method
Time Program	:B 18 % (0 min)→60 % (1.0 min) → 18 % (1.01-2.0 min)
Flowrate	:1.2 mL/min
Column Temp.	:40 ºC
Injection Volume	:5 µL
Detection	:UV Absorbance Detector at 360 nm (Ref. Correction 450 nm)
Cell	:Semi-micro Cell

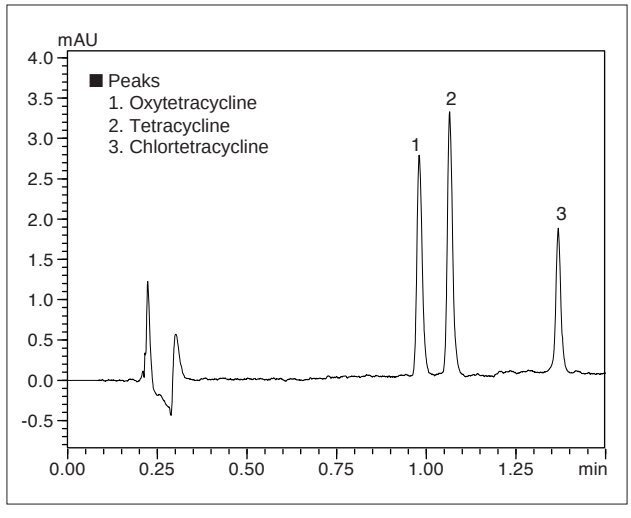


Fig. 11.8.2 Chromatogram of a Standard Mixture of Tetracycline, Oxytetracycline and Chlortetracycline

11.9 Simultaneous Analysis of Sulfa Drugs (1) - LC/MS

■Explanation

Sulfa drugs are synthetic antibacterial agents used primarily as feed additives and veterinary medicines for improved productivity of agricultural and fishery products. In the positive list system for food analysis which has been implemented in Japan since May 29, 2006, the criteria range for agricultural and fishery -products is set to 0.02 to 0.1 mg/kg, with a quantitation limit of 0.01 mg/kg. Until recently, testing was conducted using HPLC, however, LC/MS is now also being used for the analysis. Here we present an example of 9 sulfa drugs analyzed by LC/MS. In the case of sulfa drugs, the protonated molecule $[M+H]^+$ is detected as the base peak using electrospray ionization (ESI) in the positive ion mode. Fig. 11.9.1 shows the SIM chromatograms obtained from analysis of a standard solution of 9 sulfa drugs using the protonated molecules as SIM selection ions. The 9 compounds were eluted within 12 minutes, and were detected with excellent sensitivity. SDD (peak 3) has the same mass as the isomer SMPD (peak 4), so chromatographic separation of these 2 compounds is necessary. Using methanol as the mobile phase, the two compounds can be easily separated. The standard solution consisted of a commercial standard solution prepared for food analysis, and was diluted with an aqueous solution of 35 % methanol.

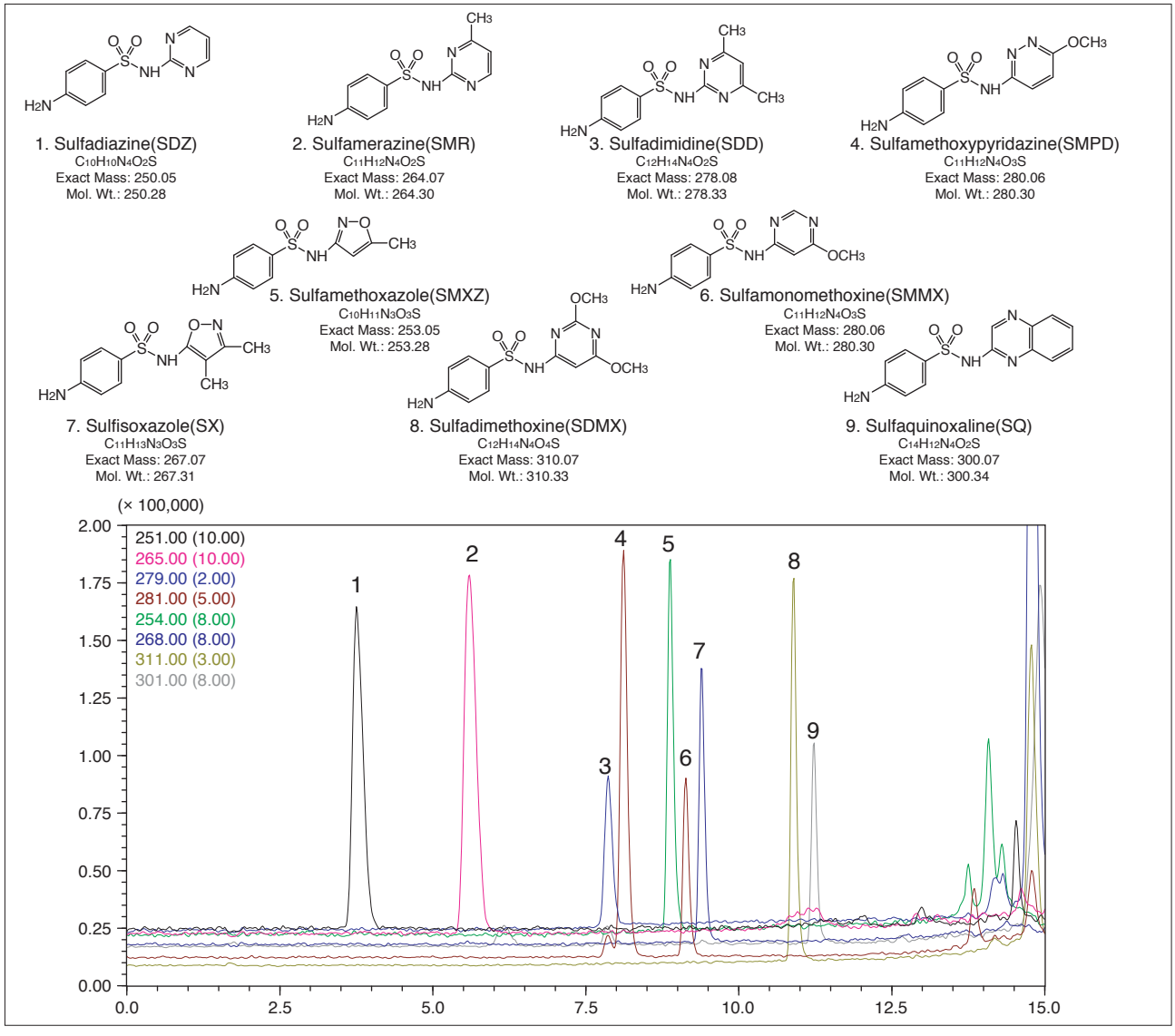


Fig. 11.9.1 SIM Chromatograms of Standard Solution (0.01 mg/L each)

11.9 Simultaneous Analysis of Sulfa Drugs (2) - LC/MS

Fig. 11.9.2 shows the calibration curves of 4 representative compounds. Excellent linearity is demonstrated in the range of 0.0005 to 0.05 mg/L, with a coefficient of determination -greater than 0.999. Similar calibration curves were also obtained for the other compounds. Fig. 11.9.3 shows the analytical results obtained from actual samples. Pretreatment of the swine fat was conducted according to the "HPLC Simultaneous Analysis of Agricultural Products Method Ⅱ (agricultural and fishery products)" of the positive list system test method. After the sample was passed through the ODS -column in the final purification process, a 40 % methanol fraction that was eluted from this ODS column was collected, then dissolved in 5 % aqueous methanol, and this sample solution was then spiked with the standard sulfa solution to obtain a final concentration 0.01 mg/L. The SIM chromatograms obtained from analysis of the sample not spiked with the sulfa drugs are shown in Fig. 11.9.3 (a), and those from the spiked swine extract are shown in Fig. 11.9.3 (b). Contaminant peaks are also present in the vicinity of the SMMX (6) and SQ (9) peaks, but these types of contaminants are considered to be common in fats, and do not affect the analysis.

■Analytical Conditions

Column	: Shim-pack FC-ODS (150 mmL. × 2.0 mm I.D.)
Mobile Phase A	: 5 mmol/L Ammonium Formate-Water with 0.1 % Formic Acid
Mobile Phase B	: Methanol
	Gradient Elution Method
Time Program	: B 20 % (0 min) → 95 % (10 min) → 20 % (10.01 min-25 min)
Flowrate	: 0.2 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 5 µL
Probe Voltage	: +4.5 kV (ESI-Positive Mode)
CDL Temp.	: 250 ºC
CDL Voltage	: C-Mode
Drying Gas Pressure	: 0.1 MPa
Nebulizing Gas Flow	: 1.5 L/min
BH Temp.	: 200 ºC
Drying Gas Flow	: 20 L/min
Q-array DC/RF Voltage	: Scan Mode

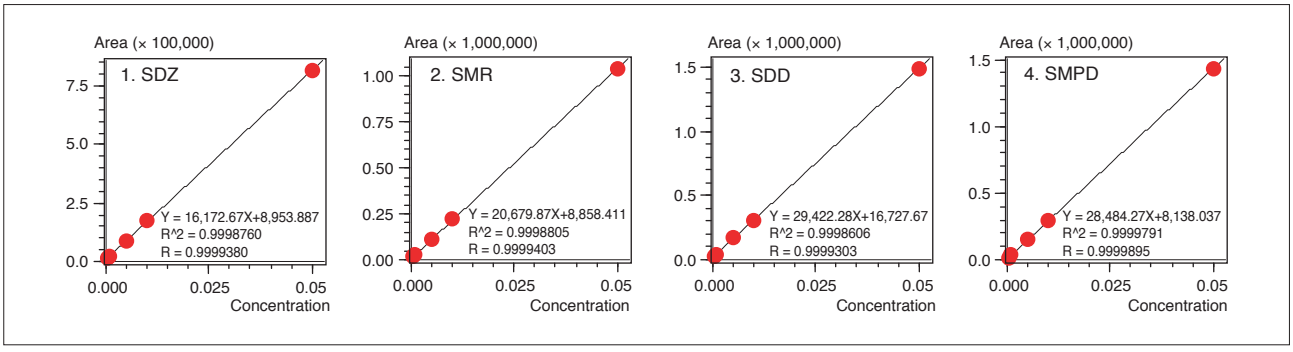


Fig. 11.9.2 Calibration Curves

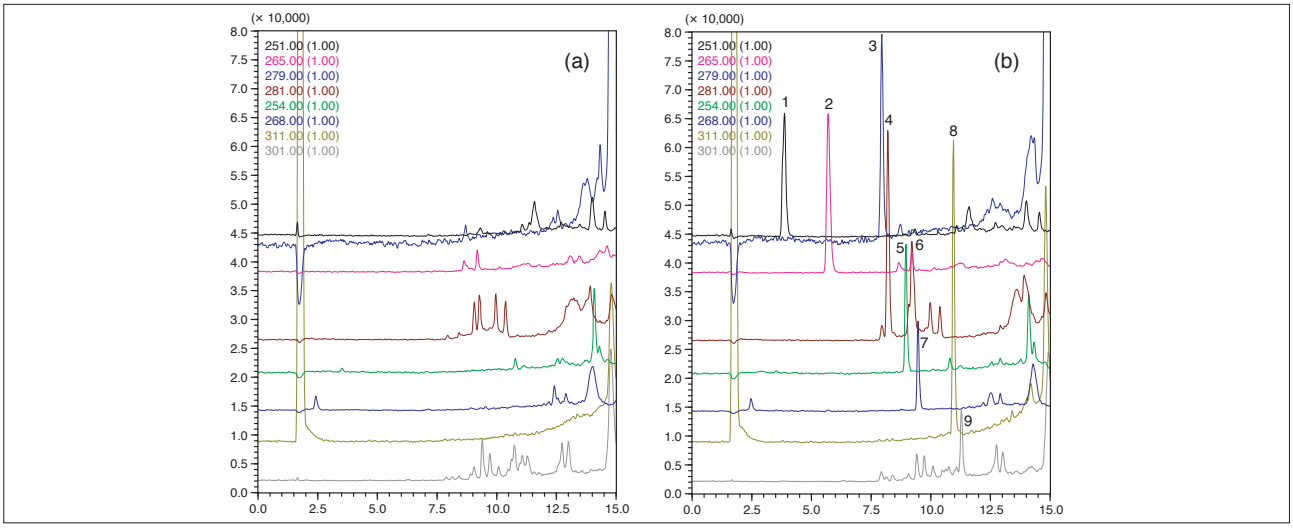


Fig. 11.9.3 (a) SIM Chromatograms of Swine Fat Extract
(b) SIM Chromatograms of Sulfa Drugs in Swine Fat Extract (each spiked at 0.01 mg/L)

11.10 Analysis of Malachite Green Using a Triple Quadrupole LC/MS/MS (1) - LC/MS/MS

■Explanation

Malachite green, besides being used as a dye in the textile and paper industries in Japan, is also used as a synthetic antibacterial drug to treat diseases such as water mold disease in aquarium fish. Due to concern related to its carcinogenicity and genotoxicity, not to mention the persistence of its metabolite, leucomalachite green, application of malachite green with aquaculture animals is prohibited under the Pharmaceutical Affairs Act. The United States in 1981, and the European Union and China in 2002 prohibited its use with all food-related items. However, due to its low price, effectiveness, and easy availability, cases of its detection in eel, salmon and other farmed fish continue to appear, resulting in strengthened worldwide monitoring. Here, we show the quantitative analysis of malachite green and leucomalachite green using the LCMS-8030. In addition, we report the results of spiked-recovery measurements conducted using a salmon extract solution as an actual sample.

■MRM Optimization and Quantitative Analysis

Optimization was conducted to determine the product ions (quantitation and reference ions) and collision energies for malachite green, malachite green-d5, leucomalachite green and leucomalachite green-d6. Fig. 11.10.1 and Fig. 11.10.2 show the respective product ion mass spectra and the calibration curves generated using the internal standard method. Excellent linearity was obtained over the range of 0.5-50 ng/mL.

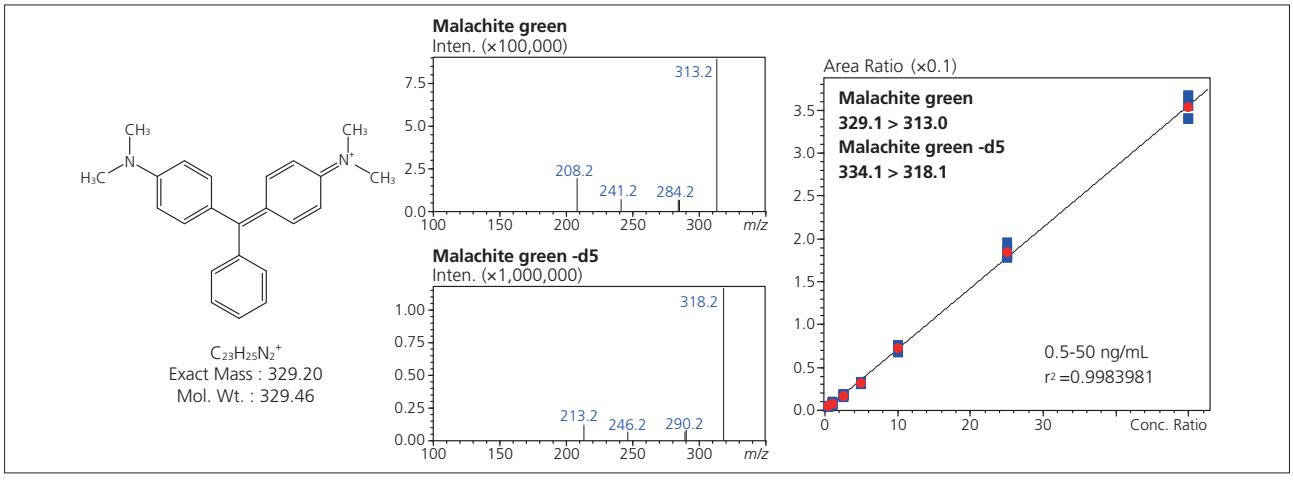


Fig. 11.10.1 Product Ion Mass Spectra and Calibration Curve for Malachite Green

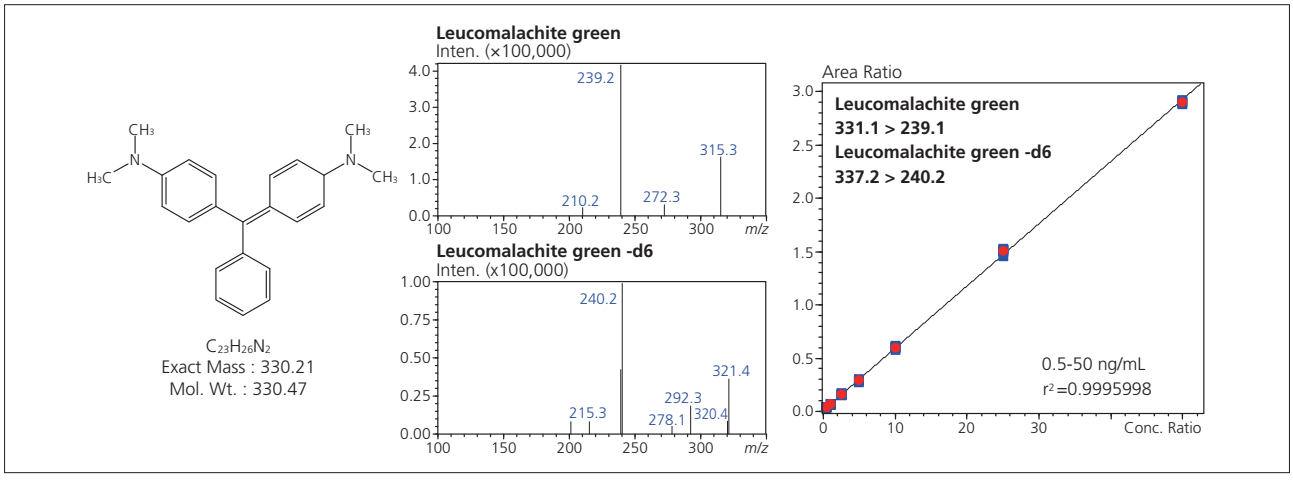


Fig. 11.10.2 Product Ion Mass Spectra and Calibration Curve for Leucomalachite Green

11.10 Analysis of Malachite Green Using a Triple Quadrupole LC/MS/MS (2) - LC/MS/MS

Salmon extracts were prepared according to the "Malachite Green Analytical Method" specified by Japan's Ministry of Health, Labour, and Welfare. Each extract was spiked with standard samples of malachite green and leucomalachite green at concentrations corresponding to 10 ng/mL and was then analyzed by LC/MS/MS. Fig. 11.10.3 shows the MRM chromatograms of the standard sample spiked salmon extract. Table 11.10.1 shows the peak area ratios of the standard sample and the salmon extract solution spiked with the standard (n=6), in addition to the respective rates of recovery. Excellent recovery was obtained with little variation, permitting quantitation at 10 ng/mL without any adverse effects from the matrix.

■Analytical Conditions

Column	: Shim-pack XR-ODS Ⅱ (75 mmL. × 2.0 mm I.D., 2.2 µm)
Mobile Phase A	: 10 mmol/L Ammonium Acetate - Water
Mobile Phase B	: Acetonitrile
	Gradient Elution Method
Time Program	: B 10 % (0 min) → 100 % (2–5 min) → 10 % (5.01–8 min)
Flowrate	: 0.2 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 2 µL
Probe Voltage	: +4.5 kV (ESI-Positive Mode)
Nebulizing Gas Flow	: 3.0 L/min
Drying Gas Flow	: 10 L/min
DL Temp.	: 250 ºC
BH Temp.	: 400 ºC
DL Voltage/ Q-array Voltage	: Default Values

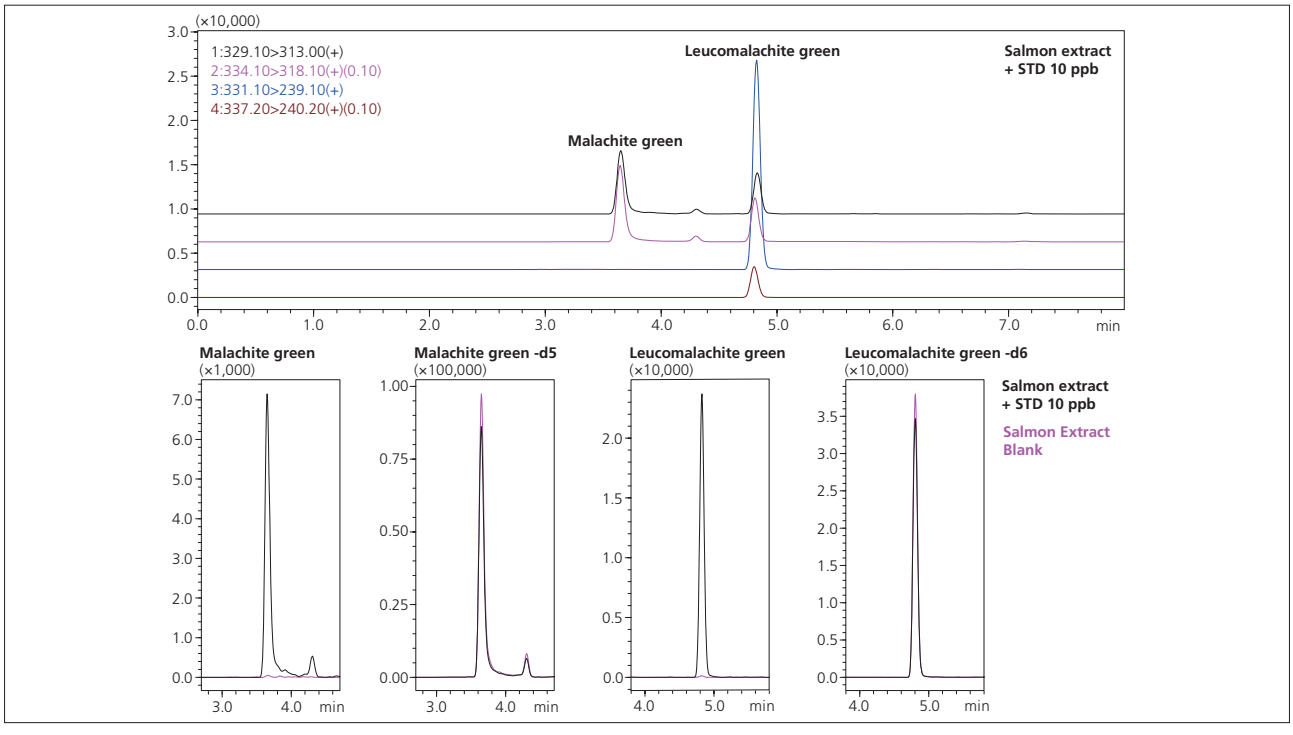


Fig. 11.10.3 MRM Chromatograms of Salmon Extract Blank and Salmon Extract Spiked with 10 ppb STD

Table 11.10.1 Recovery Ratio of Salmon Extract Spiked with 10 ppb STD

Malachite green

	Area ratio								Recovery (%)
	1	2	3	4	5	6	Average	%RSD	
STD 10 ppb	0.0750	0.0756	0.0732	0.0673	0.0699	0.0728	0.0723	4.39	
Sarmon + STD 10 ppb	0.0845	0.0851	0.0823	0.0847	0.0824	0.0837	0.0838	1.41	115.9

Leucomalachite green

	Area ratio								Recovery (%)
	1	2	3	4	5	6	Average	%RSD	
STD 10 ppb	0.6149	0.5795	0.5929	0.6105	0.6064	0.5985	0.6004	2.1611	
Sarmon + STD 10 ppb	0.6794	0.6732	0.6693	0.6931	0.6701	0.6689	0.6757	1.3938	112.5

11.11 Analysis of Isometamidium - LC

■Analysis of Isometamidium

An octyl silylated silica gel column was used as the analytical column and the substance was detected by absorptiometry. Since isometamidium in the solution state is less stable than other veterinary drugs, care must be taken during pretreatment.

■Analysis Conditions

Column	: Shim-pack CLC-C8(M) (150 mmL. × 4.6 mm I.D.)
Mobile Phase	: A: 30 mmol/L Citrate Buffer containing 5 mmol/L Sodium 1-Heptanesulfonic Acid B: Acetonitrile A/B = 7/3(v/v)
Flowrate	: 1.2 mL/min
Column Temp.	: 40 ˚C
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M10A*VP* at 380 nm

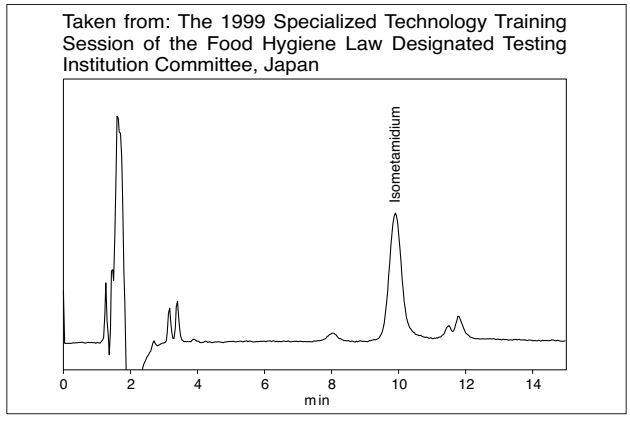


Fig. 11.11.1 Bovine Liver Extract (0.5 ppm isometamidium added)

11.12 Analysis of Triclabendazole - LC

■Analysis of Triclabendazole

When triclabendazole is metabolized within the body, the 2-methylthio group is converted into methylsulfinyl and additionally into methylsulfonyl groups. In the analysis of triclabendazole, triclabendazole and these metabolites oxidized with hydrogen peroxide are detected by absorptiometry. The oxidation is performed using a pre-column reaction.

■Analysys Conditions

Column	: Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase	: A: 25 mmol/L Sodium Dihydrogen Phosphate Buffer B: Acetonitrile A/B= 1/1(v/v)
Flowrate	: 1.0 mL/min
Column Temp.	: 40 ˚C
Detection	: UV Absorbance Detector at 295 nm (Precolumn Derivatization Method)

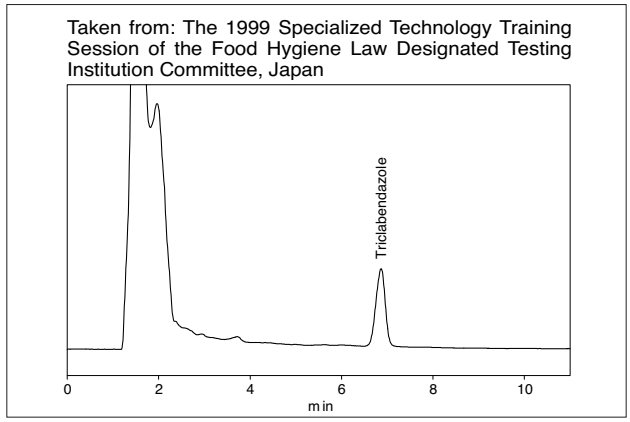


Fig. 11.12.1 Bovine Liver Extract (0.3 ppm triclabendazole added)

11.13 Analysis of Ivermectin and Moxidectin - LC

■Analysis of Ivermectin and Moxidectin

Ivermectin and moxidectin were detected by florescence after the pre-column reaction with a fluorescence derivatization reagent.

■Analysis Conditions

Column	: Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)
Mobile Phase	: Water/ Methanol = 3/ 97(v/v)
Flowrate	: 1.0 mL/min
Column Temp.	: 40 ˚C
Detection	: Fluorescence Detector (Ex: 360 nm, Em: 460 nm) (Precolumn Derivatization Method)

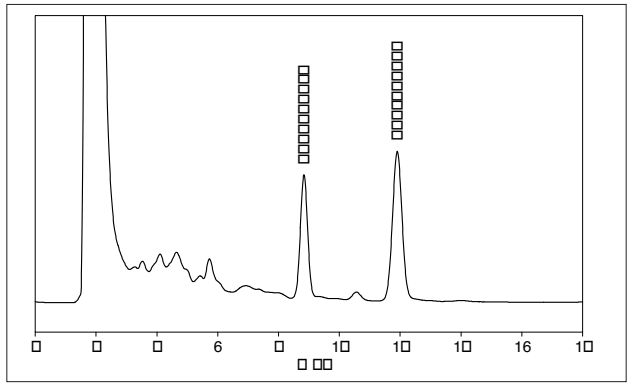


Fig. 11.13.1 Bovine Muscle Extract (0.04 ppm ivermectin and 0.02 ppm oxidectin added)

11.14 Analysis of Antiparasitic Agents (1) - LC/MS

■Explanation

Veterinary pharmaceuticals including antibiotics and hormones are used to prevent disease in livestock, promote growth, and enhance the feed efficiency. Antiparasitic agents are also widely used to eliminate parasites from the alimentary canal. Residual standards are being established for antiparasitic agents as residual levels in meat present similar health problems to antibiotics and hormones.

The four components used for this test were 5-hydroxythiabendazole, thiabendazole, flubendazole, and albendazole. Their structures are shown in Fig. 11.14.1. A residual standard is set for each of these components in food. HPLC is prescribed for the analysis of these components but LC/MS permits analysis with extremely high selectivity and sensitivity.

Fig. 11.14.2 shows the LC/MS analysis results for the four antiparasitic agents. Each component could be positively identified using mass chromatography at the mass number of the protonated molecule of each component. Fig. 11.14.3 shows their mass spectra. Selected ion monitoring (SIM) permits highly sensitive analysis. Fig. 11.14.4 shows the calibration curves in the range from 10 to 1000 ppb. Each curve shows good linearity.

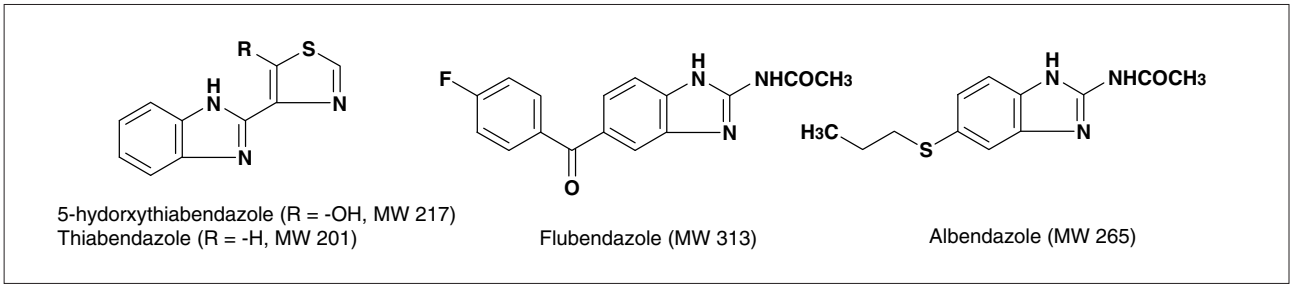


Fig. 11.14.1 Structures of Antiparasitic Agents

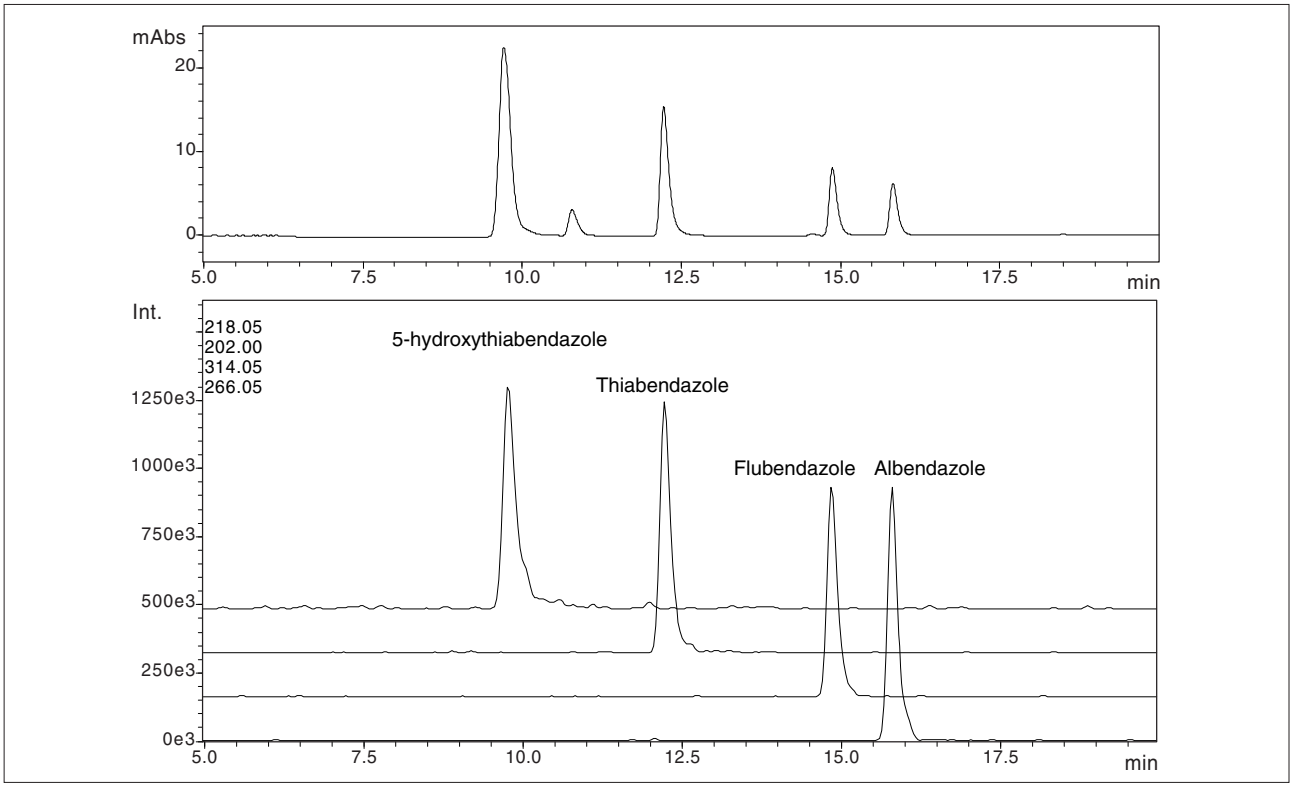


Fig. 11.14.2 UV Absorption (305nm) and Mass Chromatograms of Antiparasitic Agents

11.14 Analysis of Antiparasitic Agents (2) - LC/MS

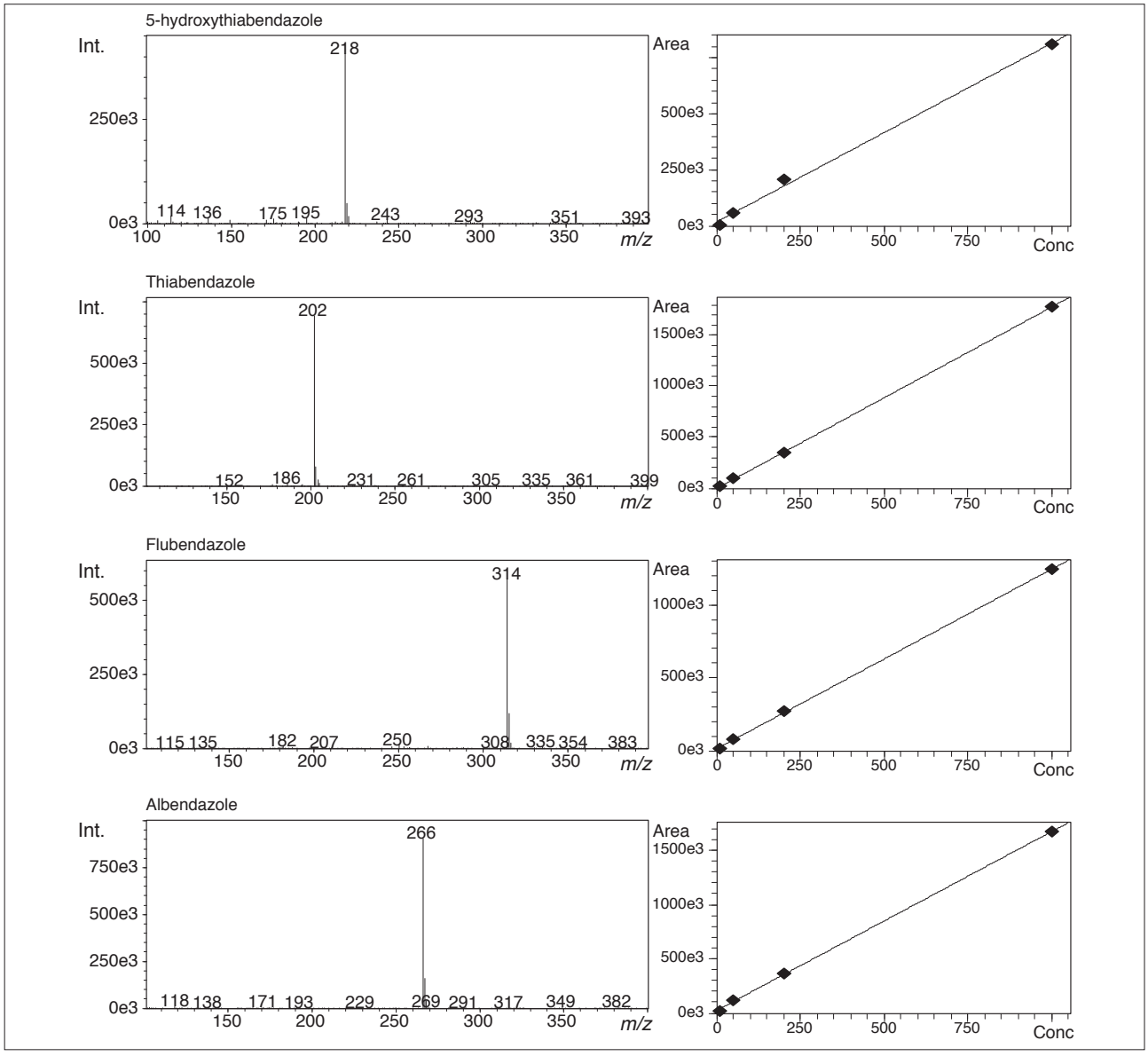


Fig. 11.14.3 Mass Spectra of Antiparasitic Agents

Fig. 11.14.4 Calibration Curves (10-1000 ppb)

■Analytical Conditions

Column	: Inertsil ODS-2 (150 mmL. × 2.0 mm I.D.)
Mobile Phase A	: 5 mmol/L Acetic Acid-Ammonium Acetate Buffer
Mobile Phase B	: Acetonitrile
Gradient Program	: B 0 % (0 min) → 100 % (20 min)
Flowrate	: 0.2 mL/min
Column Temp.	: 40 ˚C
Probe Voltage	: +4.5 kV (ESI-Positive Mode)
Nebulizing Gas Flow	: 4.5 L/min
CDL Voltage	: -40 V (0 - 14 min), -50 V (14.01 - 25 min)
DEFs Voltage	: +40 V (0 - 14 min), +45 V (14.01 - 25 min)
Scan Range	: *m/z* 100 - 400

11.15 Analysis of Hormone Agents (1) - LC/MS

■Explanation

We consume fish and meat as part of our normal daily diets and healthy human life would be impossible without nutrition from such foodstuffs. Consequently not only a stable production and supply but safety of farm products, meat, and fish are demanded. Many agricultural chemicals and veterinary pharmaceuticals are currently used to increase productivity, but their residues in food are a problem.

This report describes an analysis example of hormones used for livestock. Hormones are used to promote growth in livestock and their safety is investigated in the same way as antibiotics and antiparasitics. The residual regulation values are established according to the level harmless to humans.

The structure of the hormones used in this analysis are shown in Fig. 11.15.1. Atmospheric-pressure chemical ionization (APCI) was used for the ionization in LC/MS. Figs. 11.15.2 and 11.15.3 show their mass chromatograms and mass spectra. α-and β-trenbolone exhibit a mass spectrum with the protonated molecule as the base peak, while zeranol was detected with the dehydrated ion of the protonated molecule as the base peak.

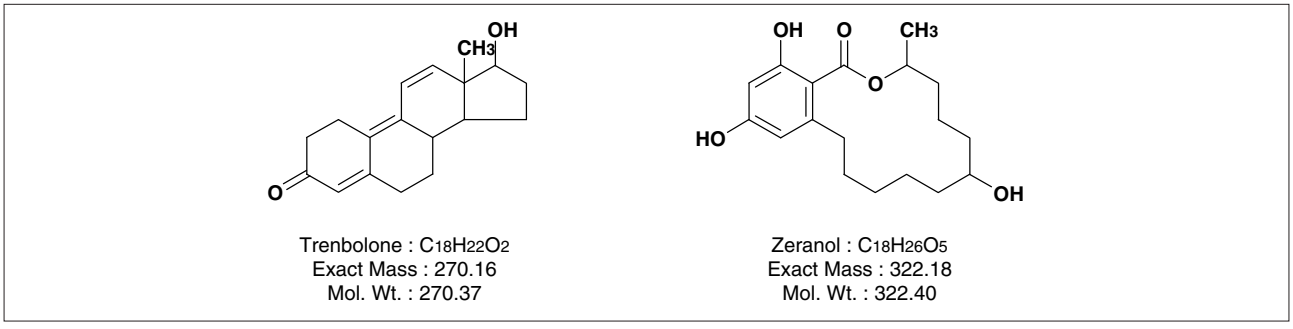


Fig. 11.15.1 Structures of Trenbolone and Zeranol

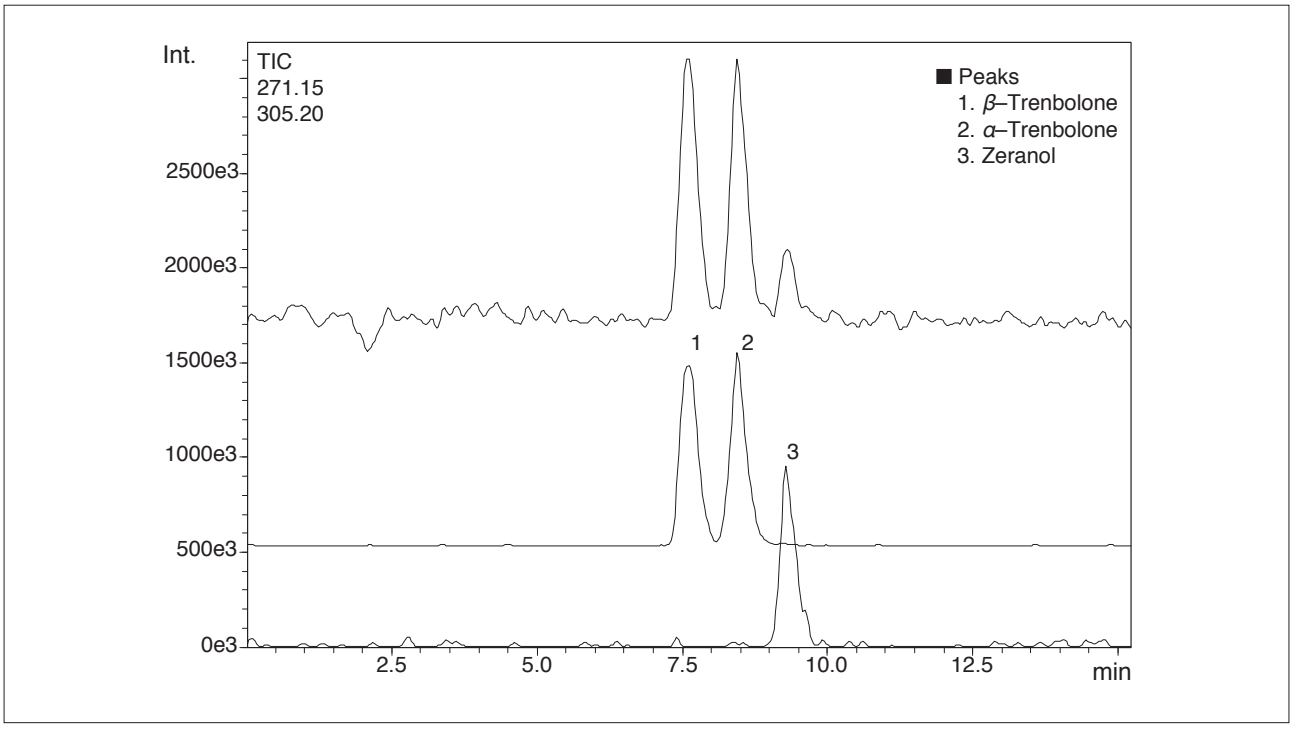


Fig. 11.15.2 Mass Chromatograms of Hormone Agents

11.15 Analysis of Hormone Agents (2) - LC/MS

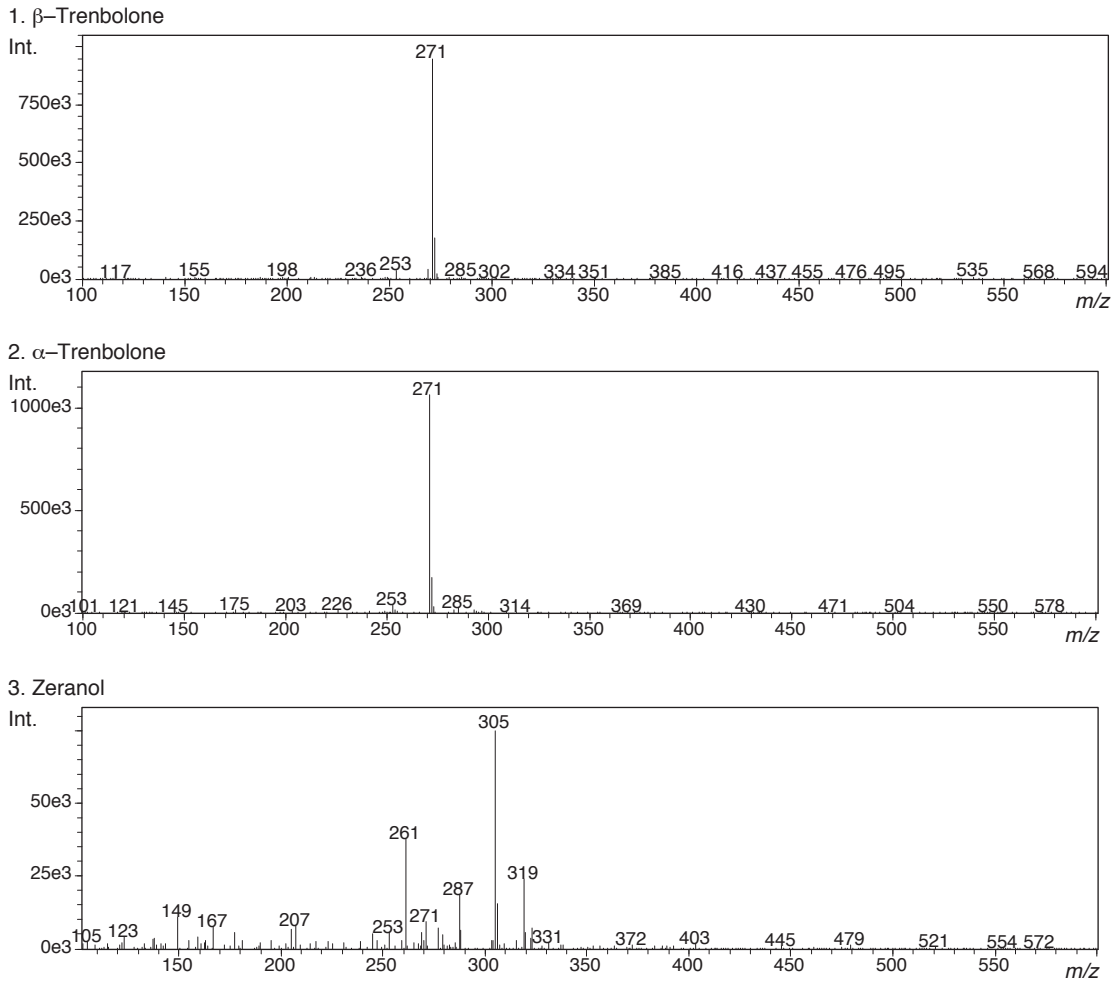


Fig. 11.15.3 Mass Spectra of Hormone Agents

■Analytical Conditions

Column	: STR ODS-II (150 mm L. × 2.0 mm I.D.)
Mobile Phase	: 60 % Methanol - Water containing 0.3 % Acetic Acid
Flowrate	: 0.2 mL/min
Column Temp.	: 40 ˚C
Probe Voltage	: +4.5 kV (APCI-Positive Mode)
CDL Temp.	: 230 ˚C
Probe Temp.	: 400 ˚C
Nebulizing Gas Flow	: 2.5 L/min
CDL Voltage	: -30 V
DEFs Voltage	: +47 V
Scan Range	: *m/z* 100 - 500

11.16 Analysis of Canthaxanthin and Astaxanthin - LC

■Explanation

Canthaxanthin and astaxanthin are carotenoid pigments that are specified as feed additives to enforce the effect of coloring, for example, for farmed fish. Their usage targets and quantities are regulated. As to canthaxanthin, residue limits related to dairy and marine food were established (applied since February 1st, 2005) and at the same time the test method was notified in Japanese Food Safety Bulletin 1126002. A simultaneous analysis of canthaxanthin and astaxanthin using the Prominence Photodiode Array UV-VIS Detector SPD-M20A will be introduced here. Fig. 11.16.1 shows the structures of canthaxanthin and astaxanthin. Fig. 11.16.2 shows the chromatogram of a mixture of canthaxanthin and astaxanthin standard solutions (20 mg/L) in accordance with the canthaxanthin test method notified in Food Safety Bulletin 1126002.

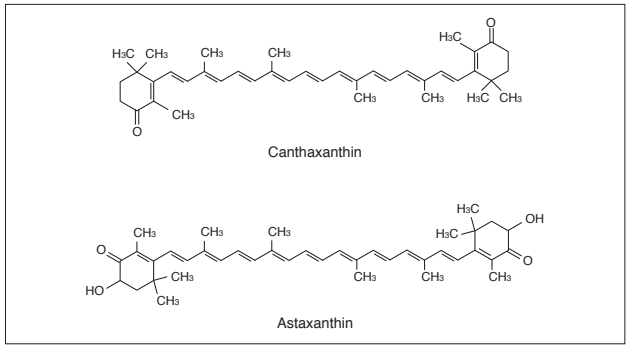


Fig. 11.16.1 Structures of Canthaxanthin and Astaxanthin

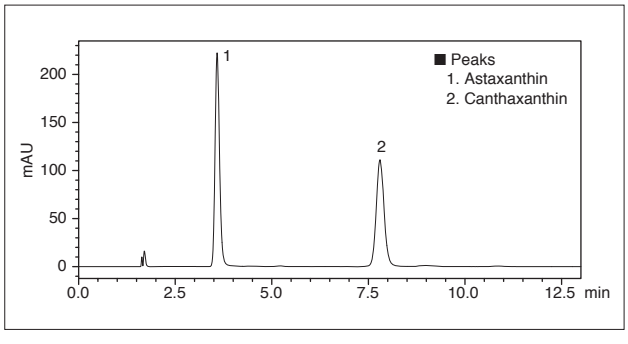


Fig. 11.16.2 Chromatogram of a Standard Mixture of Canthaxanthin and Astaxanthin (20 mg/L each, 10 µL inj.)

■Analytical Conditions

Column	: Shim-pack VP-ODS (150 mmL. × 4.6 mm I.D.)
Guard Column	: Shim-pack GVP-ODS (10 mmL. × 4.6 mm I.D.)
Mobile Phase	: 0.05 % Trifluoracetic Acid / Methanol = 3/97 (v/v)
Flowrate	: 1.2 mL/min
Column Temp.	: 40 ºC
Injection Volume	: 10 µL
Detection	: Photodiode Array UV-VIS Absorbance Detector SPD-M20A at 475 nm

■Repeatability at Low Concentration

Fig. 11.16.3 shows the chromatogram obtained by injecting 10 µL of a mixture of canthaxanthin and astaxanthin standard solutions (0.05 mg/L each) (equivalent to 0.1 mg/kg in actual sample). Table 11.16.1 shows the results of six repeated analyses of the same standard solution and the verified repeatability of peak area values. Favorable results were obtained for both canthaxanthin and astaxanthin.

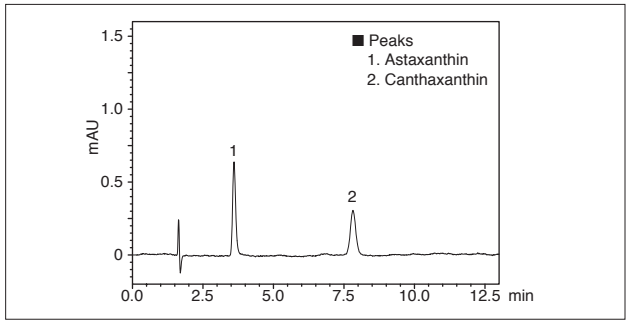


Fig. 11.16.3 Chromatogram of a Standard Mixture of Canthaxanthin and Astaxanthin (0.05 mg/L, 10 µL inj.)

Table 11.16.1 Repeatability of Peak Area

	Peak Area	
	Canthaxanthin	Astaxanthin
1st	3980	4904
2nd	4080	4775
3rd	4113	5052
4th	4006	5082
5th	3909	4932
6th	4064	4943
AVE	4025	4948
CV (%)	1.86	2.23

(0.05 mg/L, 10 µL inj.)

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