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C190-E133

RF-20A Fluorescence Detector Basics and Applications

Technical Report vol.36

1. Basics of Fluorescence Detection Technique

When light of a specific wavelength (excitation wavelength) is irradiated onto a substance, that substance emits light that usually has a longer wavelength (emission wavelength), and is referred to as fluorescence. Unlike an ultraviolet (UV) detector, which measures the amount of light absorbed by a substance at a specific wavelength, the light that is emitted from the sample due to fluorescence is split into a spectrum by a monochromator, and the intensity of light at a specific emission wavelength is measured. Fluorescence detection has the following features:

1) Selectivity is high because measurement is conducted using specific excitation and emission wavelengths specific to target substance.
2) Fluorescent substances can be detected with high sensitivity.
3) Sensitivity and selectivity can be improved by using the derivatization techniques.

Fluorescence detection is generally used for analysis when sensitivity and selectivity are required, especially when the analyte has little or no UV absorbance and can be derivatized to produce fluorescence. Although LC/MS is increasingly being used for high-sensitivity analysis, fluorescence detection by HPLC is often the officially regulated method. In addition, compared to LC/MS, fluorescence detection is more economical and maintenance is easier. Here we describe the fundamental principles of the fluorescence detector, and present actual examples of analysis using the Prominence RF-20Axs to illustrate the innovations that provide stable and high sensitivity analysis.

1-2. Principle of Fluorescence

Fluorescent substances conform to the following principle of fluorescence. The substance enters a state of excitation when it is irradiated with excitation light. Due to the instability of this excited state, the substance quickly returns to its original state (ground state). The energy emitted at this time is referred to as fluorescence, and a fluorescence detector measures the intensity of the emitted fluorescence. Since excitation is conducted at a specific wavelength, and measurement of the fluorescence is conducted at a specific wavelength, selectivity is higher than with absorbance and other types of detectors (Fig. 1).

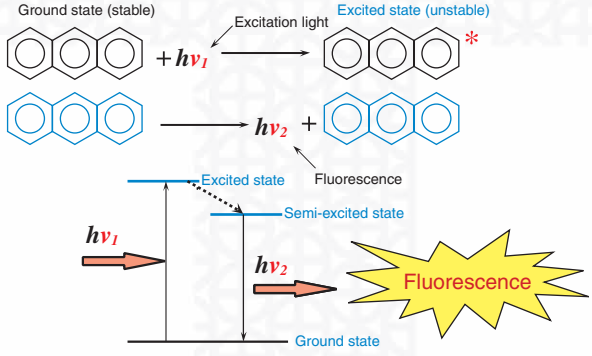


Fig. 1 Mechanism of Fluorescence from a Substance

1-3. Characteristics of Fluorescence Emission

Fluorescence generates a spectrum like that shown in Fig. 2. It is important to note that upon irradiation with the excitation wavelength, excitation scattered light and Raman scattered light will always occur in the vicinity of the excitation wavelength. Therefore, if the excitation wavelength and fluorescence (emission) wavelength are too close to one another, it may be difficult to obtain a valid chromatogram. In addition, background noise will increase if fluorescence is generated by the solvent. For this reason, it is important to use HPLC-grade water and solvent for the mobile phase.

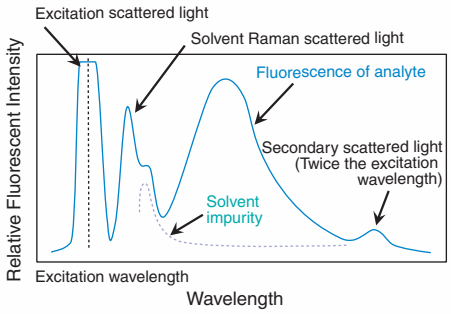


Fig. 2 Characteristics of Fluorescence Spectrum

1-4. Sensitivity and Selectivity of Fluorescence Detectors

As explained in the principle of fluorescence, two settings are made in the fluorescence detector: the excitation wavelength and the emission wavelength. This is what allows the fluorescence detector to provide greater selectivity than other detectors. The data below are chromatograms obtained from analysis of vitamin B_2 in a food product. In the chromatogram of Fig. 3 obtained using absorbance detection, the contaminant peaks overlap the analyte peak due to low selectivity. Furthermore, the weak UV absorbance intensity of vitamin B_2 makes quantitation difficult. On the other hand, as shown in Fig. 4, a fluorescence detector provides highly selective detection in vitamin B_2 analysis, because contaminants in the sample are not detected at the excitation and fluorescence wavelengths used for vitamin B_2 detection. In addition, since the background can be suppressed to a low level by using a non-fluorescent mobile phase, detection with high selectivity as well as high sensitivity is possible. The typical levels of sensitivity of various detection methods are shown in Table 1.

Table 1 Typical Levels of Sensitivity of Various Detection Methods

Detection Method	Typical Sensitivity
Absorbance	ng
Fluorescence	pg
Differential refractive index	μg
Electrochemical	pg
Electroconductivity	ng

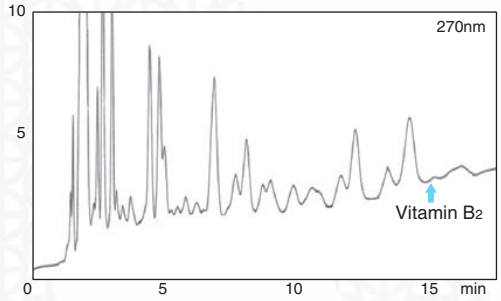


Fig. 3 Absorbance Detection Technique

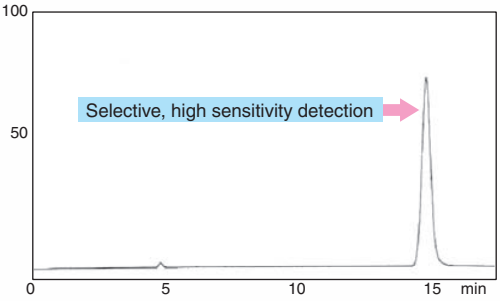


Fig. 4 Fluorescence Detection Technique

2. Factors that Influence Fluorescence Detection

In analysis using fluorescence detection, the expected peak intensity or repeatability may not be obtained due to specific environmental influences. One of these is due to dissolved oxygen in the mobile phase which has the effect of diminishing peak intensity. Another is due to temperature, which greatly affects fluorescent intensity. Ambient temperature that is either high or unstable can cause decreased peak intensity or poor area repeatability. Here we describe actual examples of these effects.

2-1. Effect of Dissolved Oxygen in Mobile Phase

When the mobile phase contains dissolved oxygen, fluorescent intensity decreases. This is referred to as "quenching." In particular, the intensity may change due to unstable degassing of the mobile phase or prolonged analysis using only offline degassing, either of which could adversely affect peak area repeatability (Fig. 5). Stable degassing can be maintained using the DGU-20A3/A5 on-line degasser in addition to offline ultrasonic degassing to allow analyses that are highly repeatable with excellent sensitivity.

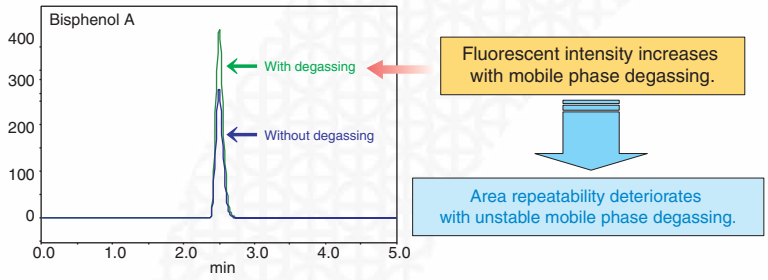


Fig. 5 Change in Peak Intensity due to Dissolved Oxygen in Mobile Phase

2-2. Affect of Ambient Temperature

Intermolecular collisions increase as the ambient temperature rises, causing a loss of potential energy that could be used for fluorescence. As a result, fluorescent intensity weakens due to the rise in temperature.
There are examples of intensity fluctuation as high as 5% with just 1°C fluctuations in ambient temperature. Therefore, by maintaining the temperature of the detector cell at a constant and cooled temperature, sensitivity and peak area repeatability can be ensured.

2-3. Examples of Temperature Influence in Amino Acid Analysis

We conducted actual amino acid analysis using detector cell temperatures of 25°C, 30°C, 35°C, and 40°C. The chromatogram below (Fig. 6) shows the overlaid results at each temperature. Fig. 7 plots the relative area values (%) for each temperature assuming area values of 100% at 25°C. The peak area values clearly decrease as the detector temperature increases. By increasing cell temperature from 25°C to 40°C, the fluorescent intensities of some of the amino acids decrease by 25% or more. Even when comparing the results at 25°C and 30°C, which is closer to an actual analysis environment, there are substances like Ala which show a decrease in area value of 10% or more, emphasizing the importance of maintaining a constant detector cell temperature from the standpoints of both sensitivity and repeatability.

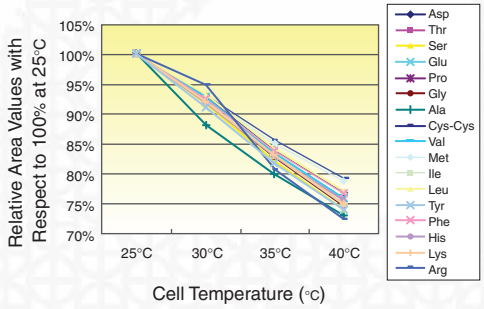


Fig. 7 Relative Area Values (%) at Each Temperature Assuming 100% at 25°C

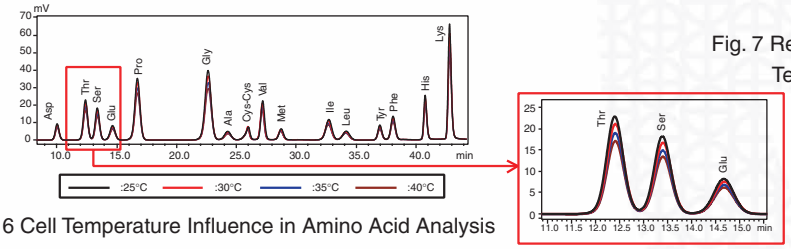


Fig. 6 Cell Temperature Influence in Amino Acid Analysis

3. Features of RF-20Axs Fluorescence Detector

The Prominence RF-20Axs is equipped with a temperature-controlled cell to ensure stable analysis even if the ambient temperature fluctuates. Further, temperature control (with a cooling function) is provided not only for the detector cell, but for the photomultiplier tube (PMT) as well. Thus, even if the ambient temperature fluctuates widely, the detector is maintained at a constant, near-ambient temperature which provides excellent repeatability without loss of sensitivity. In addition to the improved sensitivity and stability provided with this temperature control in the vicinity of ambient temperature, even further improved sensitivity is achieved with the newly designed optical system and detector cell in the RF-20Axs. As a result, the quantity of light through the flow cell is about 100 times that compared with the previous RF-10AxL model, offering a water Raman S/N ratio of at least 2000 for the RF-20Axs. These features are what make this detector a powerful tool, especially when trace-level detection is required.

3-1. Ultra-High Sensitivity Analysis of Anthracene

Fig. 8 shows the results of trace-level analysis of anthracene. An S/N ratio of 21.5 was achieved with an injection of 10.48 fg anthracene (RF-20Axs). This is equivalent to a limit of detection of about 1.5 fg (S/N ratio = 3), demonstrating excellent sensitivity.

Table 2 Analytical Conditions for Anthracene Analysis

Mobile Phase	Water / acetonitrile = 30 / 70 (v/v)
Flowrate	0.6 mL/min
Column	Shim-pack XR-ODS 50 mmL. x 2.0 mmI.D., 2.2 µm
Temperature	40°C
Detection	Excitation wavelength: 250 nm, emission wavelength: 400 nm

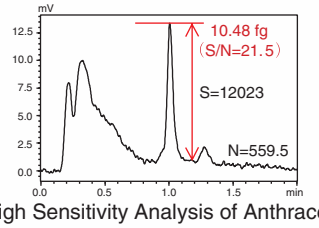


Fig. 8 Ultra-High Sensitivity Analysis of Anthracene Using RF-20Axs

3-2. Temperature Control (with Cooling) for Highly Stable Analysis

Fig. 9 shows chromatograms that illustrate the effectiveness of cell temperature control when the ambient temperature is fluctuating. By controlling the temperature of the cell, peak intensity barely changes even when the ambient temperature fluctuates. Table 4 shows just how much change occurs in the peak area value at 30°C after the temperature is increased from 25°C relative to a standard area value at 25°C. The peak area repeatability values when the temperature is increased from 25°C to 30°C are also shown. As indicated in the results, excellent repeatability is achieved with the RF-20Axs and its cell temperature control, showing no effect due to the change in ambient temperature.

Table 3 Analytical Conditions for Acridine Analysis

Mobile Phase	Water / acetonitrile = 30 / 70 (v/v)
Flowrate	0.6 mL/min
Column	Shim-pack XR-ODS 50 mmL. x 2.0 mmI.D., 2.2 µm
Temperature	40°C
Detection	Excitation wavelength: 250 nm, emission wavelength: 400 nm
Sample	Acridine

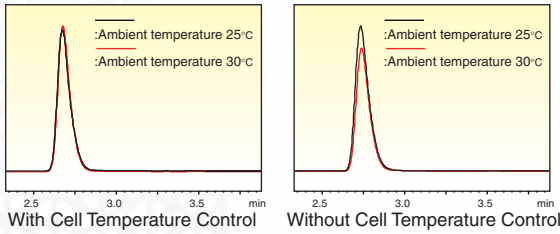


Table 4 Effectiveness of Cell Temperature Control During Ambient Temperature Fluctuation

	Peak Area Change (%)	%RSD During Temperature Fluctuation
With Cell Temperature Control	0.64	0.29
Without Cell Temperature Control	-17.45	6.30

Fig. 9 Comparison of Chromatograms With and Without Cell Temperature Control

- Peak area repeatability improves with temperature control.
- Intensity increases at lower detector temperature.
- Temperature control near ambient temperature using cooling function is effective.

4. Support for Ultra Fast Analysis

Fast response is required to keep up with the sharp peaks obtained in ultra fast analysis. The 10 ms (100 Hz) response of the RF-20A/20Axs permits ultra fast analysis without any loss of resolution. Also, to prevent diminished peak separation due to peak diffusion outside the column during ultra fast analysis, a detector cell having a small capacity is used. The user must be aware of the fact that in fluorescence detection, sensitivity depends on the capacity of the cell. The ultra-high sensitivity capability built into the RF-20Axs permits high sensitivity analysis even during ultra fast analysis with a semi-micro cell. With the RF-20Axs, one unit is all that is required to obtain high performance in all types of analysis, from conventional to ultra fast analysis. Fig. 10 shows an example of the migration of tocopherol analysis from conventional to ultra fast analysis.

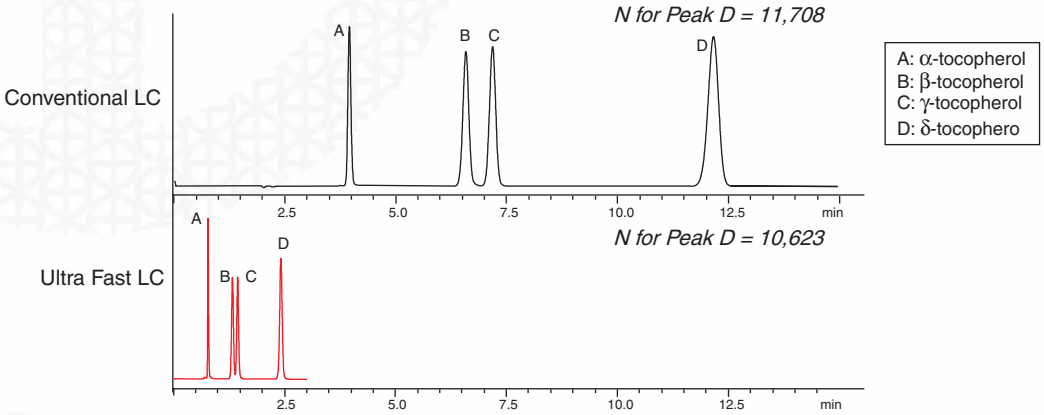


Fig. 10 Migration of Tocopherol Analysis from Conventional LC to Ultra Fast LC

Table 5 Analytical Conditions for Conventional LC

Mobile Phase	Hexane / 2-propanol = 100 / 0.5 (v/v)
Flowrate	1.0 mL/min
Column	Shim-pack CLC-SIL (M) 150 mmL. x 4.6 mmI.D., 5 µm
Temperature	30°C
Detection	Excitation wavelength: 298 nm, emission wavelength: 325 nm

Table 6 Analytical Conditions for Ultra Fast LC

Mobile Phase	Hexane / 2-propanol = 100 / 0.5 (v/v)
Flowrate	1.2 mL/min
Column	Shim-pack XR-SIL 75 mmL. x 3 mmI.D., 2.2 µm
Temperature	30°C
Detection	Excitation wavelength: 298 nm, emission wavelength: 325 nm

5. Application Examples

5-1. High-Sensitivity Analysis of Enrofloxacin

Enrofloxacin is one of the new quinolone synthetic anti-bacterial agents (Fig. 11). As a veterinary pharmaceutical product, it is used to treat bovine and swine pneumonia, and as a preventative and remedy for E. coli diarrheal syndrome. In Japan, it is not permitted for use with farm-raised fish, and all lots of specific imported products are designated for inspection. Here we conducted high-sensitivity analysis of enrofloxacin using the officially specified analytical method, and we also measured the limit of detection (LOD). The results of analysis using the RF-20Axs indicate an LOD of 0.05 µg/L, as shown in Fig. 12, demonstrating that high-sensitivity detection is possible. In addition, Fig.13 shows the calibration curve generated using concentrations from 0.05 µg/L to 1000 µg/L. This clearly shows the wide dynamic range, achieving high repeatability and linearity from ultra-trace levels to high concentrations.

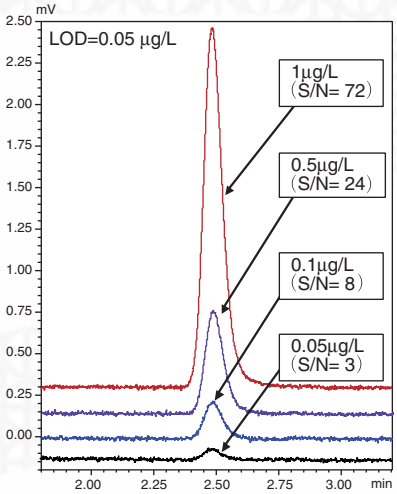


Fig. 12 Chromatograms of Enrofloxacin at Different Concentratio

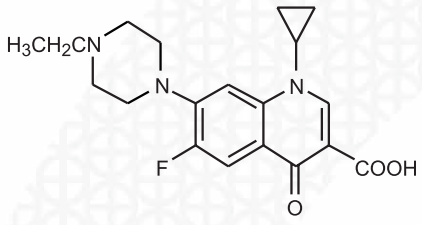
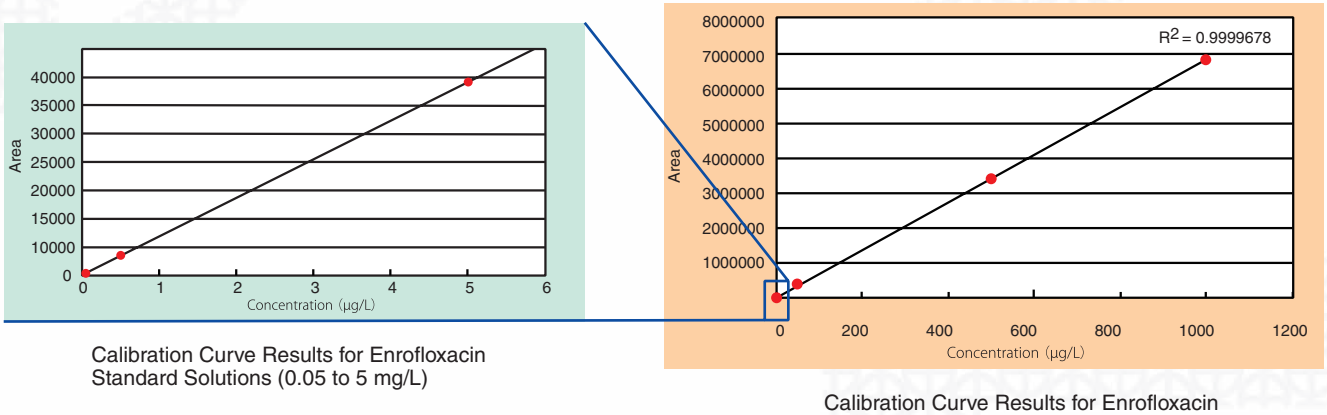


Fig. 11 Structural Formula of Enrofloxacin

Table 7 Analytical Conditions for Enrofloxacin Analysis

Mobile Phase	McIlvaine buffer solution (pH 3.0) / acetonitrile = 85 / 15 (v/v)
Flowrate	0.5 mL/min
Column	Shim-pack XR-ODS 75 mmL. x 2.0 mmI.D., 2.2 µm
Temperature	40°C
Detection	Excitation wavelength: 285 nm, emission wavelength: 460 nm
Injection Volume	2 µL



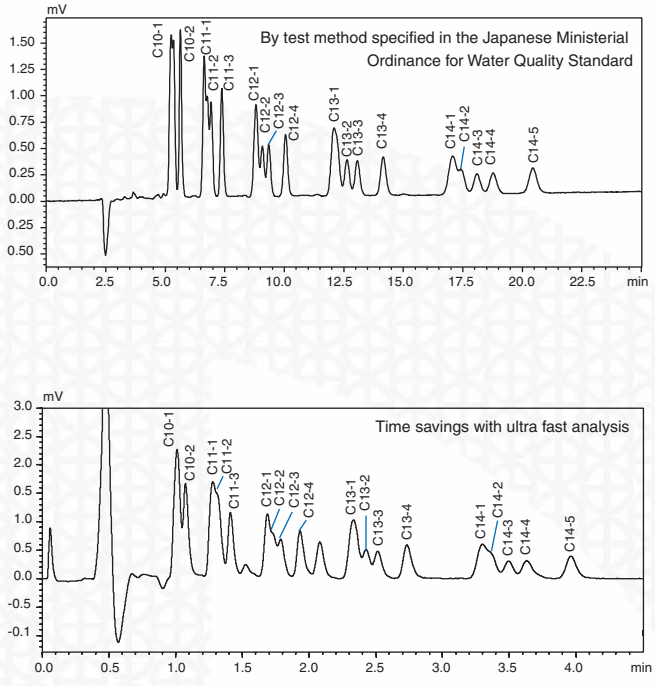
Calibration Curve Results for Enrofloxacin Standard Solutions (0.05 to 5 mg/L)

Calibration Curve Results for Enrofloxacin Standard Solutions (0.05 to 1000 mg/L)

Fig. 13 Enrofloxacin Calibration Curve Data (at left is magnification of the ultra-trace level region)

5-2. Conventional and Ultra Fast Analysis of Anionic Surfactant (LAS)

We added 0.04 mg/L of LAS to tap water, and analyzed the sample as is without concentrating the sample. The Japanese Ministerial Ordinance for Water Quality Standard specifies a criterion value for a total of 5 LAS compounds (C10 to C14 in Fig. 14, each 0.04 mg/L, for a total of 0.2 mg/L). The test method specifies HPLC for analysis of the water sample, using a 250x concentration of the solution. However, in this analysis, it is clear that a direct injection can be used without performing any kind of concentration operation. In addition, the time savings achieved using ultra fast analysis allowed the analysis time to be shortened to about 1/5.



About 5 times faster

Fig. 14 Comparison of LAS Analysis by Conventional Analysis and Ultra Fast Analysis

Table 8 Analytical Conditions for Conventional LC Analysis

Mobile Phase	Water / acetonitrile = 35 / 65 (v/v) (including 0.1 mol/L sodium perchlorate)
Flowrate	1.0 mL/min
Column	Shim-pack VP-ODS 250 mmL. x 4.6 mmI.D., 4.6 µm
Temperature	40°C
Detection	Excitation wavelength: 221 nm, emission wavelength: 284 nm
Sample	Anionic surfactant standard solution, 20 µL injected

Table 9 Analytical Conditions for Ultra Fast LC Analysis

Mobile Phase	Water / acetonitrile = 35 / 65 (v/v) (including 0.1 mol/L sodium perchlorate)
Flowrate	1.0 mL/min
Column	Shim-pack XR-ODS 100 mmL. x 3.0 mmI.D., 2.2 µm
Temperature	40°C
Detection	Excitation wavelength: 221 nm, emission wavelength: 284 nm
Sample	Anionic surfactant standard solution, 8 µL injected

5-3. Simultaneous Analysis of Polycyclic Aromatic Hydrocarbons (PAHs)

The United States Environmental Protection Agency (EPA) specifies 16 polycyclic aromatic hydrocarbons (PAHs) as "priority pollutants," and designates them as restricted substances. Here we conducted multi-wavelength, simultaneous analysis of 15 of these 16 substances. The optimum excitation and emission wavelengths are different for each of the PAH substances, making it necessary to conduct wavelength switching during analysis. However, in high-speed analysis using multiple wavelengths, on-time wavelength switching becomes extremely critical due to the narrow intervals between peaks. The 100 Hz high-speed sampling provided with the RF-20A/20Axs detectors reliably ensures on-time switching, demonstrating their powerful effectiveness in multi-wavelength, high-speed analysis of multiple compounds such as these.

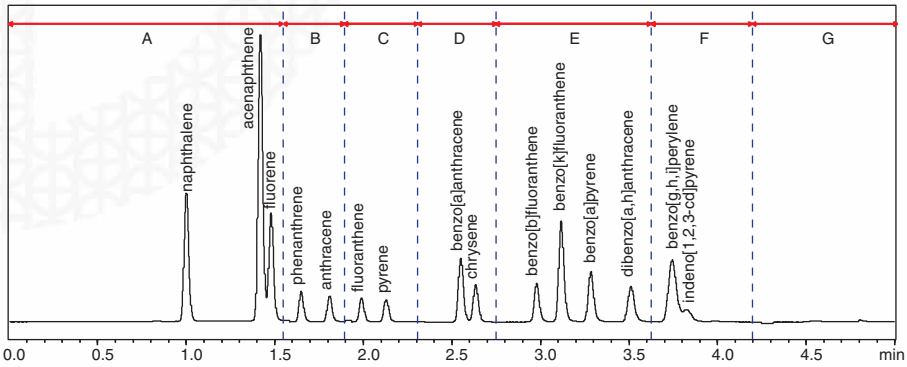


Fig. 15 Simultaneous High-Speed Analysis of 15 Polycyclic Aromatic Hydrocarbons

Table 10 Analytical Conditions for Polycyclic Aromatic Hydrocarbons

Mobile Phase	A: Water, B: acetonitrile
Gradient	B Conc. = 50% (0-0.5min) , 50-88% (0.5-3.0min) , 88% (3.0-4.2min) , 100% (4.21-4.5min) , 50% (4.51-5.0min)
Flowrate	3.0 mL/min
Column	SUPELCOSIL LC-PAH 50 mmL. x 4.6 mmI.D., 3 µm
Temperature	40°C
Detection	Excitation and emission wavelength switching at each interval (Table 11)

Table 11 Wavelength Settings at Each Interval

A	Excitation wavelength: 270 nm, emission wavelength: 330 nm
B	Excitation wavelength: 250 nm, emission wavelength: 370 nm
C	Excitation wavelength: 330 nm, emission wavelength: 430 nm
D	Excitation wavelength: 270 nm, emission wavelength: 390 nm
E	Excitation wavelength: 290 nm, emission wavelength: 430 nm
F	Excitation wavelength: 370 nm, emission wavelength: 460 nm
G	Excitation wavelength: 270 nm, emission wavelength: 330 nm

5-4. Amino Acid Analysis

This is an amino acid automatic analysis system with which we conducted post-column fluorescence derivatization detection using o-phthalaldehyde (OPA) / N-acetylcysteine as the reaction reagent. Since we used Shimadzu's proprietary N-acetylcysteine (odorless substance) reagent, not only is handling easier than with the normally used mercaptoethanol, sensitivity for imino acids such as proline is improved. Also, because the mobile phase and reaction reagents are included in a kit, time-consuming preparation of reagents is unnecessary. Fig. 16 shows a comparison of the chromatograms and S/N ratios for each analyte using the former model RF-10AXL and the RF-20AXS. The results demonstrate a 4 to 5 times increase in sensitivity with the RF-20AXS.

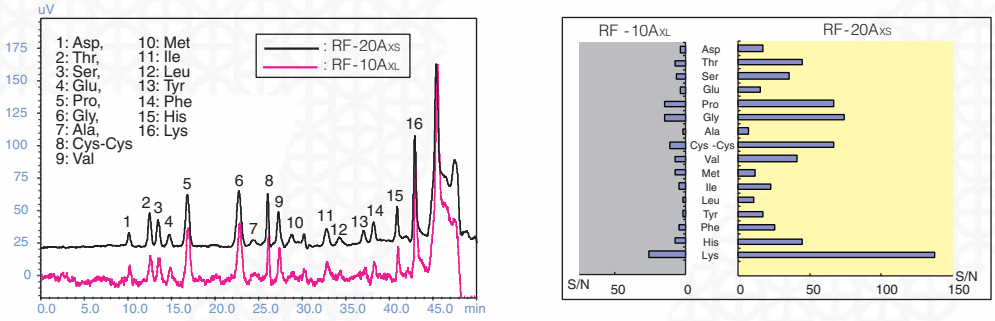


Fig. 16 Comparison of Amino Acid Analysis Using the RF-10AXL and RF-20AXS

Table 12 Analytical Conditions for Amino Acid Analysis

Mobile Phase	Amino acid analysis mobile phase kit
Flowrate	0.4 mL/min, high-pressure gradient analysis
Column	Shim-pack Amino-Na 100 mmL. x 6.0 mmI.D.
Temperature	60°C
Detection	Excitation wavelength: 350 nm, emission wavelength: 450 nm
Sample	Amino acid standard sample mixture, 10 µL injected

Table 13 Amino Acid Post-Column Reaction Conditions

Reaction Reagent	Amino acid analysis OPA reagent kit
Flowrate	Each 0.2 mL/min
Reaction Temperature	60°C

5-5. Repeatability in Carbamate Analysis

This is an N-methyl carbamate pesticide automatic analysis system which we used for post-column fluorescence derivatization detection with o-phthalaldehyde (OPA) as the reaction reagent. This system provides excellent accuracy in analysis of N-methyl carbamate pesticides in foods and tap water. N-methyl carbamate pesticides are often used in agricultural insecticides. Accordingly, residue standards and target values have been established in relation to various restrictions, including the Japanese Positive List System and water quality management target substances, etc. When conducting analysis by HPLC, the post-column fluorescence derivatization method is used. Shimadzu's Prominence Carbamate Analysis System is designed specifically for analyses that comply with the individual test methods specified in the Positive List System and water quality management target substance test methods. Fig. 17 shows the results of continuous analysis of various carbamates. From the repeatability shown for each substance in Table 16, it is clear that excellent repeatability can be obtained with this system.

Table 14 Analytical Conditions for Carbamate Analysis

Mobile Phase	Water / methanol
Flowrate	1.0 mL/min, high-pressure gradient analysis
Column	Shim-pack FC-ODS 75 mmL. x 4.6 mmI.D., 3 µm
Temperature	50°C
Detection	Excitation wavelength: 339 nm, emission wavelength: 445 nm
Injection Volume	10 µL

Table 15 Carbamate Post-Column Reaction Conditions

Reaction Reagent	A: Aqueous sodium hydroxide B: OPA, borate buffer solution containing β-mercaptopropionic acid
Flowrate	Each 0.5 mL/min
Reaction Temperature	100°C and 50°C

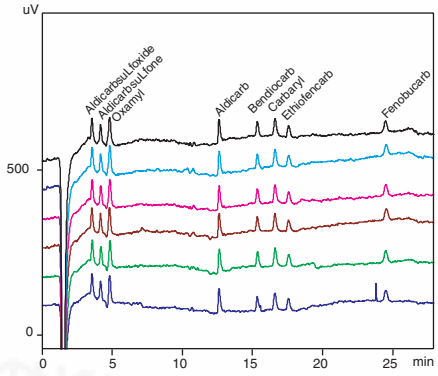


Fig. 17 Results of Continuous Analysis of Carbamates

Table 16 Repeatability for Each Compound (n = 6)

Compound	Area Value Repeatability (%RSD)	Compound	Area Value Repeatability (%RSD)
Aldicarb suLfoxide	2.63	Bendiocarb	2.44
Aldicarb suLfone	2.53	Carbaryl	1.62
Oxamyl	2.57	Ethiofencarb	2.69
Aldicarb	1.42	Fenobucarb	1.96

5-6. Analysis of Reducing Sugars

Various techniques are used for the separation and detection of sugars. For detection, differential refractive index detectors and evaporative light scattering detectors are commonly used. However, quantitation can be difficult with these detectors from the standpoints of separation conditions, sensitivity, and selectivity. Shimadzu's Prominence Reducing Sugar Analysis System is specially designed for highly selective detection of reducing sugars. After separation of sugars by a ligand exchange, borate complex anion exchange, use of HILIC column, etc., the sugars are combined with a reaction reagent containing borate and arginine under high temperature to form fluorescent derivatives. This system allows analysis of reducing sugars in samples containing many contaminants, with excellent selectivity and sensitivity (Fig. 18). Here we compare data obtained using the former RF-10AXL model and the RF-20AXS (Table 19), in which nearly all of the constituents are detected at more than 10 times the original sensitivity when using the RF-20AXS. The limit of detection with a 10 µL injection was 0.1 to 0.3 µmol/L (about 1µmol/L for sucrose), and the quantitation limit was 0.4 to 1 µmol/L (about 4µmol/L for sucrose).

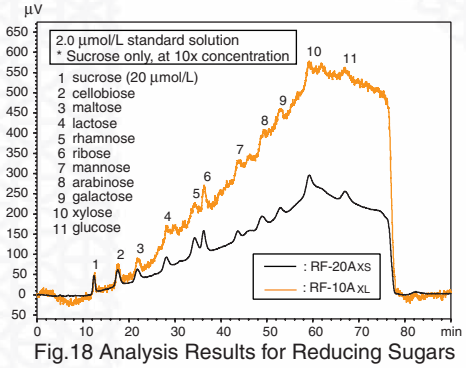


Fig.18 Analysis Results for Reducing Sugars

Table 17 Analytical Conditions for Reducing Sugar Analysis

Mobile Phase	Potassium borate buffer solution
Flowrate	0.6 mL/min, gradient elution analysis
Column	Shim-pack ISA-07/S2504 250 mmL. x 4.0 mmI.D.
Temperature	65°C
Detection	Excitation wavelength: 320 nm, emission wavelength: 430 nm
Injection Volume	10 µL

Table 18 Reducing Sugar Post-Column Reaction Conditions

Reaction Reagent	Arginine, aqueous borate solution
Flowrate	Each 0.5 mL/min
Reaction Temperature	150°C

Table 19 Sensitivity Comparison Between RF-20AXS and RF-10AXL

	Peak #	1	2	3	4	5	6	7	8	9	10	11
RF-20A XS	S/N	46.01	40.66	27.82	28.89	49.22	59.92	20.33	27.82	20.33	44.94	23.54
RF-10A XL	S/N	3.37	3.09	2.59	2.73	2.30	4.09	2.23	2.44	2.01	2.94	1.80
	Sensitivity Ratio	13.6	13.2	10.8	10.6	21.4	14.6	9.1	11.4	10.1	15.3	13.1

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