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Application News

High Performance Liquide Chromatograph Nexera™ XR

Improved Selectivity of Skin Sensitization Test ADRA with RF-20Axs and i-PDeA II

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User Benefits

- Separation of co-eluting component without changing analytical conditions by using i-PDeAII
- Higher selectivity and sensitivity can be obtained by using a fluorescence detector.

■ Introduction

ADRA, as a skin sensitization test, that evaluates the allergic reaction of chemicals to the skin using N- (2- (1-naphthyl) acetyl) -L-cysteine (NAC) and α-N- (2- (1-naphthyl) acetyl) -L-lysine (NAL) which contains naphthalene rings and cysteine or lysine. An example analysis according to the OECD Test Guideline is given in AN _ 01-00258. Depending on the test substance, co-elution peaks may appear near the NAC and the NAL peaks. In this case, LC separation conditions should be considered. The ADRA test must be performed within 72 hours from sample preparation. If the co-elution peak appears, the test must be repeated from the beginning.
In this report, we introduce two methods to avoid the interferences of co-elution peaks by increasing the selectivity of detection without changing the analysis. One example is the use of a fluorescence detector (RF), which is more selective than a UV detector, to avoid the interferences of co-elution. Another example is an analysis using a photodiode array detector (PDA) with the deconvolution function i-PDeA II to obtain separated peaks. *)

■ Analytical Conditions

Table 1 shows the analytical conditions. ADRA kit(FUJIFILM Wako Pure Chemical Corporation) was used for preparation of reaction reagents. For more information about this analysis, refer to AN_01-00258.

Table1 Analytical conditions

System	: Nexera XR
Column	: Shim-pack™ Velox C18 *1 (150 ㎜ × 3.0 ㎜ I.D., 2.7 µm)
Mobile Phase	: A) 0.1%TFA in Water B) 0.1%TFA in Acetonitrile
Time Program for NAC	: B conc. 30% (0 min) ->55% (9.5 min) -> 100% (10-13 min) ->30% (13.5-20 min)
Time Program for NAL	: B conc. 20% (0 min) ->45% (9.5 min) -> 100% (10-13 min) ->20% (13.5-20 min)
Flow Rate	: 0.3 mL/min
Column Temp.	: 40 ℃
Injection Vol.	: 10 µL
96-well Plate	: TORAST 96well 500 RU*2 NAL-96 sealing film (USA Scientific) *3
Detection	: SPD-M40 at 281 nm RF-20Axs (Ex:284 nm, Em:335 nm)

*1 P/N: 227-32010-04、*2 P/N: 370-04010-01
*3 P/N: 2923-5000

■ Example of a Fluorescence Detector

There are some reports that Chloramine T may co-elute with NAL in this analytical conditions. Figure 1 shows the chromatograms and the UV spectra of the NAL solution, the chloramine T solution, and the NAL-chloramine T mixed solution. The upper part of Figure 1 shows the chromatogram of water+Chloramine T mixed solution, it was found that a peak is detected on the NAL eluted position. but this is a false peak because the UV spectrum is different(middle part of Figure 1). The chromatograms of RF detection are shown at the lower part of Figure 1. There is no peak near NAL position in water+Chloramine T mixed solution.

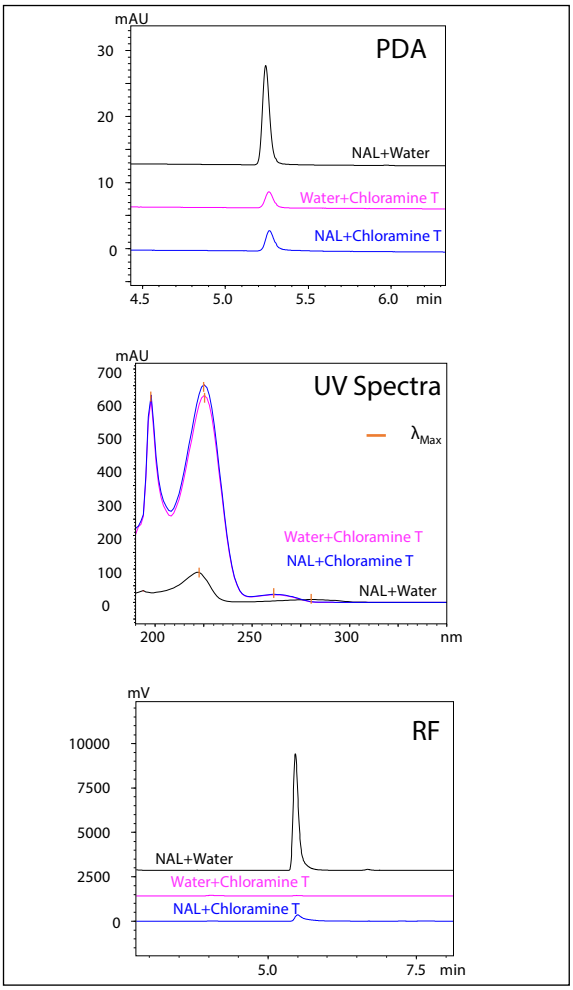

Fig. 1 Results of chloramine T as a test substance and NAL (Upper) UV Chromatograms, (Middle) UV Spectra, (Lower)RF Chromatograms

■ Proficiency testing with RF

Table 2 shows the results of the reference control (RC), and Figure 2 shows the calibration curves for NAC and NAL. Table 3 shows the depletion and standard deviation results of positive control and proficiency substances. The RC results, calibration curve linearity, and depletion of the proficiency substances all met the acceptance criteria.

*For i-PDeAII, the correct result may not be obtained depending on the differences of peak intensities or the resolutions between the impurity peak and the target peak. We recommend that you use this function as one of the methods for checking duplicate peaks. For details on i-PDeAII, please refer to the Shimazu Technical Report (C 191-0078).

Table 2 Reference Control (RC) Results with RF detection

		Acceptance Criteria	NAC	NAL
RC-A	Conc.	3.2-4.4 µM	3.7	4.0
RC-B, RC-C (Acetonitrile)	CV (%) (n=9)	<10	<0.1	<0.1
RC-C (Water)	Conc.	3.2-4.4 µM	3.6	3.7
	CV (%) (n=3)	<10	0.1	0.1
RC-C (Acetonitrile)	Conc.	3.2-4.4 µM	3.8	4.0
	CV (%) (n=3)	<10	<0.1	<0.1
RC-C (Acetone)	Conc.	3.2-4.4 µM	3.9	3.9
	CV (%) (n=3)	<10	<0.1	<0.1

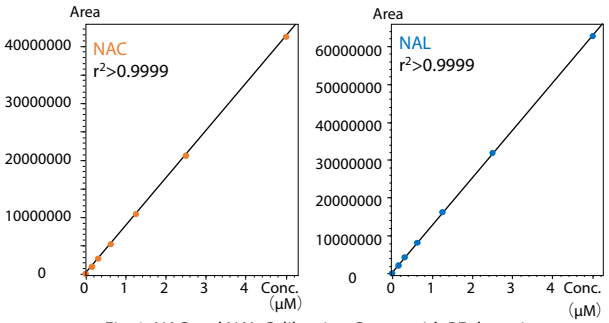

Fig. 2 NAC and NAL Calibration Curves with RF detection

■ Example of i-PDeAll peak deconvolution function

Using the i-PDeAll function of LabSolutions™ (workstation), which deconvolutes unresolved peaks, an approximate solution of a single chromatogram of each component can be obtained by a simulation based on the spectral information obtained by the PDA. Figure 3 shows the chromatograms of NAC with *trans*-2 hexenal as the test substance, and Figure 4 shows the chromatograms of NAL with 2, 3 butanedione as the test substance. The co-elution peak and the NAC/NAL peak, which were detected as a single peak, are separated. Quantification is possible.

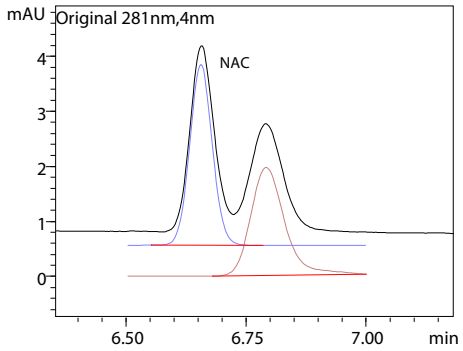

Fig.3 Chromatograms for NAC- *trans*-2-hexenal mixed solution (deconvolved from 6.5 minutes to 7.0 minutes)

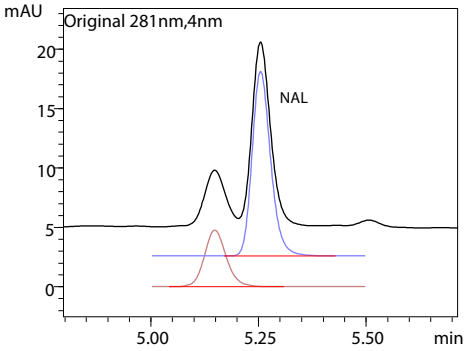

Fig.4 Chromatograms for NAL-2,3-butanedione mixed solution (deconvolved from 5.0 minutes to 5.5 minutes)

■ Conclusion

Using a fluorescence detector for ADRA analysis, higher selectivity and sensitivity are obtained in comparison with the UV detector. As with the UV detector (AN _ 01-00258), the results obtained in the proficiency test met the acceptance criteria. The peak deconvolution function i-PDeAll of the LabSolutions software is useful to prevent the influence of the co-elution peak and to obtain the estimated peak area.

- We would like to thank Yusuke Yamamoto, Masaharu Fujita and Toshihiko Kasahara from Fujifilm Corporation for their generous support in this work.

- Refer to AN _ 01-00258 for reference materials.

Table 3 Percent Depletion and Standard Deviation (n = 3) for Positive Control (PC) and Proficiency Substances NAC and NAL

No.	Substance	Solvent	NAC Depletion (%)			NAL Depletion (%)		
			Criteria	Result	SD	Criteria	Result	SD
PC	Squaric acid diethyl ester	Acetonitrile	15-40	17.8	0.1	40-85	84.8	3.1
1	*p*-Benzoquinone	Acetonitrile	90-100	100	< 0.1	40-70	56.4	0.1
2	Diphenylcyclopropenone	Acetonitrile	15-45	23.7	1.2	≦10	0.0	0.1
3	2-Methy-2*H*-isothazol-3-one	Water	80-100	99.2	< 0.1	≦7	0.3	0.3
4	Palmitoyl Chloride	Acetonitrile	≦10	5.7	0.6	50-100	51.3	1.0
5	Imidazolidinyl urea	Water	10-45	32.3	3.2	≦10	0.3	0.1
6	Farnesal	Acetonitrile	20-40	30.6	1.7	≦15	1.3	0.3
7	Glycerol	Water	≦7	0.0	< 0.1	≦7	0.0	< 0.1
8	Isopropanol	Water	≦7	0.3	0.6	≦7	0.7	0.5
9	Dimethyl isophthalate	Acetonitrile	≦7	0.9	1.5	≦7	0.0	< 0.1
10	Propyl paraben	Acetonitrile	≦7	1.1	1.5	≦7	0.0	< 0.1

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