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Application News

High Performance Liquid Chromatograph Nexera™ XR

Evaluating Technical Proficiency of ADRA Skin Sensitization Testing and High Speed Analysis

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User Benefits

- Provides ADRA results with high reproducibility and good calibration curve linearity.
- Using high-speed conditions, HPLC analysis is completed in 18 hours instead of about 50 hours.
- Solvent consumption and analytical time can be reduced without compromising quantitative accuracy, even for high-speed conditions.

■ Introduction

Skin sensitization tests evaluate the allergic reactivity of skin to chemical substances. For animal welfare reasons, non-animal alternative methods are being developed for these tests. Chemical substances can induce an inflammatory reaction if they bind to cysteine or lysine in epidermal proteins. International test guidelines for evaluating chemical substances, published by the Organisation for Economic Co-operation and Development (OECD), include the Direct Peptide Reactivity Assay (DPRA) and Amino Acid Derivative Reactivity Assay (ADRA) as alternative *in chemico* test methods based on the properties indicated above.

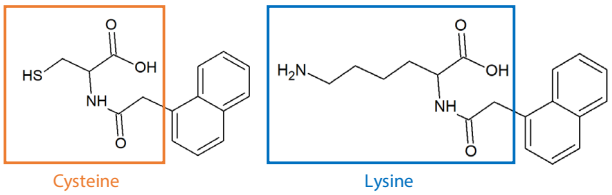
ADRA evaluates the binding characteristics of test substances using *N*-(2-(1-naphthyl)acetyl)-*L*-cysteine (NAC) or α-*N*-(2-(1-naphthyl)acetyl)-*L*-lysine (NAL), which are synthesized by inducing cysteine or lysine, respectively, to bind with naphthalene rings. ADRA sample solutions are analyzed at 1/100 of the concentration analyzed for DPRA. The presence of naphthalene rings enables detection at a 281 nm UV wavelength, where effects from any detection of contaminants are minimal.

The ADRA test method is specified in OECD TG 442C Appendix II, Annex 2. Annex 2 also specifies methods for system suitability testing and proficiency testing. Proficiency tests are performed to assess the technical proficiency of testing personnel and the given testing environment in terms of the binding characteristics of NAC and NAL (NAC and NAL percent depletion) with respect to ten types of test substances. The system suitability tests and proficiency tests described in this article were all performed using a Nexera XR high performance liquid chromatograph. The test results were within the specified ranges for all indicated substances.

High-speed conditions were also analyzed. Those results were no different from those obtained with the conditions specified by OECD.

■ ADRA

The structural formulas of NAC and NAL used to evaluate the protein reactivity by ADRA are shown in Fig. 1. After letting the test substance solution react with the NAC and NAL solutions, the tendency of the test substances to bind with cysteine and lysine is evaluated by using HPLC to measure the percent depletion of NAC or NAL.



Cysteine
***N*-(2-(1-naphthyl)acetyl)-*L*-cysteine**
NAC

Lysine
α-*N*-(2-(1-naphthyl)acetyl)-*L*-lysine
NAL

Fig. 1 Structural Formulas of NAC and NAL

■ Analytical Conditions

The analytical conditions are indicated in Table 1, and the pretreatment procedure in Fig. 2. The reagents were prepared using an ADRA kit (FUJIFILM Wako Pure Chemical).

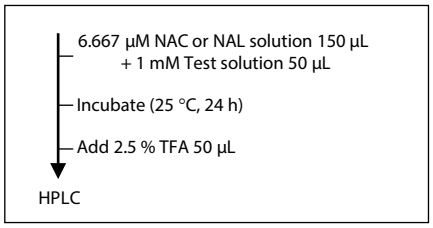


Fig. 2 Pretreatment Procedure

Table 1 Analytical Conditions

System:	Nexera XR
Column:	Shim-pack™ Velox C18*1 (150 mm × 3.0 mm 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)
Flowrate:	0.3 mL/min
Column Temp.:	40 °C
Injection Vol.:	10 µL
96-Well Plate:	TORAST 96 well 500 RU*2 NAL-96 sealing film (USA Scientific)*3
Detection	SPD-M40 at 281 nm (UHPLC cell)

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

■ Analyzing Standard Solutions and Reference Control Solutions

Conditions are also specified for ADRA validity, such as calibration curve linearity criteria for standard solutions and the use of reference control samples to check system stability. NAC and NAL calibration curves are shown in Fig. 3. Seven-point calibration curves were prepared for concentrations from zero (blank) to 5 µM. Both curves indicated good linearity with an r^2 contribution rate greater than the specified 0.990 value.

Test results for reference control (RC) samples are shown in Table 2. Acetonitrile was used instead of the test substance for samples RC-A and B, with the solvent used for the test substance solutions added for RC-C. RC-A is used to verify that NAC and NAL concentrations are within the specified range and RC-B is used to verify system stability during the analysis period, based on NAC and NAL concentration results and area repeatability from three successive measurements at the beginning and end of batch analysis. RC-C is used to verify the reactivity of the solvent used for NAC, NAL, and each test substance, and also as a reference for calculating percent depletion values.

Table 2 Reference Control (RC) Results

		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	0.0	0.0
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	0.0	0.0
RC-C (Acetone)	Conc.	3.2-4.4 µM	3.9	3.9
	CV (%) (n=3)	<10	0.0	0.0

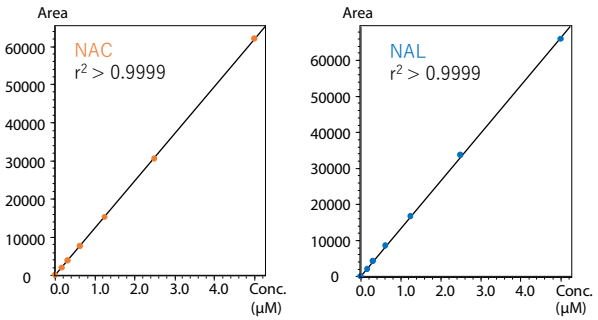


Fig. 3 NAC and NAL Calibration Curves (OECD Conditions)

$$\text{NAC or NAL depletion (\%)} = \left(1 - \frac{\text{NAC or NAL peak area in replicate injection}}{\text{Mean NAC or NAL peak area in RC-C}}\right) \times 100$$

Fig. 4 Calculation of NAC and NAL Percent Depletion

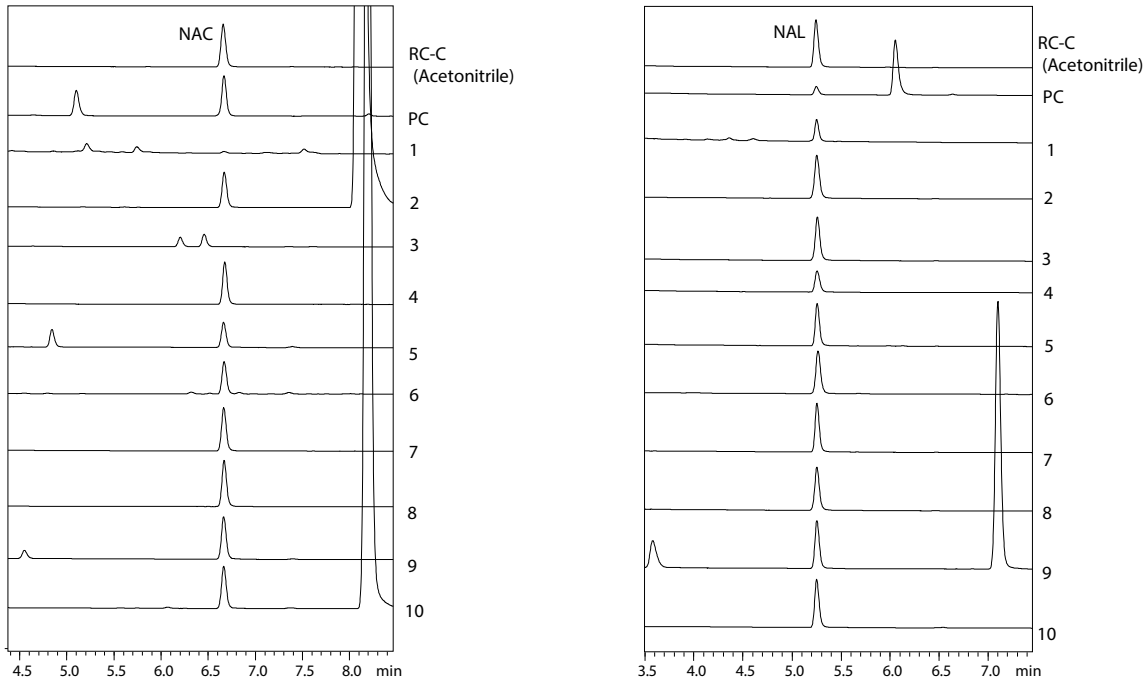


Fig. 5 Chromatograms of Positive Control (PC) and Proficiency Substances (NAC on left and NAL on right)
(Refer to Table 3 for substances corresponding to numbered chromatograms)

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	15.0	0.2	40-85	84.9	3.0
1	*p*-Benzoquinone	Acetonitrile	90-100	96.2	0.4	40-70	55.2	0.1
2	Diphenylcyclopropenone	Acetonitrile	15-45	23.8	1.4	≤ 10	0.0	0.1
3	2-Methyl-2*H*-isothiazol-3-one	Water	80-100	100	0.0	≤ 7	0.5	0.4
4	Palmitoyl Chloride	Acetonitrile	≤ 10	5.9	0.5	50-100	51.6	0.9
5	Imidazolidinyl urea	Water	10-45	33.5	3.8	≤ 10	1.0	0.1
6	Farnesal	Acetonitrile	20-40	31.2	1.5	≤ 15	1.9	0.5
7	Glycerol	Water	≤ 7	0.0	0.0	≤ 7	0.0	0.0
8	Isopropanol	Water	≤ 7	0.4	0.7	≤ 7	0.7	0.4
9	Dimethyl isophthalate	Acetonitrile	≤ 7	0.7	1.3	≤ 7	0.0	0.0
10	Propyl paraben	Acetonitrile	≤ 7	0.8	1.2	≤ 7	0.0	0.0

■ Analyzing Proficiency Substances

Proficiency was evaluated for the ten test substances specified by OECD. Skin sensitization to test substances is determined based on the percent depletion of NAC and NAL. The formula used to calculate percent depletion values is shown in Fig. 4. According to the procedure for evaluating technical proficiency, a passing result requires that the percent depletion for both NAC and NAL must be within the specified ranges and the standard deviation (SD) for n = 3 samples is 10 % or less. For system suitability, the procedure requires that reference values are satisfied for at least 8 of the 10 proficiency substances. Chromatograms for the positive control (PC) and proficiency substances are shown in Fig. 5, with the corresponding depletion rates and standard deviation values listed in Table 3. The results satisfied reference values for all ten proficiency substances.

■ High-Speed Analysis

The OECD recommends a column with a 2.7 µm particle size intended for high-speed analysis, but OECD analytical conditions are specified assuming pressures within the pressure capacity range of a conventional HPLC system, which requires an analysis time of about 20 minutes per sample. For example, testing ten substances, including system suitability testing and calibration curve preparation/testing, would require 50 hours. For this article, high-speed analysis was considered using the same column, but with the pressure capacity range of the Nexera XR (max. 70 MPa).

The analytical conditions are listed in Table 4. These conditions resulted in seven minutes per analysis. Chromatograms for NAC and NAL standard solutions are shown in Fig. 6 and calibration curves in Fig. 7. The results show nice linearity. Also, the system suitability results from RC-B and RC-C (acetonitrile) solutions were equivalent to the results obtained using the analytical conditions specified by the OECD.

Table 4 Analytical Conditions

System:	Nexera XR
Column:	Shim-pack Velox C18 (150 mm × 3.0 mm 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 % (3.5 min) → 100 % (3.6-5.0 min) → 30 % (5.1-7.0 min)
Time Program for NAL:	B conc. 20 % (0 min) → 45 % (3.5 min) → 100 % (3.6-5.0 min) → 20 % (5.1-7.0 min)
Flowrate:	1.0 mL/min
Column Temp.:	40 °C
Injection Vol.:	10 µL
96-Well Plate:	TORAST 96 well 500 RU NAL-96 sealing film
Detection:	SPD-M40 at 281 nm (UHPLC cell)

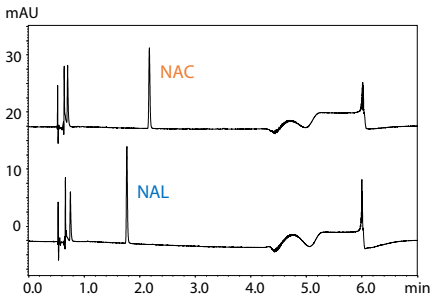


Fig. 6 Chromatograms for NAC and NAL Standard Solutions (5 µM)

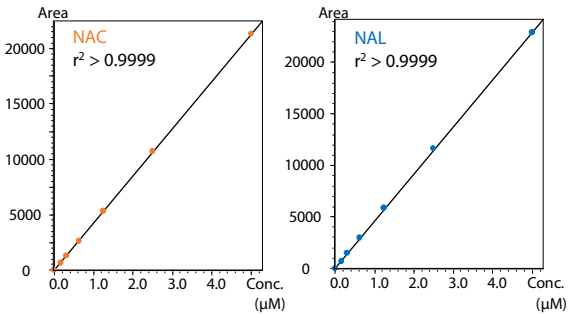


Fig. 7 Calibration Curves for NAC and NAL (High-Speed Conditions)

■ Conclusion

The Nexera XR system was used for OECD-compliant ADRA testing. Good reproducibility and calibration curve linearity were achieved. Using conventional analytical standards it took 50 hours to analyze ten types of substances, but high-speed conditions shortened the analysis time to 18 hours.

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- Testing protocols and data analysis procedures were prepared based on the OECD TG 442C test guidelines, the Fujifilm presentation on protocols and techniques at the 2019 ADRA Technology Seminar, and the ADRA Kit Instruction Manual.

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