Within- and between-laboratory reproducibility and predictive capacity of amino acid derivative reactivity assay (ADRA) using a 0.5 mg/ml test chemical solution: Results of the study for reproducibility confirmation implemented in five participating laboratories

Short title

WLR, BLR and predictive capacity of ADRA (0.5 mg/ml)

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Keywords
Amino acid Derivative Reactivity Assay, ADRA (0.5 mg/mL), NAC, NAL, reproducibility, predictive capacity, within-laboratory reproducibility, between-laboratory reproducibility, proficiency substances

Abbreviations:
ADRA: amino acid derivative reactivity assay
ADRA (1 mM): ADRA using 1 mM test chemical solution
ADRA (0.5 mg/ml): ADRA using 0.5 mg/ml test chemical solution
NAC: *N*-(2-(1-naphthyl)acetyl)-L-cysteine
NAL: α-*N*-(2-(1-naphthyl)acetyl)-L-lysine
WLR: within-laboratory reproducibility
BLR: between-laboratory reproducibility
SADE: squaric acid diethyl ester

Abstract

The amino acid derivative reactivity assay (ADRA) is an *in chemico* alternative assay for skin sensitization listed in OECD test guideline 442C. ADRA evaluates the reactivity of sensitizers to proteins, which is key event 1 in the skin sensitization adverse outcome pathway.

Although the current key event 1 evaluation method is a simple assay that evaluates nucleophile and test chemical reactivity, mixtures of unknown molecular weights cannot be evaluated because a constant molar ratio between the nucleophile and test chemical is necessary. In addition, because the nucleophile is quantified by HPLC, the frequency of co-eluting the test chemical and nucleophile increases when measuring multi-component mixtures. To solve these issues, test conditions have been developed using a 0.5 mg/ml test chemical solution and fluorescence-based detection. Since the practicality of these methods has not been substantiated, a validation test to confirm reproducibility was conducted in this study.

The 10 proficiency substances listed in the ADRA guidelines were tested three times at five different laboratories. The results of both within- and between-laboratory reproducibility were 100%, and the results of ultraviolet- and fluorescence-based measurements were also consistent. In addition to the proficiency substances, a new positive control, squaric acid diethyl ester, was tested three times at the five laboratories. The results showed high reproducibility with *N*-(2-(1-naphthyl)acetyl)-L-cysteine depletion of 37%–52% and α-*N*-(2-(1-naphthyl)acetyl)-L-lysine depletion of 99%–100%.

Thus, high reproducibility was confirmed in both evaluations of the 0.5 mg/ml test chemical and the fluorescence-based measurements, validating the practicability of these methods.

Short abstract

We implemented a study to validate a new amino acid derivative reactivity assay (ADRA) using a 0.5 mg/ml test chemical solution by determining within-laboratory and between-laboratory reproducibility in five participating laboratories. The aim of this study was to verify reproducibility with 10 proficiency substances and 1 positive control. The results showed sufficiently high reproducibility and predictive capacity. We plan to submit this new ADRA test method to the OECD test guideline group in the near future.

1. Introduction

In the safety evaluation of chemicals, the adverse outcome pathway (AOP) has been proposed as a concept portraying the mechanism from the molecular initiation event to the adverse outcome to link the data obtained in various assays to each endpoint (Ankley et al., 2009). The test methods listed in the OECD test guidelines must be based on the AOP. Four key events were defined in the AOP for skin sensitization (OECD, 2014). Each key event is defined as follows: key event 1: covalent binding of chemicals to proteins (haptenization); key event 2: activation of keratinocytes; key event 3: activation of dendritic cells; and key event 4: activation and proliferation of antigen-specific T cells.

As an alternative to animal testing for the evaluation of key event 1, our laboratory has developed the amino acid derivative reactivity assay (ADRA) (Fujita et al., 2014; Yamamoto et al., 2015). ADRA uses two nucleophilic reagents (*N*-(2-(1-naphthyl)acetyl)-L-cysteine (NAC) and α-*N*-(2-(1-naphthyl)acetyl)-L-lysine (NAL)) that possess naphthalene rings at the N-termini of cysteine and lysine, respectively; it evaluates protein-binding potential by measuring the reaction between NAC/NAL and chemicals. NAC and NAL have a naphthalene ring with high UV absorbance, which allows testing at nucleophilic reagent concentrations that are 100-fold lower than those used in the previously developed direct peptide reactivity assay (DPRA; Gerberick et al., 2007; OECD TG442C., 2021a) (Yamamoto et al., 2015). In addition, our test method was improved to include EDTA to increase the stability of NAC, as listed in the OECD test guidelines (Fujita et al., 2019a; OECD TG442C., 2021b). By verification using a test chemical solution prepared at a specific weight concentration, it was confirmed that the predictive capacity of using 0.5 mg/ml of a test chemical solution is equal to or higher than that using a test chemical solution prepared at a conventional molar concentration. This allowed the evaluation of chemicals with unknown molecular weights using ADRA (Yamamoto et al., 2019). Furthermore, taking advantage of the fact that the naphthalene ring in NAC and NAL is fluorescent, a highly selective measurement method using fluorescence detection has been established, and it has been confirmed that substances that are likely to co-elute, such as those contained in mixtures, can still be evaluated (Fujita et al., 2019b; Wanibuchi et al., 2019). Thus, ADRA can technically evaluate mixtures consisting of multiple components by combining a test method that uses a test substance prepared at a specific weight concentration with fluorescence detection. This test method is particularly useful for evaluating cosmetic raw materials, including many substances and mixtures of unknown composition, such as botanical extracts.

Therefore, the purpose of this study was to validate the reproducibility of this test at multiple laboratories to confirm the practicality of the methods. For ADRA using a 1 mM test substance solution (ADRA (1 mM)), a validation study on within-laboratory reproducibility (WLR) for 10 chemicals and between-laboratory reproducibility (BLR) for 40 chemicals has already been conducted. As a result, ADRA (1 mM) 's WLR was 100% (10/10), 100% (7/7), 90% (9/10), and 100% (10/10) in the four participating laboratories. The BLR for the 40 test chemicals calculated based on the results from three participating laboratories was 91.9% (Fujita et al., 2019c; OECD, 2019a). In this way, ADRA (1 mM) achieved high reproducibility both within and between laboratories, and its reliability was guaranteed by an independent peer review (OECD, 2019b). Thus, for the test method using 0.5 mg/ml test chemical solution (ADRA (0.5 mg/ml)) and fluorescence detection (HPLC-FL), which used the same test conditions as ADRA (1 mM) except for the test chemical concentration and detection method, the reproducibility confirmation study was conducted at five laboratories using the 10 proficiency substances described in the test guidelines.

Phenylacetaldehyde was specified as a positive control in the OECD test guidelines for ADRA. This aldehyde is not suitable for long-term use because it may deteriorate depending on the storage conditions. In addition, because a positive control is required for each test, it is preferable that this control is easily available. Thus, if the user can select a positive control from a plurality of chemicals and not be limited to one chemical, the range of acquisition means is widened and the test can be conducted more easily. Therefore, in the latest OECD test guidelines, squaric acid diethyl ester (SADE) was newly added as a positive control substance in addition to the conventional phenylacetaldehyde (OECD TG442C, 2021). In the present study, the reproducibility of ADRA (0.5 mg/ml) and HPLC-FL for SADE was also confirmed at the five laboratories in the same way as for the 10 proficiency substances.

2. Materials and Methods

2.1. Materials

The reagents in the ADRA test (NAC, NAL, and the sodium phosphate buffer) are included in the ADRA kit for skin sensitization testing (Catalog No. 296-80901; Fujifilm Wako Pure Chemical Corporation, Osaka, Japan), which was used in this study. The proficiency substances for ADRA described in the test guidelines (OECD TG442C, 2021) were used as test chemicals. The suppliers of the test chemicals and the positive control are shown in Table 1. These test chemicals and positive controls were acquired, aliquoted, and shipped by the lead laboratory (Lab.5).

2.2. Outline of the ADRA test method

All tests were performed following the procedures specified by the lead laboratory with reference to the ADRA test method using a test chemical solution prepared as a weight concentration (Yamamoto *et al*., 2019). The test samples were prepared in triplicate by adding 50 µl of 0.5 mg/ml test chemical solution or positive control to 150 µl of the 6.667 µM NAC or NAL stock solution in a 96-well microplate. Control samples containing 50 µl of solvent without test chemicals were also prepared. The 96-well microplate was sealed using a TORAST 96well Seal F Type (SHIMADZU GLC Ltd., Tokyo, Japan), shaken gently, and then incubated in the dark for 24 h at 25°C. After incubation, 20 µl of test sample was added to 180 µl of 0.5% trifluoroacetic acid (TFA) in water in a new 96-well plate for HPLC-FL. In addition, 45 µl of 2.5% TFA in water was added to the remaining 180 µl of the test sample for HPLC ultraviolet detection (HPLC-UV). Standard solutions for defining the calibration curve were prepared for NAC and NAL at concentrations ranging from 0.02 to 0.6 µM for HPLC-FL and 5.0 to 0.156 µM for HPLC-UV. The 96-well microplates were then resealed and gently shaken.

2.3. HPLC analysis of nucleophilic reagents

HPLC analysis was performed according to the method described by Yamamoto *et al*. (2015). Mobile phases (A) and (B) were prepared with 0.1% TFA in water and 0.1% TFA in acetonitrile, respectively. NAC and NAL in all samples and standards were quantified using an HPLC system (Shimadzu Scientific Instruments, Kyoto, Japan; Hitachi High-Tech Science Corporation, Tokyo, Japan; OSAKA SODA CO., LTD., Osaka, Japan; GL Sciences Inc., Tokyo, Japan) on a Wakopak Core C18 ADRA (φ3.0 × 150 mm, FUJIFILM Wako Pure Chemical Corporation). The flow rate was 0.3 ml/min, and the temperatures of the column oven and auto-sampler were maintained at 40°C and

4°C, respectively. The volume of each sample injected (8 to 20 µl) depended on the HPLC system and detector. The analysis was performed using a linear gradient from 30% B to 55% B over 9.5 min for NAC and from 20% B to 45% B over 9.5 min for NAL, followed by rapidly increasing to 100% B for 0.5 min, holding at 100% B for 3.5 min, and then returning to the initial conditions (30% B for NAC and 20% B for NAL). The total run time was 20 min per sample. Finally, NAC and NAL were quantified using ultraviolet detection at 281 nm as well as fluorescence detection with an excitation wavelength of 284 nm and an emission wavelength of 333 nm (Fujita et al., 2019b; Wanibuchi, et al., 2019), except for one laboratory that used only fluorescence detection (Lab.4 in Tables 2, 3, and 4).

2.4. Prediction models

Reactivity of the test chemical with NAC and NAL was expressed as percent depletion calculated from the NAC and NAL peak areas of replicate injections and the mean NAC and NAL areas in the three relevant controls (in the appropriate solvent) using the following formula:

NAC or NAL depletion (% depletion) = [1 − (NAC/NAL peak area in replicate injection / mean NAC or NAL peak area in reference controls C)] × 100.

The mean percentage depletion was calculated from the depletion of NAC and NAL, and the test chemical was predicted to be either a sensitizer or a non-sensitizer as follows. If the mean percentage depletion of a test chemical was <4.9%, the test chemical was categorized as a non-sensitizer. If the mean percentage depletion of a test chemical was ≥4.9%, the test chemical was categorized as a sensitizer.

2.5. Data analysis

The reproducibility of the test method was evaluated by calculating WLR and BLR. The testing for this study was conducted at five participating laboratories. Since BLR is normally calculated using results from three participating laboratories, the results from five laboratories were evaluated using the following method.

Step 1. Reproducibility was calculated using test results of 3 laboratories selected from the 5 participating laboratories (total 10 patterns as a combination; .i.e. Lab.1-2-3, Lab. 1-2-4, Lab.1-2-5, and so on).

Step 2. BLR was calculated as an average value of the reproducibility of each of the 3 laboratories as calculated in Step 1.

The predictive parameters sensitivity, specificity, positive and negative predictive value and accuracy were derived from 2×2 contingency tables to describe the predictive capacity: sensitivity: (True positive (TP) / [TP + False Negative (FN)] × 100); specificity: (True negative (TN) / [TN + False positive (FP)] × 100); positive predictive value: (TP / [TP + FP] × 100); negative predictive value:((TN / [TN + FN] × 100); and accuracy: ([TP + TN] / [TN + TP + FP + FN] × 100) with FN being the number of false negative calls, FP the number of the false positive calls, TN the number of the true negative calls and TP the number of the true positive calls.

3. Results

This study was conducted to confirm the reproducibility of the method using a test chemical solution prepared at 0.5 mg/ml (ADRA (0.5 mg/ml)) and fluorescence detection (i.e., HPLC-FL). Under these test conditions, the WLR and BLR as well as the predictive capacity were evaluated. Moreover, SADE, which was adopted in the test guidelines as a new positive control, was tested with ADRA (0.5 mg/ml) and HPLC-FL to confirm the consistency of depletion.

3.1 Between-laboratory reproducibility

Tables 2 and 3 show the NAC and NAL depletion, respectively, measured by HPLC-UV and HPLC-FL for the 10 proficiency substances in the three tests at each laboratory. Since Lab.4 did not perform UV measurements due to infrastructure reasons, only the results of fluorescence measurements are shown. With regard to NAC depletion in each laboratory, the variability for farnesal was slightly high by both HPLC-UV and HPLC-FL, and the standard deviation (SD) in the three tests in Lab.3 and Lab.5 by HPLC-UV and Lab.4 by HPLC-FL was more than 10%. In addition, although the SD in the three tests was more than 10% for diphenylcyclopropenone in Lab.4, palmitoyl chloride in Lab.3, and imidazolidinyl urea in Lab.2 by HPLC-FL, there was almost no variation in the other results. There was no significant difference in depletion between HPLC-UV and HPLC-FL at the four laboratories that performed both HPLC-UV and HPLC-FL.

The variability in NAL depletion for farnesal was slightly high, similar to NAC, and the SD in the three tests in Lab.5 by HPLC-UV was more than 10%, but there was almost no variation in the other laboratories and test chemicals. There was also no significant difference in NAL depletion between HPLC-UV and HPLC-FL at the four laboratories that performed both HPLC-UV and HPLC-FL, as demonstrated with NAC.

Table 4 shows the mean depletion of NAC and NAL and the prediction of skin sensitization. As a result, the predictions of the three tests were in agreement at all laboratories, and the BLR was 100%.

3.2 Within-laboratory reproducibility

First, focusing on the NAC depletion in all five laboratories, the SD was greater than 15% for diphenylcyclopropenone and farnesal, but almost no variation was observed for the other test chemicals (Table 2). For NAL depletion, variability across the five laboratories was less than 10% for all test chemicals (Table 3).

Regarding the prediction of skin sensitization, the judgments of the five laboratories were in agreement for all 10 test chemicals, and the WLR was 100%.

3.3 Predictive capacity

The 10 proficiency substances used in this study consisted of 6 sensitizers and 4 non-sensitizers. As shown in Table 4, the prediction of each laboratory for these 10 test chemicals using both HPLC-UV and HPLC-FL was consistent with the *in vivo* results. From the above results, the accuracy of the local lymph node assay (LLNA) was 100%.

3.4 Reproducibility of new positive control

Fig. 1 shows the depletion of NAC and NAL in the three tests at each laboratory for SADE prepared using two solvents, water and acetonitrile. First, when solvent-dependent depletion was compared, there was almost no difference between water and acetonitrile. However, when the reproducibility of each solvent was compared, the NAC depletion with water solvent was approximately 28%–65%, while that with acetonitrile solvent was approximately 37%–52%; thus, the variation was smaller with acetonitrile. Similarly, NAL depletion was approximately 79%–97% with water solvent, whereas it was approximately 99%–100% with acetonitrile solvent; thus, the variation was smaller with acetonitrile. There was no difference in the depletion between HPLC-UV and HPLC-FL.

4. Discussion

The current ADRA test guidelines stipulate that chemicals with known molecular weights should be tested using a test chemical solution prepared at 1 mM, and the reproducibility of this test has been confirmed by the validation study in at four laboratories. However, there is a concern that only chemicals with known molecular weights can be evaluated under these test conditions. To address this issue, a novel (additional) test method using a test chemical solution prepared at a weight concentration of 0.5 mg/ml (ADRA (0.5 mg/ml)) and a measurement method using fluorescence detection (HPLC-FL), which can be applied to the evaluation of chemicals and mixtures of unknown molecular weights, has been developed. Therefore, in this study, the reproducibility of ADRA (0.5 mg/ml) and HPLC-FL was validated by five laboratories, as in the conventional test method.

Because the test method verified in this study differs from the conventional ADRA (1mM) only in the prepared concentration of the test chemical and the HPLC detection, it was expected that the same reproducibility could be obtained with both methods. As a result, in the prediction for the 10 test chemicals at five laboratories, both the BLR and WLR were 100%, confirming high reproducibility. However, although the predictions for sensitizers/non-sensitizers were all in agreement, some chemicals showed variability within or between laboratories in the depletion of NAC and NAL. Specifically, the SD within or between laboratories for diphenylcyclopropenone, palmitoyl chloride, imidazolidinyl urea, and farnesal with NAC, as well as farnesal with NAL was more than 10%. The factors involved in the variation in depletion of these chemicals are discussed below.

First, the NAC depletion for diphenylcyclopropenone in four laboratories (Lab.1 to Lab.3 and Lab.5) was approximately 47%–61%, which was a small variation, but the depletion in Lab.4 was 99.8% for the first set, 99.7% for the second set, and 50.0% for the third set, which was a large variation. In addition, the depletions for the first and second sets in Lab.4 are higher than those in Lab.1 to Lab.3 and Lab.5. In contrast, *p*-benzoquinone and 2-methyl-2*H*-isothiazol-3-one adjacent to diphenylcyclopropenone in the arrangement on the 96-well plate both had a high depletion of more than 91% in all laboratories. From these data, it is possible that the depletion for the first and second sets was high in Lab.4 due to contamination of *p*-benzoquinone or 2-methyl-2*H*-isothiazol-3-one in the diphenylcyclopropenone wells. If this is the case, there was an error in the test operation, but it was judged that there was no need for retesting because these test data met all acceptance criteria.

Next, for palmitoyl chloride by HPLC-FL in Lab.3, the SD of NAC depletion in the three tests was greater than 10%. The NAC depletion was 10.2% for the first set, 10.9% for the second set, and 28.5% for the third set; thus, the depletion for the third set was high. However, the depletion of the third set as assessed by HPLC-UV was 19.8%, which was approximately 9% lower than that by HPLC-FL, and the SD of the NAC depletion in the three tests using HPLC-UV was 4.5%, which was not variable. In this study, the reaction solution was diluted 10-fold before measurement by HPLC-FL because there was a concern regarding signal saturation and reduced assay quantitation due to the high fluorescence intensity of NAC and NAL. Therefore, the depletion could have been increased because the low amount of the reaction solution was added in the operaton of diluting the reaction solution when preparing the sample for HPLC-FL. Similarly, the reason the SD of NAC depletion exceeded 10% in the three tests for imidazolidinyl urea by HPLC-FL in Lab.2 may have been related to the dilution operation because the HPLC-FL value was 16.4% higher than that of HPLC-UV in the depletion of the third set. However, it has been reported that the results for 82 chemicals, including depletion, are almost the same as those for HPLC-UV using the same method as in this study, in which the reaction solution was diluted 10-fold before fluorescence measurement (Wanibuchi *et al*., 2019). Therefore, although the variation was not frequent enough to impact our conclusions, we believe that the depletion may fluctuate to some extent owing to variation in the dispensing amount in the dilution operation. It has been confirmed that the fluorescence detectors on the market have a large dynamic range, and no peak saturation occurs even when the undiluted reaction solution is measured (data not shown). Therefore, since the above-mentioned dilution operation of the reaction solution will not be necessary in the future, the variation in depletion caused by this operation should no longer be a concern.

For farnesal, the SD of depletion in the three tests exceeded 10% in Lab.5, Lab.3, and Lab.4 for NAC and in Lab.5 for NAL. In addition, the depletion between laboratories varied widely, with NAC depletion ranging from 25.1% to 88.8% and NAL depletion ranging from 1.9% to 28.0%. Farnesal is an aldehyde that changes to a non-reactive alcohol or carboxylic acid by reduction or oxidation, respectively. Therefore, it is possible that some of the farnesal changed depending on the storage conditions and the environment when handling the test chemical in each laboratory, which affected the reactivity. In addition, since there was no variation between facilities in the validation study for ADRA (1 mM), the possibility of differences in storage temperature depending on the season is also a concern. Despite these possibilities, although there

were variations in depletion, the results of all the laboratories were clearly positive, and there was no effect on the test reproducibility with ADRA (0.5 mg/ml).

From the above, most chemicals with variability in NAC or NAL depletion are believed to be caused by accidental test errors and chemical stability, and all the results in which there was no mean depletion around the criteria clearly distinguished between negative and positive. In addition, since the negative/positive judgments were 100% in agreement for all the test chemicals within-/between-laboratories, it was confirmed that ADRA (0.5 mg/ml) and HPLC-FL possessed the same highly reproducible test conditions as ADRA (1 mM).

In the ADRA test procedure, water is the first priority solvent in the solvent selection for test chemicals. Therefore, in the ADRA (1 mM) validation study, water was selected as the solvent for SADE in all laboratories. However, because SADE can be hydrolyzed in an aqueous solvent, the test was conducted in acetonitrile in addition to water in this study.

As described in the Results section, the variation in depletion for SADE was larger in the aqueous solvent than in the acetonitrile solvent for both NAC and NAL. The reason for this was that depletion of the aqueous solvent in the second set from Lab.2 was low, and there was almost no variation in the other depletion results. Although the exact cause of the low depletion of NAC and NAL in the second test from Lab.2 is unknown, it is possible that the test chemical solution was prepared incorrectly or the reactivity decreased due to hydrolysis of the test chemical. There was no difference in depletion between HPLC-UV and HPLC-FL in any of the solvents.

Thus, high reproducibility was confirmed both within and between laboratories in both aqueous and acetonitrile solvents, except for the result of the second test from Lab.2. Although high reproducibility was obtained in this study, when using SADE as a positive control, it is preferable to dissolve it in an acetonitrile solvent since SADE may be hydrolyzed in an aqueous environment as mentioned above.

5. Conclusion

As a result of three tests performed at five laboratories using 10 proficient substances and 1 new positive control, high WLR and BLR were confirmed in both the evaluation by ADRA (0.5 mg/ml) and the measurement by HPLC-FL, and the practicality of these test methods was validated.

KeratinoSens and the human cell line activation test (h-CLAT), which evaluate key events 2 and 3 in the skin sensitization AOP, use test substance solutions prepared by weight and can be utilized to evaluate mixtures. If ADRA (0.5 mg/ml) and HPLC-FL,

whose applicability has been confirmed in this study, are adopted in the OECD test guidelines, it will be possible to evaluate mixtures that could not be evaluated by conventional ADRA or DPRA. This makes it possible to evaluate the mixture using three alternative methods with different key events, and it is expected that a more accurate prediction of skin sensitization will be possible.

Conflict of interest

The authors declare that there is no conflict of interest.

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Figure Legends

Fig. 1. NAC and NAL depletion for squaric acid diethyl ester in different solvents

The depletion of NAC and NAL for squaric acid diethyl ester when using test chemical solutions prepared with two different solvents, water and acetonitrile is shown. The graph on the left shows the depletion of NAC, and the graph on the right shows the depletion of NAL. Black circles (●) indicate the depletion measured by HPLC-UV and white circles (○) indicate the depletion measured by HPLC-FL.

Table 1. Test chemicals

No.	Test chemical	CAS No.	Source	LLNA		Solvent
				Category	EC3	
Proficiency substancies						
1	*p* -Benzoquinone	106-51-4	FUJIFILM Wako	Extreme	0.0099	Water
2	Diphenylcyclopropenone	886-38-4	FUJIFILM Wako	Extreme	0.003	Acetonitrile
3	2-Methyl-2*H* -isothiazol-3-one	2682-20-4	FUJIFILM Wako	Strong	0.4	Water
4	Palmitoyl chloride	112-67-4	FUJIFILM Wako	Moderate	8.8	Acetone
5	Imidazolidinyl urea	39236-46-9	Sigma-Aldrich	Weak	24	Water
6	Farnesal	19317-11-4	Frinton	Weak	12	Acetonitrile
7	Glycerol	56-81-5	FUJIFILM Wako	Non-sensitizer	NC	Water
8	Isopropanol	67-63-0	FUJIFILM Wako	Non-sensitizer	NC	Water
9	Dimethyl isophthalate	1459-93-4	TCI	Non-sensitizer	NC	Acetonitrile
10	Propyl paraben	94-13-3	TCI	Non-sensitizer	NC	Acetonitrile
Positive control						
	Squaric acid diethyl ester	5231-87-8	TCI	Strong	0.9	Water / Acetonitrile

Frinton, Frinton Laboratories, Inc., Hainesport, NJ, USA; Sigma-Aldrich, Sigma-Aldrich Corporation, St. Louis, MO, USA; TCI, Tokyo Chemical Industry Co. Ltd., Tokyo, Japan; FUJIFILM Wako, FUJIFILM Wako Pure Chemical Industries Ltd., Osaka, Japan.

Table 2. NAC depletion for the 10 proficiency substances at the five laboratories

Test chemical	Depletion of NAC (%)																										
	Lab. 1					Lab. 2					Lab. 3					Lab. 4					Lab. 5					Over all	
	1st set	2nd set	3rd set	Mean	SD	1st set	2nd set	3rd set	Mean	SD	1st set	2nd set	3rd set	Mean	SD	1st set	2nd set	3rd set	Mean	SD	1st set	2nd set	3rd set	Mean	SD	Mean	SD
Result of UV detection																											
p-Benzoquinone	100.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	100.0	0.0	-	-	-	-	-	97.5	100.0	100.0	99.2	1.4	99.8	0.7
Diphenylcyclopropenone	50.1	49.9	55.3	51.8	3.1	47.2	49.6	48.2	48.3	1.2	62.2	56.0	60.1	59.4	3.2	-	-	-	-	-	53.2	51.6	52.2	52.3	0.8	53.0	4.7
2-Methyl-2H-isothiazol-3-one	100.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	100.0	0.0	94.1	93.3	91.8	93.0	1.2	-	-	-	-	-	93.0	92.6	92.8	92.8	0.2	96.5	3.7
Palmitoyl Chloride	9.7	14.4	20.8	15.0	5.6	0.0	5.8	6.0	3.9	3.4	12.2	11.8	19.8	14.6	4.5	-	-	-	-	-	14.5	12.5	13.5	13.5	1.0	11.8	5.8
Imidazolidinyl urea	31.1	38.5	46.0	38.5	7.5	23.8	25.2	22.6	23.9	1.3	36.6	39.4	33.7	36.6	2.8	-	-	-	-	-	37.1	33.8	35.3	35.4	1.7	33.6	6.9
Farnesal	34.2	36.8	45.6	38.9	6.0	54.0	53.5	54.6	54.0	0.6	77.5	88.8	66.2	77.5	11.3	-	-	-	-	-	25.1	46.8	45.7	39.2	12.3	52.4	18.1
Glycerol	1.0	2.9	0.0	1.3	1.5	0.0	0.5	1.3	0.6	0.6	2.5	0.4	0.1	1.0	1.3	-	-	-	-	-	1.5	0.1	0.1	0.6	0.8	0.9	1.0
Isopropanol	1.2	0.0	0.0	0.4	0.7	0.0	0.0	0.3	0.1	0.2	1.6	2.0	0.0	1.2	1.0	-	-	-	-	-	0.3	0.0	0.3	0.2	0.2	0.5	0.7
Dimethyl isophthalate	0.0	3.6	1.2	1.6	1.8	1.6	0.4	0.2	0.7	0.7	3.5	4.2	3.3	3.6	0.5	-	-	-	-	-	1.8	0.9	0.0	0.9	0.9	1.7	1.5
Propyl paraben	0.0	3.7	0.7	1.5	2.0	2.8	0.0	1.2	1.4	1.4	7.5	3.9	3.2	4.9	2.3	-	-	-	-	-	1.2	0.2	0.0	0.5	0.6	2.0	2.3
Result of FL detection																											
p-Benzoquinone	100.0	100.0	100.0	100.0	0.0	100.0	100.0	99.5	99.8	0.3	100.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	100.0	0.0	100.0	0.1
Diphenylcyclopropenone	50.0	51.8	56.6	52.8	3.4	50.1	49.2	47.2	48.9	1.5	61.2	54.3	58.0	57.9	3.5	99.8	99.7	50.0	83.2	28.7	52.5	51.4	52.5	52.2	0.6	59.0	17.0
2-Methyl-2H-isothiazol-3-one	98.9	98.9	100.0	99.3	0.6	98.0	97.9	97.7	97.9	0.2	100.0	100.0	100.0	100.0	0.0	98.4	98.1	98.7	98.4	0.3	99.1	99.2	99.1	99.1	0.1	98.9	0.8
Palmitoyl Chloride	25.5	19.4	24.0	23.0	3.2	4.7	4.4	4.9	4.6	0.3	10.2	10.9	28.5	16.5	10.4	3.9	15.8	16.9	12.2	7.2	16.7	13.3	14.9	15.0	1.7	14.3	7.9
Imidazolidinyl urea	31.3	31.3	44.7	35.8	7.7	18.2	27.2	39.0	28.1	10.4	25.7	27.5	28.7	27.3	1.5	19.9	31.2	24.2	25.1	5.7	29.6	27.0	26.5	27.7	1.7	28.8	6.6
Farnesal	47.3	45.8	60.1	51.1	7.9	51.7	49.4	55.5	52.2	3.1	75.6	85.7	75.5	79.0	5.9	48.5	56.9	72.9	59.5	12.4	29.1	46.0	46.4	40.5	9.8	56.4	15.0
Glycerol	0.7	0.6	2.3	1.2	1.0	0.1	1.6	4.0	1.9	2.0	1.0	0.9	1.3	1.1	0.2	0.0	2.4	0.4	0.9	1.3	6.3	0.5	0.1	2.3	3.4	1.5	1.7
Isopropanol	1.3	3.1	2.4	2.3	0.9	2.5	0.7	0.3	1.1	1.2	0.4	4.7	0.6	1.9	2.4	0.0	3.3	0.7	1.3	1.8	0.6	0.3	0.1	0.3	0.3	1.4	1.4
Dimethyl isophthalate	5.5	0.0	1.7	2.4	2.8	1.5	0.1	1.8	1.1	0.9	3.1	2.3	0.0	1.8	1.6	0.0	3.1	0.0	1.0	1.8	3.2	0.7	1.0	1.7	1.4	1.6	1.6
Propyl paraben	3.4	3.2	0.7	2.4	1.5	1.4	0.4	3.2	1.7	1.4	7.3	2.6	0.0	3.3	3.7	0.0	5.2	0.0	1.7	3.0	3.0	0.3	1.3	1.5	1.4	2.1	2.1

Table 3. NAL depletion for the 10 proficiency substances at the five laboratories

Test chemical	Depletion of NAL (%)																										
	Lab. 1					Lab. 2					Lab. 3					Lab. 4					Lab. 5					Over all	
	1st set	2nd set	3rd set	Mean	SD	1st set	2nd set	3rd set	Mean	SD	1st set	2nd set	3rd set	Mean	SD	1st set	2nd set	3rd set	Mean	SD	1st set	2nd set	3rd set	Mean	SD	Mean	SD
Result of UV detection																											
p-Benzoquinone	100.0	91.4	89.6	93.7	5.6	100.0	100.0	89.6	96.5	6.0	93.1	94.2	89.9	92.4	2.3	-	-	-	-	-	90.8	91.3	89.7	90.6	0.8	93.3	4.3
Diphenylcyclopropenone	1.9	9.1	7.0	6.0	3.7	2.4	2.6	1.4	2.2	0.7	2.0	8.2	6.8	5.7	3.2	-	-	-	-	-	4.3	3.2	3.2	3.6	0.6	4.4	2.7
2-Methyl-2H-isothiazol-3-one	0.2	0.0	1.3	0.5	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.5	1.5	0.7	0.8	-	-	-	-	-	0.0	0.0	0.0	0.0	0.0	0.3	0.5
Palmitoyl Chloride	98.2	100.0	100.0	99.4	1.0	100.0	100.0	100.0	100.0	0.0	100.0	100.0	100.0	100.0	0.0	-	-	-	-	-	100.0	100.0	100.0	100.0	0.0	99.9	0.5
Imidazolidinyl urea	2.0	1.8	2.1	2.0	0.2	2.5	0.0	0.0	0.8	1.4	0.8	0.7	2.4	1.3	1.0	-	-	-	-	-	6.3	1.2	1.1	2.8	3.0	1.7	1.7
Farnesal	4.4	9.8	16.0	10.1	5.8	6.8	8.5	14.9	10.1	4.3	7.3	14.6	16.1	12.7	4.8	-	-	-	-	-	16.8	4.9	1.9	9.1	10.1	10.5	5.8
Glycerol	0.0	0.3	0.0	0.1	0.2	0.0	2.3	0.0	0.8	1.3	0.0	0.0	1.3	0.4	0.7	-	-	-	-	-	2.5	0.6	0.3	0.3	0.3	0.4	0.7
Isopropanol	0.2	0.8	0.0	0.3	0.4	0.0	0.2	0.0	0.1	0.1	0.0	0.1	0.5	0.2	0.2	-	-	-	-	-	0.0	0.6	0.0	0.2	0.3	0.2	0.3
Dimethyl isophthalate	0.0	0.2	4.1	1.4	2.3	0.0	0.0	0.0	0.0	0.0	0.0	5.1	4.6	3.2	2.8	-	-	-	-	-	0.0	0.3	0.0	0.1	0.2	1.2	2.1
Propyl paraben	0.0	4.9	4.5	3.1	2.7	0.0	0.0	0.0	0.0	0.0	0.0	4.5	4.8	3.1	2.7	-	-	-	-	-	0.0	0.5	0.0	0.2	0.3	1.6	2.3
Result of FL detection																											
p-Benzoquinone	89.1	90.5	90.3	90.0	0.8	89.8	90.0	90.6	90.1	0.4	93.1	92.9	91.6	92.5	0.8	91.5	88.7	86.3	88.8	2.6	92.4	90.4	92.1	91.6	1.1	90.6	1.8
Diphenylcyclopropenone	0.0	6.7	6.8	4.5	3.9	3.6	2.3	2.6	2.8	0.7	2.9	8.0	4.1	5.0	2.7	0.0	7.6	0.8	2.8	4.2	4.4	3.8	6.6	4.9	1.5	4.0	2.7
2-Methyl-2H-isothiazol-3-one	0.0	0.8	5.2	2.0	2.8	1.6	0.2	0.1	0.6	0.9	0.3	0.2	3.3	1.3	1.7	0.0	11.0	0.0	3.7	6.3	0.2	0.6	1.8	0.9	0.8	1.7	3.0
Palmitoyl Chloride	98.2	99.9	99.9	99.3	1.0	97.6	100.0	100.0	99.2	1.4	100.0	100.0	100.0	100.0	0.0	98.8	92.9	99.9	97.2	3.8	100.0	100.0	100.0	100.0	0.0	99.1	1.9
Imidazolidinyl urea	2.0	0.0	5.4	2.5	2.7	0.0	0.0	0.0	0.0	0.0	2.6	1.3	7.7	3.9	3.4	0.0	9.2	0.0	3.1	5.3	4.1	0.7	1.9	2.2	1.7	2.3	3.0
Farnesal	5.9	5.2	17.7	9.6	7.0	9.3	9.1	17.0	11.8	4.5	10.3	20.5	22.5	17.8	6.5	11.1	20.3	19.3	16.9	5.0	28.0	8.9	10.7	14.7	8.5	14.1	6.3
Glycerol	1.4	0.0	4.0	1.8	2.0	0.0	4.8	0.0	1.6	2.8	1.0	0.1	7.5	2.9	4.1	0.0	0.3	0.0	0.1	0.2	0.0	0.7	1.4	1.3	0.5	1.5	2.2
Isopropanol	0.0	0.6	4.0	1.5	2.2	1.6	1.0	1.3	1.3	0.3	1.7	2.0	0.8	1.5	0.7	0.0	0.3	0.0	0.1	0.2	0.4	1.0	1.3	0.9	0.4	1.1	1.0
Dimethyl isophthalate	0.0	0.0	6.5	2.2	3.8	0.0	0.0	0.0	0.0	0.0	1.7	3.2	5.2	3.4	1.7	0.1	3.5	0.0	1.2	2.0	0.3	1.2	3.1	1.4	1.5	1.6	2.2
Propyl paraben	0.5	4.4	5.7	3.5	2.7	0.0	0.0	0.0	0.0	0.0	1.3	3.1	1.7	2.0	0.9	0.0	2.3	0.0	0.8	1.3	0.0	0.9	3.2	1.4	1.6	1.5	1.8

Table 4. Mean depletion for the 10 proficiency substances at the five laboratories

Test chemical	LLNA category	Mean depletion (%)														
		Lab.1			Lab.2			Lab.3			Lab.4			Lab.5		
		1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set	1st set	2nd set	3rd set
Result of UV detection																
p-Benzoquinone	Extreme	100.0	95.7	94.8	100.0	100.0	94.8	96.6	97.1	94.9	-	-	-	94.1	95.7	94.9
Diphenylcyclopropenone	Extreme	26.0	29.5	31.2	24.8	26.1	24.8	32.1	32.1	33.5	-	-	-	28.7	27.4	27.7
2-Methyl-2H-isothiazol-3-one	Strong	50.1	50.0	50.7	50.0	50.0	50.0	47.0	46.9	46.7	-	-	-	46.5	46.3	46.4
Palmitoyl Chloride	Moderate	54.0	57.2	60.4	50.0	52.9	53.0	56.1	55.9	59.9	-	-	-	57.3	56.3	56.8
Imidazolidinyl urea	Weak	16.6	20.2	24.1	13.2	12.6	11.3	18.7	20.0	18.1	-	-	-	21.7	17.5	18.2
Farnesal	Weak	19.3	23.3	30.8	30.4	31.0	34.8	42.4	51.7	41.2	-	-	-	20.9	25.9	23.8
Glycerol	NS	0.5	1.6	0.0	0.0	1.4	0.6	1.3	0.2	0.7	-	-	-	2.0	0.4	0.2
Isopropanol	NS	0.7	0.4	0.0	0.0	0.1	0.1	0.8	1.0	0.2	-	-	-	0.2	0.3	0.2
Dimethyl isophthalate	NS	0.0	1.9	2.7	0.8	0.2	0.1	1.7	4.6	3.9	-	-	-	0.9	0.6	0.0
Propyl paraben	NS	0.0	4.3	2.6	1.4	0.0	0.6	3.8	4.2	4.0	-	-	-	0.6	0.3	0.0
Result of FL detection																
p-Benzoquinone	Extreme	94.6	95.3	95.2	94.9	95.0	95.0	96.6	96.5	95.8	95.8	94.4	93.1	96.2	95.2	96.0
Diphenylcyclopropenone	Extreme	25.0	29.3	31.7	26.9	25.7	24.9	32.1	31.2	31.1	49.9	53.7	25.4	28.4	27.6	29.6
2-Methyl-2H-isothiazol-3-one	Strong	49.5	49.9	52.6	49.8	49.1	48.9	50.1	50.1	51.6	49.2	54.5	49.4	49.7	49.9	50.5
Palmitoyl Chloride	Moderate	61.9	59.7	62.0	51.1	52.2	52.4	55.1	55.4	64.3	51.3	54.3	58.4	58.3	56.7	57.5
Imidazolidinyl urea	Weak	16.7	15.7	25.1	9.1	13.6	19.5	14.2	14.4	18.2	10.0	20.2	12.1	16.9	13.9	14.2
Farnesal	Weak	26.6	25.5	38.9	30.5	29.2	36.2	43.0	53.1	49.0	29.8	38.6	46.1	28.6	27.5	28.6
Glycerol	NS	1.1	0.3	3.2	0.0	3.2	2.0	1.0	0.5	4.4	0.0	1.4	0.2	3.1	0.6	0.8
Isopropanol	NS	0.7	1.9	3.2	2.0	0.8	0.8	1.0	3.4	0.7	0.0	1.8	0.3	0.5	0.6	0.7
Dimethyl isophthalate	NS	2.8	0.0	4.1	0.8	0.0	0.9	2.4	2.7	2.6	0.0	3.3	0.0	1.8	1.0	2.1
Propyl paraben	NS	2.0	3.8	3.2	0.7	0.2	1.6	4.3	2.8	0.8	0.0	3.8	0.0	1.5	0.6	2.2

Judged as positive; Judged as negative

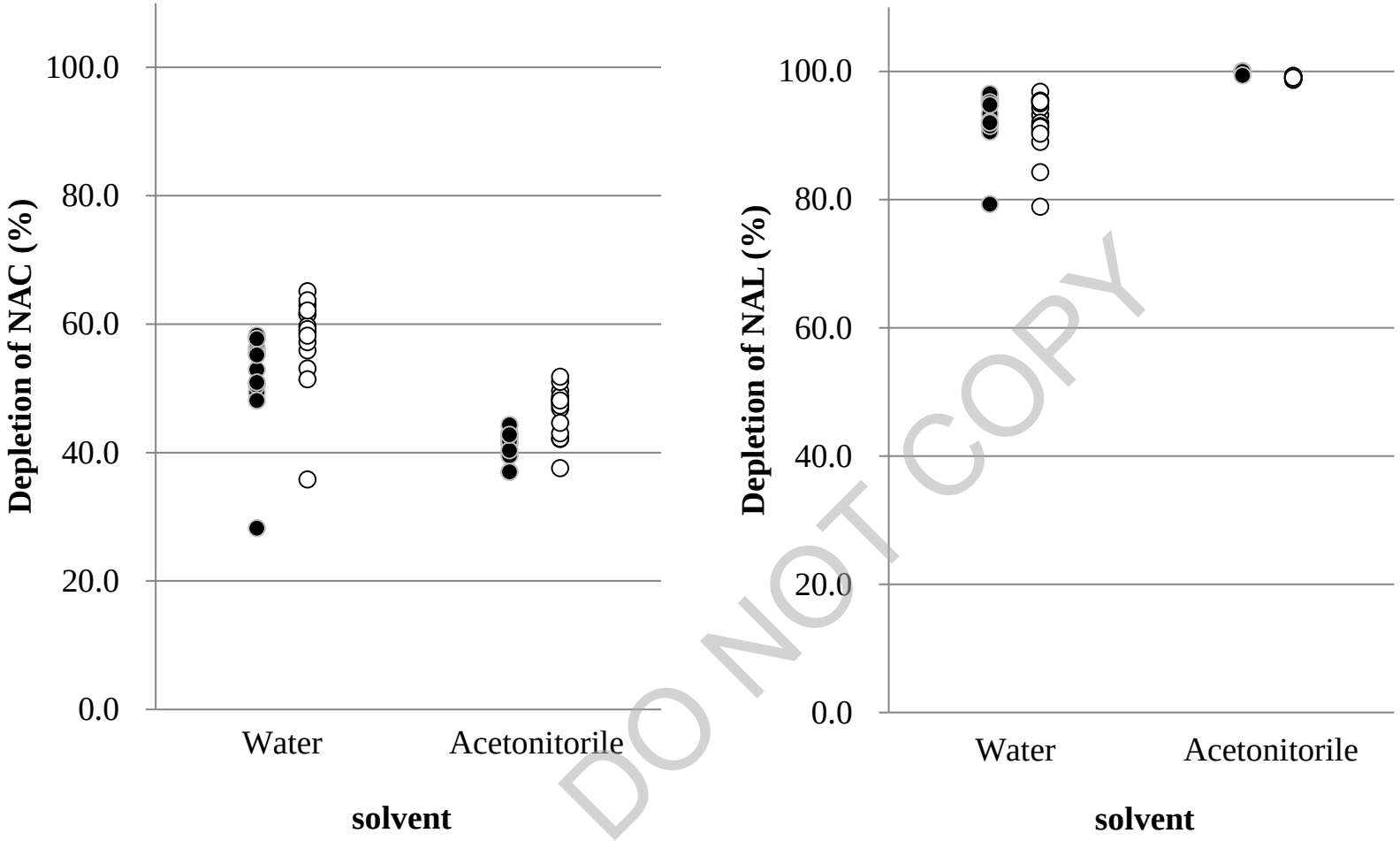

Fig. 1. NAC and NAL depletion for squaric acid diethyl ester in different solvents