



Assessment of skin sensitization under REACH: A case report on vehicle choice in the LLNA and its crucial role preventing false positive results

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ABSTRACT

In the EU, chemicals with a production or import volume in quantities of one metric ton per year or more have to be tested for skin sensitizing properties under the REACH regulation. The murine Local Lymph Node Assay (LLNA) and its modifications are widely used to fulfil the data requirement, as it is currently considered the first-choice method for *in vivo* testing to cover this endpoint.

This manuscript describes a case study highlighting the importance of understanding the chemistry of the test material during testing for 'skin sensitization' of MCDA (mixture of 2,4- and 2,6-diamino-methylcyclohexane) with particular focus on the vehicle used. While the BrdU-ELISA modification of the LLNA using acetone/olive oil (AOO) as vehicle revealed expectable positive results. However, the concentration control analysis unexpectedly revealed an instability of MCDA in the vehicle AOO. Further studies on the reactivity showed MCDA to rapidly react with AOO under formation of various imine structures, which might have caused the positive LLNA result.

The repetition of the LLNA using propylene glycol (PG) as vehicle did not confirm the positive results of the LLNA using AOO. Finally, a classification of MCDA as skin sensitizer according to the Globally Harmonized System (GHS) was not justified.

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1. Introduction

Allergic Contact Dermatitis (ACD) is an inflammatory skin disease caused by skin contact with either an irritant or an allergenic chemical. In contrast to acute irritant contact dermatitis, ACD is characterized by skin lesions, redness, as well as itching usually following more than one skin contact with an allergenic chemical. It is well known that the skin response of ACD is immunologically mediated, delayed, and consists of varying degrees of edema, erythema, and vesiculation. Allergenic chemicals are usually small molecules with a molecular weight of less than 400 g/mol (low molecular weight, LMW) capable to penetrate the skin (Zhai et al., 2008). Typically, they are characterized as incomplete allergens (haptens) until they bind to nucleophilic proteins forming a

complete allergen. This is the so-called molecular initiating event of skin sensitization transforming a non-immunogenic molecule into a potential allergen. The protein reactivity of the substance may be inherent or can develop following metabolic or abiotic transformations of the pro- or pre-haptens, respectively (Gerberick et al., 2007). However, several studies have shown that currently available *in vitro* methods to assess skin sensitization are predicting pro- or pre-haptens better than expected (Urbisch et al., 2016; Patlewicz et al., 2016). The first significant exposure to a hapten may activate the immune system. This induction may take a few days to a few weeks. Following entry into the skin, the allergenic chemical may bind to dendritic cells (Langerhans cells), that direct the allergen to a regional lymph node, where an expansion of the T-lymphocyte population takes place. The next contact with the same allergen then results in an elicitation of a skin response.

It has been reported, that ACD accounts for up to 95% of occupational skin diseases, with epoxy resins implicated as one of the most common causes (Lushniak, 2000). Efficient methods for

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Abbreviations

ACD	Allergic Contact Dermatitis
AOO	acetone:olive oil (4:1, v/v)
AOP	Adverse Outcome Pathway
BrdU-ELISA	5-bromo-2-deoxyuridine based enzyme-linked immunosorbent assay
CE	capillary electrophoresis
CMR	substances that are carcinogenic, mutagenic, or reprotoxic
DPBS	Dulbecco's Phosphate Buffered Saline
DPRA	Direct Peptide Reactivity Assay
EC	estimated concentration
ECHA	European Chemicals Agency
EGDMA	ethylene glycol dimethacrylate
ESI	Electrospray Ionization
EU	European Union

GHS	Globally Harmonized System
GPMT	Guinea Pig Maximization Test
IATA	Integrated Approaches to Testing and Assessment
i.p	intraperitoneally
LLNA	Local Lymph Node Assay
LMW	low molecular weight
LOD	limit of detection
MAO	monoamine oxidase
MCDA	methyl cyclohexyldiamine (mixture of 2,4- and 2,6-isomers)
mmu	milli mass unit.
PG	propylene glycol
REACH	Registration, Evaluation, Authorisation and Restriction of Chemicals
SI	Stimulation Index
TG	test guideline
TMB	tetramethylbenzidine

identification of substances that may pose hazardous risks in the workplace as well as in consumer area is therefore crucial.

The Registration, Evaluation, Authorization and Restriction of Chemicals (REACH) regulation requires that substances produced or imported into the European Union (EU) in quantities of one metric ton per year or more have to be tested for skin sensitization properties (EU, 2006).

In the 1990s, the murine Local Lymph Node Assay (LLNA) was developed as an alternative *in vivo* test suitable for skin sensitization hazard identification and characterization fulfilling the 3R criteria (ICCVAM, 2009). In particular, the LLNA and its modifications allow the use of a reduced number of animals for detection of skin sensitizers compared to the number of guinea pigs necessary in the OECD TG 406 (OECD, 1992). In the standard LLNA (OECD TG 429; OECD, 2002), radio-isotopic thymidine is used to measure lymphocyte proliferation after an induction period. Modified protocols of the LLNA avoiding the use of a radiolabel for lymphocyte proliferation have been proposed including the 5-bromo-2-deoxyuridine based enzyme-linked immunosorbent assay (LLNA: BrdU-ELISA, OECD TG 442B).

Research on the underlying molecular and cellular mechanisms concerning skin sensitization has resulted in the publication of a document by OECD in 2012 formally describing the key events leading to skin sensitization (Adverse Outcome Pathway, AOP) (OECD, 2012a; OECD, 2012b). Recently, *in chemico* as well as *in vitro* assays addressing the first three key events of this AOP were validated and have been adopted as OECD test guidelines: firstly, the Direct Peptide Reactivity Assay (DPRA; OECD, 2015a), secondly the ARE-Nrf2 Luciferase Test (LuSens; OECD, 2015b), and thirdly the human Cell Line Activation Test (h-CLAT; OECD, 2016a). It is well accepted that an individual *in vitro* test will not suffice to cover the endpoint skin sensitization and hence several tests shall be used in so-called Integrated Approaches to Testing and Assessment (IATAs) to investigate skin sensitization potentials without the need for animal testing (Basketter et al., 2015; Rovida et al., 2015; OECD, 2016b). The DPRA addresses the molecular initiating event of skin sensitization, the alteration of a protein/peptide. In the DPRA, the electrophilic reactivity of a test item towards synthetic cysteine (C)- or lysine (K)-containing peptides is evaluated. Chemical reactivity has been shown to be well associated with allergenic potency (Gerberick et al., 2007). Within this context, measuring the amount of proteins with nucleophilic side chains such as cysteine or lysine residues after incubation with potential allergens may serve as

surrogate markers.

From a regulatory point of view, known skin sensitizers are discussed as substances of equivalent concern under article 57f REACH to carcinogenicity, mutagenicity, or reprotoxicity (CMR). The European Chemicals Agency (ECHA) defined a process, which allows the assessment of such substances and identifies a regulatory option to manage potential risk under REACH via authorization, restriction or substance evaluation, respectively (ECHA, 2015). Therefore, a comprehensive evaluation of all available data as well as the correct selection of the most appropriate experimental conditions are crucial for the assessment of skin sensitization.

This manuscript describes a case study highlighting the importance of understanding the chemistry of the test material during testing for 'skin sensitization' of MCDA (mixture of 2,4- and 2,6-diamino-methylcyclohexane, structure see Fig. 1) under REACH.

2. Material and methods

2.1. Animals

All animals were maintained in accordance with national animal protection standards. Female mice (CBA/CaOlaHsd) were adapted to housing conditions over five days at least and only those without any visible signs of illness were used for the *in vivo* studies. The animals were purchased from Harlan Winkelmann GmbH (Borchen, Germany) at an age between 8 and 9 weeks and were individually housed in Makrolon type II cages. Identification was performed by cage number. The experiments were conducted under standard laboratory conditions at a room temperature of $22 \pm 3^\circ\text{C}$ and a relative humidity in the range of 30%–70%. The day-night rhythm was set to 12 h - 12 h (6.00 a.m. - 6.00 p.m./6.00 p.m. - 6.00 a.m.). Feeding (STANRAB (P) SQC, Altrip, Germany) and tap

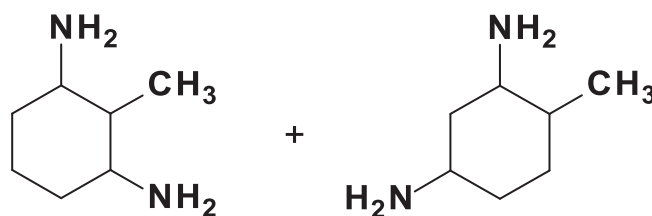


Fig. 1. Structure of MCDA.

water were provided *ad libitum*.

2.2. Chemicals

Acetone (purity: 99%, manufacturer: Merck KGA (Darmstadt, Germany)), propylene glycol (purity: 99.5%, manufacturer: Merck KGA (Darmstadt, Germany)), and olive oil used as vehicles in the *in vivo* tests were purchased from VWR International GmbH (Darmstadt, Germany) and Carl Roth GmbH & Co. KG (Karlsruhe, Germany), respectively. Bromodeoxyuridine (BrdU) as well as the Cell Proliferation ELISA Kit were obtained from Roche Applied Science (Mannheim, Germany). The positive control substance for the LLNA α -hexyl cinnamaldehyde (purity: $\geq 95.0\%$) and the reagents for preparing the buffers used in the *in vitro* experiment or for instrumental analysis (acetic acid; 1,4-dioxane (purity 99.5%); *N,N*-dimethylacetamide (purity: 99.8%); 4-aminopyridine; sodium phosphate, monobasic monohydrate; sodium phosphate, dibasic heptahydrate; ammonium acetate and ammonium hydroxide, 28%–30%) were supplied by Sigma Aldrich (Taufkirchen, Germany). The peptides are custom material (RS Synthesis, Louisville KY, USA) containing phenylalanine to aid in detection and either cysteine or lysine as the reactive center:

- Cysteine- (C-) containing peptide (Ac-RFAACAA-COOH, $M_W = 751.9$ g/mol)
- Lysine- (K-) containing peptide (Ac-RFAAKAA-COOH, $M_W = 776.2$ g/mol)

The positive control substance for the *in vitro* test ethylene glycol dimethacrylate (EGDMA) as well as acetonitrile and trifluoroacetic acid were also purchased from Sigma Aldrich. All other chemicals were available from Merck (HCl, 1 M; H_2SO_4 , 95%–97%; 2-propanol), Alfa Aesar (methylamine x HCl), or Bernd Kraft (ammonia solution, 25%).

2.3. Protocols

2.3.1. *In vivo* experiments: Local Lymph Node Assay (LLNA: BrdU-ELISA)

The studies were carried out in accordance with OECD Guideline 442B under GLP conditions.

According to the recommendations given by this guideline, two pre-experiments were necessary for each study: a solubility test as well as an irritation pre-test to determine the highest non-irritant test concentration to be used in the main study. Therefore, mice were treated by topical (epidermal) application to the dorsal surface of each ear with different test item concentrations in comparison to the respective vehicle once daily on three consecutive days. Prior to the first application of the test item and before sacrifice, the body weight was determined. Clinical signs were recorded at least once daily. Putative signs of local irritation were documented, and a score was used to grade a possible reddening of the ear skin. Furthermore, prior to the first application of the test item, on day 2 and before sacrifice on day 5, the ear thickness was determined using a micrometer (C110TKroeplin, Schlüchtern, Germany). Additionally, the ears were punched after sacrifice on day 5 at the apical area using a biopsy punch (Stiefel, $\varnothing$ 8 mm corresponding to 0.5 cm^2) and were immediately pooled per animal and weighed using an analytical balance. Putative ear irritation was considered to be excessive if reddening of the ear skin of a score ≥ 3 was detected at any observation time and/or if an increase in ear thickness of $\geq 25\%$ was recorded on day 2 or day 5 (exclusion criteria). The measured ear weight was also considered in this evaluation. Within the main study, each test group of mice was treated in an equivalent way as for the irritation pre-test by topical

application to the dorsal surface of each ear with respective test item concentrations in AOO (4:1, v/v) or PG. An application volume of $25\text{ }\mu\text{L/ear/day}$ was spread over the entire dorsal surface ($\varnothing$ 8 mm) of each ear once daily for three consecutive days. The control test group of mice was treated with an equivalent volume of the relevant vehicle alone. Additionally, a test group of mice was treated with 25% (w/w) α -hexyl cinnamaldehyde dissolved in the corresponding vehicle, serving as concurrent positive control.

Four days after the first topical application (day 5), the mice were injected intraperitoneally (i.p.) with 0.5 mL of a BrdU solution in DPBS (10 mg/mL corresponding to 5 mg per mouse and injection). Approximately 24 h after treatment with BrdU mice were sacrificed and the draining lymph nodes were rapidly excised, pooled for each animal, and weight was determined (XS 204, Mettler Toledo, Gießen, Germany). Single cell suspensions of pooled lymph node cells were prepared by gentle mechanical disaggregation through a nylon mesh (mesh size $70\text{ }\mu\text{m}$). The lymph node cells were re-suspended in 6 mL DPBS. After determination of the lymph node cell count using a cell counter (Casy Cell Counter, Innovatis, Reutlingen, Germany), cell suspensions' cell density were adjusted to 100.000 cells/mL. The incorporation of BrdU into lymph node cells was determined using a commercial cell proliferation assay kit: 100 μL of the lymph node cell suspension were added to the wells of a flat-bottom microplate in triplicate. After fixation and denaturation of the lymph node cells with FixDenat (included in Cell Proliferation Elisa Kit), anti-BrdU antibody was added to each well and allowed to react. Subsequently, the anti-BrdU antibody was removed by washing and the substrate solution TMB (included in Cell Proliferation Elisa Kit) was then added and allowed to produce chromogen. The substrate reaction was determined by a measurement of the absorbance at 370 nm with a reference wavelength of 492 nm (Absorbance-Reader SUNRISE, Tecan, Männergendorf, Switzerland). The determination of ear weights was achieved as described within the pre-experiment above. The proliferative response of the lymph node cells was expressed as the mean Stimulation Index (SI). The SI is derived by dividing the mean BrdU labeling index/mouse within each test substance group as well as for the positive control group by the mean BrdU labeling index for the vehicle group. A test item was regarded as a sensitizer in the LLNA, if the following criteria were fulfilled:

- exposure to at least one concentration of the test item resulted in an incorporation of BrdU at least 1.6-fold or greater than that recorded in control mice, as indicated by the SI
- data are compatible with a conventional dose response, although exemptions must be allowed (especially at high topical concentrations) for either local toxicity or immunological suppression

Furthermore, an index was calculated for the lymph node weight and -cell count as well as for the ear weight by dividing the mean values of the test item treated groups by the mean of the vehicle control group. For BALB/c mice, a cutoff-value for the lymph node cell count index of 1.55 was reported for a positive response (Ehling et al., 2005). The skin sensitization potential of a positive test substance can further be characterized by the estimated concentration (EC) 1.6, which is the cut-off concentration level above which a positive result is estimated ($SI > 1.6$). This EC 1.6 value would be calculated from the concentration response curve by linear regression, if a clear dose-response was proven.

2.3.2. Concentration control analysis of samples used in the *in vivo* experiments

In order to comply with the GLP requirements, concentration

control analyses of the three testing solutions as well as the corresponding vehicle controls used in each LLNA were performed following the *in vivo* tests. Samples of 1 mL each were taken immediately before application of the test item. The test item aliquots were stored at $\leq -20^\circ\text{C}$ until analysis.

2.3.2.1. Sample processing and analysis of MCDA in acetone/olive oil (AOO). The internal standard solutions were prepared as follows: methylamine*HCl (40 mg) was exactly weighed to the nearest 0.01 mg into a volumetric flask (50 mL), dissolved and filled up to the mark with demineralized water. For the calibration solutions, pure MCDA (20–36 mg) was also exactly weighed into volumetric flasks (50 mL) and dissolved with water and a drop of HCl. The flasks were filled up to the mark with demineralized water and five different calibration solutions were prepared to achieve a concentration range of approximately 1.6–14.4 $\mu\text{g/mL}$. An aliquot of 0.5 mL of the internal standard solution was added before dilution to the final volume.

The thawed samples were quantitatively transferred into flasks (100 mL) and filled up to the mark with acetone. A volume of 5 mL of each sample was transferred into another flask (25 or 50 mL), HCl (1 mL), and internal standard solution (0.5 mL or 1 mL, respectively) was added before the flasks were filled up to the mark with demineralized water.

To check also for stability of MCDA in the vehicle after sample preparation, the samples were stored after shaking for one hour and overnight at room temperature, respectively. Thereafter, aliquots were filtered (0.2 μm "Weißbandfilter") and analyzed. For the blank, two preparations were used. On the one hand, the vehicle was diluted with water (1:10; v/v) and injected, on the other the vehicle was prepared in an analogous way to the sample and injected.

The measurement was performed using capillary electrophoresis (CE; Beckman P/ACE MDQ automated capillary electrophoresis system, Fullerton, California, United States) with indirect UV detection (CE-UV) at 260 nm and an injection pressure of 69 mbar over 5 s. For separation, an uncoated fused silica capillary (70/60 cm total length/length to detector; 75 μm internal diameter) was used. The cathode as well as anode electrolytes were pH adjusted (pH 3.4, using H_2SO_4) 4-aminopyridine (5 mM) working with a voltage of 25 kV and a current of circa 12 μA resulting in a field intensity of 357 V/cm ($T = 25^\circ\text{C}$).

For detection of potential MCDA reaction products with the vehicle AOO (see also Table 3), a qualitative determination of the chemical structures of possible reaction products was carried out (non-GLP) using high resolution mass spectrometry (Bruker maXis time of flight (TOF) mass spectrometer, Bremen, Germany) coupled to a CE (Agilent 7100A, Waldbronn, Germany) system (CE-ESI-MS).

For the CE-ESI-MS measurement MCDA (45–50 mg) was weighed to the nearest 0.01 mg into a volumetric flask (25 mL), dissolved in acetone and filled up to volume. A volume of 1 mL was transferred into a volumetric flask (5 mL) and filled up to volume with water. To achieve a chromatographic separation, a bare silica

column (85 cm total length; 75 μm internal diameter) with an aqueous buffer solution containing NH_4 -formate (20 mg/L; pH 4.3, adjusted with formic acid), as well as an aqueous-alcoholic sheath liquid (0.1% acetic acid in water/2-propanol, 1:1, v/v) were used. The working voltage was 25 kV at a column temperature of 23°C . MS-dependent parameters were as follows: spray voltage 4.5 kV; pressure of nebulizer gas 0.3 bar (assisted by the addition of gaseous NH_3 via the source gas inlet); positive polarity; scan range 4–2000 (m/z); mass accuracy < 3 mmu; resolution 30,000. Data were evaluated by Compass Software (Compass Software 1.7; Bruker, Bremen, Germany).

2.3.2.1.1. Reactivity of MCDA towards acetone. To confirm a reactivity of MCDA towards acetone in pH neutral conditions, the following analysis was performed in duplicate. MCDA (201.8 mg and 197.2 mg) was weighed into a volumetric flask (20 mL) and was filled up with AOO (4:1, v/v) to a gross weight of 10.00 g and 10.01 g, reaching a final concentration of 2.02 g/100 g and 1.97 g/100 g, respectively. The injection volume was 2 μL .

The analytical determination was performed using gas chromatography (Agilent 6890 with autosampler) coupled with a flame ionization detector (GC-FID). The chromatographic separation was performed on a Rtx-35-Amine column (Restek, 30 m length, 0.25 mm internal diameter, 0.5 μm film thickness). GC-dependent parameters were as follows: carrier gas helium; split ratio 1:20; flow rate 1.1 mL/min; injector temperature 280°C ; detector temperature 310°C . The temperature program started with a temperature of 100°C , which was raised to 120°C (1°C/min). After reaching 120°C the temperature was further increased to 300°C (30°C/min), which was then held constant for further 10 min.

2.3.2.2. Sample preparation and analysis of MCDA in propylene glycol (PG). The internal standard solutions were prepared as follows: *N,N*-dimethylacetamide was dissolved in 1,4-dioxane to obtain a final concentration of 14.7 mg/mL. Four calibration mixtures were prepared by mixing MCDA (33.3–277.1 mg) with PG (circa 2000 mg). About 200 mg of these mixtures were dissolved in the internal standard solution (1.0 mL). The thawed samples were exactly weighted (circa 200 mg) and dissolved in the internal standard solution (1 mL).

The measurement was performed using capillary gas chromatograph equipped with a flame ionization detector (GC-FID). The chromatography was performed with a fused silica capillary column (DB 35 MS, 30 m length, 0.25 mm internal diameter, 0.25 μm film thickness). GC-dependent parameters were as follows: carrier gas nitrogen; head pressure 12 psi; split ratio 60:1; septum purge 5 mL/min; injector temperature 250°C ; detector temperature 300°C . A chromatographic separation was achieved via a temperature gradient from 80°C (2 min isothermal) to 260°C with 5°C/min .

2.3.3. *In vitro* experiment: Direct Peptide Reactivity Assay (DPRA)

The DPRA was conducted according to published protocols (Gerberick et al., 2007; OECD, 2015a). MCDA was incubated with synthetic peptides for 24 h at room temperature, and the remaining non-depleted peptide concentration was determined by high performance liquid chromatography (HPLC) with gradient elution and UV-detection at 220 nm. The peptides are custom-made material containing phenylalanine to facilitate UV-detection, and either cysteine or lysine as the nucleophilic center. MCDA was dissolved at a concentration of 100 mM in demineralized water. Three samples of this stock solution were incubated with each peptide in ratios of 1:10 for the C-peptide (100 mM phosphate buffer, pH 7.5) or 1:50 for the K-peptide (100 mM acetate buffer, pH 10.2). Additionally, triplicates of the concurrent vehicle control (negative control) were incubated with the peptides. In order to detect a possible

Table 1

Study mean values of Stimulation Indices for lymph node cell count (LNCC), lymph node weight (LNW), ear weights (EW), and BrdU labeling index.

Vehicle	MCDA concentration [%]	EW	BrdU labeling index
AOO	0.5	0.99	1.5
	1.0	1.01	1.6
	2.0	1.07	3.0
PG	2.0	1.09	0.9
	5.0	1.05	1.0
	10.0	1.04	1.1

Table 2

Mean peptide depletions of cysteine, lysine and both peptides; negative depletions were set to zero for calculation of mean peptide depletion [SD: standard deviation, PC: positive control].

	Cysteine-peptide $\pm$ SD [%]	Lysine-peptide $\pm$ SD [%]	Mean of both depletions [%]
MCDA	5.77 $\pm$ 2.28	−5.49 $\pm$ 0.81	2.88
PC EGDMA in H ₂ O	82.20 $\pm$ 4.77	14.40 $\pm$ 1.65	48.30

Table 3

CE-ESI-MS results, concentration control analysis of MCDA in AOO (4:1, v/v) and PG [n.d.: not determined].

Vehicle	Nominal concentration [g/100 g]	Storage time: 1 h [g/100 g]	Storage time: overnight [g/100 g]
AOO	2.0	0.76	1.55
	1.0	0.32	0.78
	0.5	0.14	0.33
PG	2.0	1.97	n.d.
	5.0	5.14	n.d.
	10.0	10.42	n.d.

interference of MCDA with the peptides, a co-elution control was also performed. These samples consisted of the test substance, vehicle and the respective buffer but without peptide. Moreover, the samples were analyzed by measuring UV absorbance at 258 nm and the area ratio 220/258 was calculated as a measure of peak purity.

The peptide depletion of MCDA incubated samples was compared to the peptide depletion of the negative control samples and expressed as relative peptide depletion. The mean peptide depletion of MCDA was calculated as average of C- and K-peptide depletion and used for evaluation of the chemical reactivity (Gerberick et al., 2007). Analysis of free peptides was performed by RP-HPLC (Agilent 1100 Series) with UV detection at 220 nm using a C18 (2) Phenomenex Luna[®] column (2.0 $\times$ 100 mm, 3 μ m particle size) equipped with a security guard column (C18; 2.0 $\times$ 4 mm). Separation of peptides was performed by isocratic flow with a flow rate of 0.35 mL/min (mobile phase A: 0.1% trifluoroacetic acid in demineralized water) and a linear gradient from 10% to 25% mobile phase B (0.085% trifluoroacetic acid in acetonitrile, v/v) over 10 min, followed by an increase to 90% mobile phase B.

2.4. Statistical analyses

Mean values and standard deviations were calculated using Excel (Version, 2010). A statistical analysis was conducted on the BrdU values, the ear weights, the lymph node weights and the lymph node cell count to assess whether the difference was statistically significant between test item groups and the negative control group. For all statistical calculations, Statistica (Version 10) was used. Mann-Whitney-U test for non-parametric comparison was applied as statistical test. Statistical significance was set at the five per cent level ($p < 0.05$).

3. Results

3.1. Local Lymph Node Assay (BrdU-ELISA modification) using acetone/olive oil (AOO) as vehicle

After application of MCDA in AOO at concentrations of 0.5%, 1%, and 2%, the animals did not show any relevant signs of systemic

toxicity during the course of the study and no cases of mortality were observed. Body weights recorded prior to the first application and prior to sacrifice were within the range commonly reported for animals of this strain and age (data not shown; Harlan, 2014). According to OECD guideline 442B, an increase in ear weight exceeding the threshold value of 25% was considered to be indicative for excessive local skin irritation. This threshold was not exceeded in any MCDA treated group as all ear weight Stimulation Indices (S.I.) were clearly below the threshold (data not shown).

A statistically significant increase in lymph node weights and -cell counts was observed in the 1% (S.I.: 1.3 and 1.6) and 2% (S.I.: 1.5 and 1.9) group and in the positive control (S.I.: 2.0 and 2.4) in comparison to the vehicle control group (see also Table 1). A statistically significant increase in ear weights was observed in the positive control in comparison to the vehicle control group only (S.I.: 1.1). The S.I.s for BrdU labeling were 1.5, 1.6, and 3.0 at concentrations of 0.5%, 1% and 2% (w/w), respectively. A test item is regarded as a sensitizer in the LLNA, if exposure to one or more test item concentrations results in a 1.6-fold or greater increase in incorporation of BrdU compared with concurrent controls, as indicated by the S.I. (EC1.6). Based on the S.I. obtained with 1% MCDA concentration, an EC1.6 value of 1% (w/w) was determined. MCDA was therefore considered as skin sensitizing when tested in AOO as vehicle.

3.1.1. Concentration control analysis of samples used in the LLNA with AOO as vehicle

The concentration control analyses of the samples of the LLNA using AOO as vehicle were performed via CE-UV measurement and is given as rounded mean value of two injections (see Table 3). The values found corresponded only to 28%–38% of the expected concentrations after a storage time of 1 h and 66%–78% after overnight storage of the expected concentrations showing an instability of MCDA in AOO.

To further investigate the instability of MCDA in AOO and to identify possible reaction products, an additional qualitative CE-ESI-MS analysis was performed. The putative reaction products are shown in Fig. 2. Structures were derived from the chemical structure of one of the two main components of MCDA (4-methylcyclohexane-1,3-diamine; CAS no. 13897-55-7) and elemental composition, which was calculated from the accurate masses (data not shown). Amongst others, theoretically identified structures could be 4-methylcyclohexane-1,3-diamine (1), 3-(isopropylideneamino)-4-methyl-cyclohexanamine (2 and 3: mono- and di-protonated), *N*-[3-(isopropylideneamino)-4-methyl-cyclohexyl]propan-2-imine (4), 2-[[3-(isopropylideneamino)-4-methyl-cyclohexyl]amino]propan-2-ol (5 and 6: mono- and di-protonated), and 2-methyl-*N*-(2-methylcyclohexyl)cyclohexanamine (6).

Additionally, to exclude any influence of the sample preparation due to acidification with HCl as well as of the mobile phase during chromatographic separation on the reactivity of MCDA with acetone a GC-FID analysis was performed for further confirmation. The results again confirmed that MCDA is not stable in AOO even

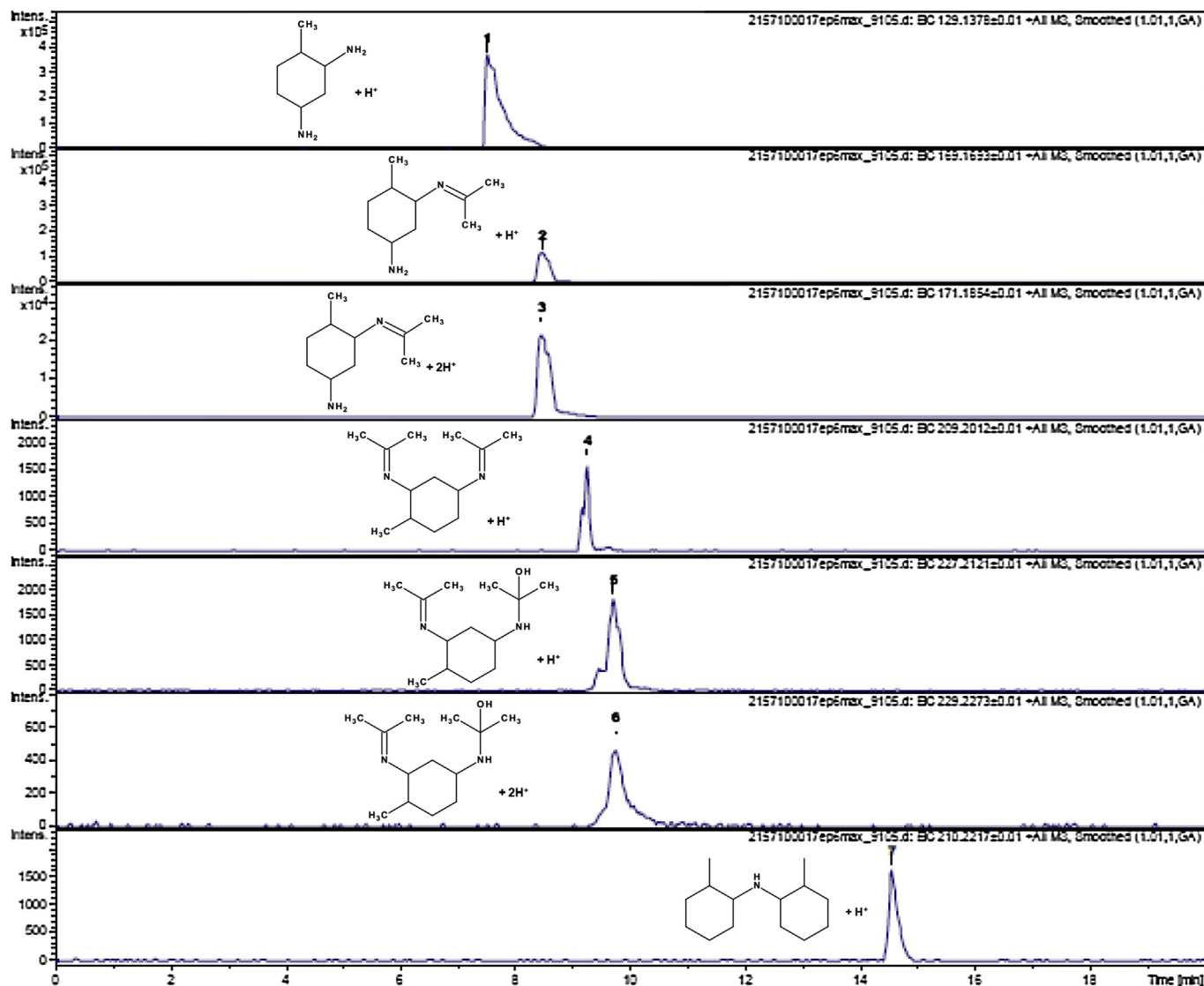


Fig. 2. CE-ESI-MS analysis after reaction of MCDA with acetone: conceivable structures.

after direct detection after mixing with the vehicle, with only 35% of the nominal concentration being found. After a storage for 80 min, the MCDA concentration was below the limit of detection (LOD).

3.2. Direct Peptide Reactivity Assay (DPRA)

When tested in the DPRA, visual observation did not reveal precipitates of MCDA in any samples of the test substance with both peptides after the 24 h incubation period. Furthermore, no co-elution of test substance and peptide occurred as demonstrated by the area ratio 220/258 and the co-elution control (data not shown). The mean peptide depletion of MCDA was calculated as average of cysteine- and lysine-peptide depletions and summarized in Table 2 (negative depletions were considered to be zero for calculation of the mean peptide depletion). MCDA showed a low reactivity with a mean peptide depletion of 2.88% and thus not indicating sufficient peptide reactivity required for skin sensitization. MCDA was therefore considered as a non-sensitizer under the conditions of this single assay. The positive control, however, revealed a mean value of 48.30%.

3.3. Local Lymph Node Assay (BrdU-ELISA modification) using propylene glycol (PG) as alternative vehicle

After application of MCDA in PG at concentrations of 2%, 5%, and 10%, the animals in the test groups did not show any relevant signs of systemic toxicity (see also chapter 3.1). However, in the positive control group, very slight to well-defined erythema, moderate swelling of the ears and scaling could be noted during the course of the study, which reflects the statistically significant and biologically relevant increase in ear weight measurement (S.I.: 1.74). Lymph node weights and -cell counts of all animals treated with the test substance were neither statistically nor biologically significant influenced by treatment in comparison to the vehicle group. In contrast, a statistical and biological relevant increase in lymph node weight and lymph node cell count was determined in the positive control (S.I.: 2.1 and 2.2). A statistically significant increase in ear weights was not observed in any MCDA treated group in comparison to the vehicle control group (see Table 1). The S.I.s for BrdU labeling were 0.9, 1.0 and 1.1 at concentrations of 2%, 5%, and 10% (w/w), respectively. As all S.I.s obtained were below the threshold of 1.6, MCDA was not considered skin sensitizing when tested in PG.

as vehicle.

3.3.1. Concentration control analysis of samples used in the LLNA with PG as alternative vehicle

The concentration control analysis of the samples from the LLNA using PG as alternative vehicle was obtained via GC-FID measurement and is given as rounded mean value of two injections (see Table 3). The values found corresponded to 99%–104% of the expected concentrations, confirming that the MCDA concentration in the vehicle was correct as well as that PG was a suitable vehicle showing MCDA to be stable over the test period.

4. Discussion

REACH requires that substances produced or imported into the EU in quantities of one metric ton per year or more have to be tested for their skin sensitization properties. Until recently, the murine Local Lymph Node Assay (LLNA) was the first-choice method for *in vivo* testing under REACH. The update of the REACH annex now made the *in vitro* assays the default requirement for skin sensitization assessment. These alternative methods focus on key events in the AOP for skin sensitization. Combinations of non-animal methods such as proposed by Urbisch and co-workers within Integrated Approaches to Testing and Assessment (IATAs) will need to fully replace the animal test (Urbisch et al., 2015; OECD, 2016b).

In this case report, good laboratory practices while closing the data gap ‘skin sensitization’ during data collection for the REACH registration of MCDA with particular focus on the vehicle used were described. In order to assess the skin sensitization potential of MCDA, the LLNA standard protocol as described in OECD test guideline 442B with the BrdU-ELISA modification was followed. The results suggested MCDA to be a skin sensitizer when tested in AOO as vehicle (see Table 1).

However, concentration control analysis of the preparations of MCDA in AOO using CE-UV indicated MCDA to be unstable under the study conditions used. After storage for 1 h the concentrations determined ranged between 28% and 38% of the nominal concentrations and after overnight storage the concentrations ranged from 66% to 78% of the nominal concentrations, respectively (see Table 3). As a likely explanation for that low recovery, a reactivity between MCDA and the whole vehicle or single vehicle components was considered. In order to detect potential MCDA reaction products with the vehicle, a qualitative CE-ESI-MS analysis of the chemical structures of possible reaction products was carried out. It could be clearly shown that the primary di-amine MCDA (structure see Fig. 1) easily reacts with acetone via exclusive formation of several Schiff bases (imines, structures see Fig. 2).

Moreover, the results of the concentration control analysis as shown in Table 3 further suggest an instability of the Schiff bases unintentionally formed, since the 1 h recovery was lower than the overnight recovery. This observation may suggest a further degradation of the adducts formed between acetone and MCDA via a reverse reaction. It is known in general, that the treatment of imines with water in the presence of a catalytic amount of acid leads to their hydrolysis to an aldehyde and an amine (Binauld and Stenzel, 2013). During the sample preparation for instrumental analysis, HCl was added for reasons of a better separation via CE. This acidification could have been sufficient for the observed MCDA concentration increase via imine hydrolysis after a prolonged storage time. Thus, in order to exclude any influence of the sample preparation, a GC-FID analysis was used, which did not require an extensive sample preparation. An additional advantage of this analysis was that the same pH conditions as those applied to the ears of mice during the LLNA could be mimicked. The GC-FID confirmed the findings of the CE-ESI-MS analysis with respect to

the reaction between MCDA and acetone. Moreover, the GC-FID analysis showed that the Schiff base formation takes place very fast, since no MCDA was detectable after a storage time of 80 min up to 24 h. A reverse reaction of the MCDA-acetone-adducts to form the free amine and the ketone was not observed, substantiating the assumption that the acidification using HCl actually triggered the imine hydrolysis.

It is well known that chemical reactivity has to be associated with allergenic potency (Gerberick et al., 2007). Briefly, an alteration of a protein/peptide plays a crucial role and is regarded as a key event in the AOP of skin sensitization. The DPRA addresses the molecular initiating event of skin sensitization via evaluation of the electrophilic reactivity of a test item towards synthetic cysteine (C)- or lysine (K)-containing peptides. In order to test a potential reactivity of MCDA towards peptides, a DPRA was performed in an aqueous vehicle. Based on the observed results and applying the prediction model proposed by Gerberick and coworkers (Gerberick et al., 2007; OECD TG 442C), it was concluded that MCDA shows only negligible chemical reactivity in the DPRA under the conditions of the test. Considering that chemical reactivity may be well associated with allergenic potency, a high response in the DPRA with MCDA might reasonably have been expected (Gerberick et al., 2007). However, the missing reactivity of MCDA towards the peptides can be explained: MCDA is a primary amine and characterized by nucleophilic reactivity. The peptides used in the DPRA contain cysteine (-SH) as well as lysine (-NH₂) residues as reactive groups, all of which carry one or more free electron pairs also allowing solely nucleophilic reactions. Therefore, a reaction between neat and unmodified MCDA and these peptides can be excluded.

Since protein reactivity of a substance can also develop following metabolic activation of a pro-hapten, an *in vivo* modification via metabolism of the chemical structure of MCDA under formation of an electrophilic moiety should also be considered (Gerberick et al., 2007). Concerning MCDA, a metabolic oxidative deamination could be taken into account. It is well known that the mitochondrial flavoenzyme monoamine oxidase (MAO) is capable to catalyze this deamination: the amine moiety binds to the active site of the enzyme, is then oxidized to the protonated imine, and then hydrolyzed via formation of an ammonium ion and the corresponding aldehyde (Edmondson et al., 2009). This aldehyde would have been the electrophilic compound able to modify proteins leading to a positive response in the LLNA.

At this stage, a final assessment on the intrinsic properties of MCDA to cause skin sensitization or not was not possible since the DPRA is not metabolic competent. On the one hand, MCDA showed a reaction with the vehicle AOO under formation of electrophilic Schiff bases or imines and on the other hand, MCDA could have been activated by metabolism via formation of an electrophilic aldehyde. Previous studies have reviewed that the choice of the vehicle may influence the results obtained in the LLNA (e.g. Jowsey et al., 2008) and is hence not further detailed here. In order to exclude any influence of the vehicle and to include metabolism into the final assessment, it was decided to conduct an additional LLNA according to the same protocol using another vehicle. In addition to AOO, the test guideline (OECD TG 442B) recommends different vehicles such as *N,N*-dimethylformamide, methyl ethyl ketone, propylene glycol (PG), and dimethyl sulfoxide. From those vehicles PG was chosen as appropriate, because MCDA also was shown to be soluble in it. Additionally, PG was inert to a reaction with MCDA up to 24 h and the maximal applicable concentration not producing excessive ear irritation was twice as high as the concentration applied in the LLNA with AOO as vehicle. The results of the LLNA with PG as vehicle suggested MCDA not to be a skin sensitizer when tested in PG as vehicle (see Table 1).

In conclusion, the positive results of the LLNA with AOO as

vehicle could be considered an artefact and thus false positive since MCDA was clearly shown to be reactive towards acetone under formation of protein/peptide modifiable imines. The positive LLNA result was not confirmed when PG was used as vehicle. This substantiates the assumption that MCDA is per se not protein reactive as proven in the DPRA as well as that oxidative deamination of MCDA via *in vivo* metabolism plays - if at all - a minor role only. On the basis of the reliable LLNA with the vehicle PG, a classification and labeling of MCDA as skin sensitizer according to the Globally Harmonized System (GHS) is not justified.

5. Conclusion

The outcome of these *in vivo* studies clearly confirm that the stability of the test substance in the vehicle plays a crucial role in the outcome of the LLNA. To our knowledge, reactivity towards the vehicle leading to an entirely different result has not been reported yet. Up to now, several LLNAs have been performed with various amines in AOO (i.e. [Gamer et al., 2008](#)). In the light of the reported findings here, a re-analysis of the results of those LLNAs especially in the light of the regulatory consequences, with special focus on contradicting results between LLNA and GPMT data, is highly envisaged.

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