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Isabelle Manière, Alizée Aubert, Céline Dubois, Cécilia Solal, Jean-Pierre Lepoittevin, Christophe Rousselle

Received: 12 July 2022 | Revised: 23 August 2022 | Accepted: 5 September 2022

DOI: 10.1111/cod.14216

ORIGINAL ARTICLE

CONTACT DERMATITIS WILEY

Sensitization properties of acetophenone azine, a new skin sensitizer identified in textile

Isabelle Manière[1] | Alizée Aubert[2] | Céline Dubois[1] | Cécilia Solal[1] | Jean-Pierre Lepoittevin[3] | Christophe Rousselle[1]

[1]Risk Assessment Department, French agency for food, environmental, ocupational health and safety, Maisons-Alfort, France

[2]Charles River Laboratories Evreux, Miserey, France

[3]Institute of Chemistry UMR 7177, University of Strasbourg, CNRS, Strasbourg, France

Correspondence
Christophe Rousselle, Risk Assessment Department, French Agency for Food, Environmental and Occupational Health & Safety (ANSES), 14 rue Pierre et Marie Curie, F-94701 Maisons-Alfort Cedex, France.
Email: christophe.rousselle@anses.fr

Funding information
Agence Nationale de Sécurité Sanitaire de l'Alimentation, de l'Environnement et du Travail; Scientific Cooperation Agreement, Grant/Award Number: ANSES/CTC 2015-CRD-22; Research and Development Cooperation Agreement, Grant/Award Number: 2017_CRD-08 P6474-H

Abstract

Background: Acetophenone azine (CAS no. 729-43-1) present in sports equipment (shoes, socks and shin pads) has been suspected to induce skin allergies. Twelve case reports of allergy in children and adults from Europe and North America were published between 2016 and 2021.

Objectives: The objective of this study was to confirm that acetophenone azine is indeed a skin sensitizer based on in vitro/ in vivo testings derived from the Adverse Outcome Pathway (AOP) built for skin sensitization by OECD in 2012.

Methods: Acetophenone azine was tested in vitro according to the human cell line activation test (h-CLAT) and the ARE-Nrf2 Luciferase Test (KeratinoSens) and in vivo using the Local Lymph Nodes Assay (LLNA).

Results: Both the h-CLAT and the KeratinoSens were positive whereas the LLNA performed at 5, 2.5 and 1% (wt/vol) of acetophenone azine, was negative.

Conclusion: Based on these results, acetophenone azine was considered as a skin sensitizer. This was recently confirmed by its classification under the CLP regulation.

KEYWORDS
acetophenone azine, case report, h-CLAT, KeratinoSens, LLNA, skin sensitization

1 | INTRODUCTION

In 2018, following complaints of skin allergies from customers using footwear or garments, ANSES (French Agency for Food Environment and Occupational Health and Safety) investigated which compounds could be responsible for these adverse effects.[1] Acetophenone azine (CAS no. 729-43-1) was suspected to be involved as it was already associated to several cases of severe contact dermatitis in children and adults since 2016. Four case reports were published in 2016 and 2017 involving three boy soccer players and an adult hockey player.[2–4] Acetophenone azine was measured in socks, sneakers, children's leather shoes, walking shoes, shin pads, acrylic fur at concentrations between 20 (sneakers) and 70 ppm (children's shoes).[2,3] No data were available at this time on the presence of acetophenone azine in other products. In the framework of a scientific cooperation agreement with the technical centre on leather funded by ANSES (ANSES/CTC 2015-CRD-22), this substance was found in 14% of the footwear articles tested by France and in particular in sports equipment like shoes, socks and shin pads.

The first case with severe allergic contact dermatitis caused by acetophenone azine present in the shin pads of a 17-year-old hockey player was reported in the United Kingdom in 2019.[5] Another case of

Abbreviations: AOP, adverse outcome pathway; h-CLAT, human cell line activation test; LLNA, local lymph nodes assay; OECD, organism of economic collaboration and development; QSAR, quantitative structure-activity relationship.

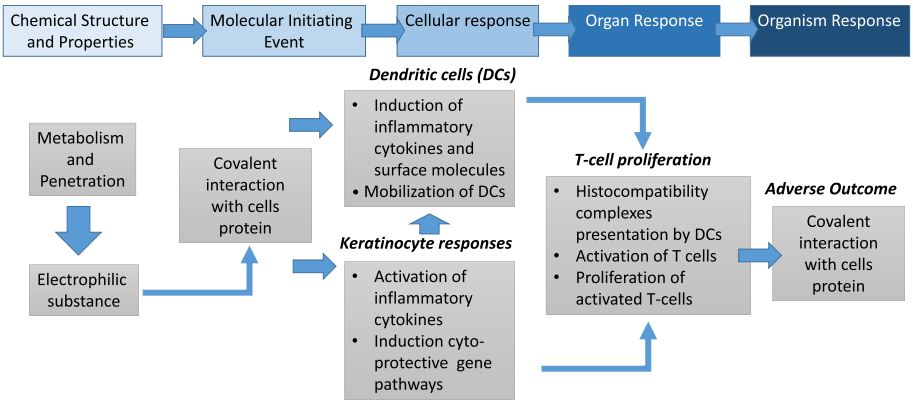

FIGURE 1 Flow diagram of the pathways associated with skin sensitization

acetophenone azine-induced shin pad and sports shoe dermatitis was reported in 2020 in a North American 6-year-old soccer player.[6]

Skin sensitization is an immunological process that follows two phases: the induction of sensitization followed by the elicitation of the immune response. The first phase includes a sequential set of events which are described in the Adverse Outcome Pathway (AOP) no. 40 (https://aopwiki.org/wiki/index.php/Aop:40) as shown in Figure 1 taken from Strickland et al.[7]

Two in vitro human cell-based assays and one in vivo experiment are described in this AOP. The objective of this work was to perform these assays in order to confirm experimentally and better characterize the skin sensitization potential of acetophenone azine.

2 | METHODS

2.1 | Chemicals

Acetophenone Azine; Chemical name: 1-phenyl-ethanone(1-phenylethylidene)hydrazine (CAS no. 729-43-1; batch number: 10138845 I) were purchased from Alfa Aesar and the purity of the compound was 97%; Cinnamic aldehyde (CA), (CAS no.14371-10-9) and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich. 2,4-Dinitrochlorobenzene (DNCB) (CAS no.97-00-7) and Nickel Sulphate ($NiSO_4$) (CAS no.10101-97-0) were purchased from Sigma-Aldrich and Merck, respectively. The purity of the compounds was reported equal to or higher than 99% by the respective suppliers.

Acetone:Olive oil 4:1 (vol/vol) mixture, *N,N*-dimethylformamide (DMF), methyl ethyl ketone, propylene glycol, dimethyl sulfoxide and 1% aqueous Pluronic PE9200 were used as standard OECD vehicles.

α-Hexylcinnamaldehyde (HCA) (CAS no.101-86-0; batch number: MKCD2910) was purchased from Sigma-Aldrich Co.

2.2 | Stability testing

The stability of acetophenone azine has been tested in a hydrolysis study which was performed to determine the rate of hydrolysis and to identify the degradation products of the substance in artificial sweat. The aim was to investigate the possibility of hydrolysis of acetophenone azine by sweat leading to the formation of urea and hydrazine. A study (study of acetophenone azine hydrolysis in artificial sweat [non-GLP]. Date: 14 August 2017) was therefore conducted to determine the degradation products from hydrolysis and to determine the hydrolysis rate of acetophenone azine in artificial sweat.[8] The stability of acetophenone-azine was examined in artificial sweat for 5 days at 37°C. Taken into consideration solubility and analytical limit, it was decided to assess the stability of acetophenone azine at 0.1%. The sampling time were 0 h–30 min, 1–3 h, 8–24 h (3 h relative to standard sweat/leather and 8 h relative to the wearing of 1, 3 and 5 days). Incubation of samples in plastic tubes (10–20 ml) at 37°C under mechanical mixing for the sampling times in three replicates (three tubes of the same sample, run in the same time). Filtration was at 0.45 microns only if required.

Analysis of samples (plus controls of a time zero sample and incubated samples without test item) by LC/QTOF MS for any degradation products of acetophenone azine. Approximate quantification of Hydrazine was based on standard for each sample.

A. Spectra to look for other chemicals that may have been produced, with rapid evaluation of probable identity of peaks other than acetophenone azine or hydrazine was examined.
B. For any peaks, the probable identity and order of magnitude of concentration (if possible) were reported including evaluation of available data, literature search and evaluation as required.

The following skin sensitization tests were then performed to investigate the four key events of the AOP no. 40. Tests were retained based on expert judgement regarding results of hydrolysis assay showing that acetophenone azine was not only hydrolysed in hydrazine but also in acetophenone, a classified substance and regarding the prediction of alerts using QSAR modelling. The following combination of three skin sensitization tests was performed according to AOP.

2.3 | In vitro skin sensitization: ARE-Nrf2 Luciferase Test Method (KeratinoSens) (OECD TG 442D)

In Vitro Skin Sensitization: ARE-Nrf2 Luciferase Test Method (KeratinoSens) (OECD TG 442D), adopted on February 2015, was

used to investigate the second key event of the skin sensitization pathway, which is the inflammatory response as well as the expression of the genes associated with the cell activation pathway of keratinocytes.

The KeratinoSens test method was considered scientifically valid to be used as part of an Integrated Approach to Testing and Assessment (IATA), to support the discrimination between skin sensitizers and non-sensitizers for hazard classification and labelling. Acetophenone azine was tested at different concentrations. The KeratinoSens cells were first plated on 96-well plates and grown for 24 h at 37°C. Then, the medium was removed and the cells were exposed to the vehicle control or to different concentrations of test item and of positive control (cinnamic aldehyde). The treated plates were then incubated for 48 h at 37°C. At the end of the treatment, cells were washed and the luciferase production was measured by flash luminescence. In parallel, the cytotoxicity was measured by an MTT reduction test and was taken into consideration in the interpretation of the sensitization results. Two independent validated runs were performed as part of this study. All acceptance criteria were met for the positive and negative controls in each run and the two runs were performed using the following concentrations: 0.49, 0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 μM in culture medium containing 1% DMSO were therefore considered as validated.

2.4 | In vitro skin sensitization: human cell line activation test (h-CLAT) (OECD TG 442E)

The h-CLAT test method, based on the OECD TG 442E '*In vitro* skin sensitization: human cell line activation test (h-CLAT)', adopted in July 2016, allows to investigate the third key event of the skin sensitization pathway by quantifying changes in the expression of cell surface markers associated with the process of activation of monocytes and dendritic cells (i.e., CD86 and CD54). The measured expression levels of CD86 and CD54 cell surface markers are then used for supporting the discrimination between skin sensitizers and non-sensitizers. However, it may also potentially contribute to the assessment of sensitizing potency when used in integrated approaches such as IATA.

Following the solubility assays, the cytotoxic potential was assessed in a Dose-Range Finding assay in order to select sub-toxic concentrations for testing in the main test. The skin-sensitizing potential of the test item was then evaluated in the main test, in three validated runs (Runs A, C and D). During this main test, treatments were performed at the following final concentrations: 139.54, 167.45, 200.94, 241.13, 289.35, 347.22, 416.67 and 500 μg/ml. In each run, the test item formulations were applied to THP-1 cells and cultured in a 24-well plate for 24 h ± 30 min at 37°C, 5% CO_2 in a humidified incubator. A set of control wells was also added in each plate to guarantee the validity of each run: DMSO as a negative control and DNCB as an extreme sensitizer and Nickel Sulphate (NiSO4) as a moderate sensitizer. At the end of the incubation period, cells from each well were distributed to three wells of 96-well plate: the first well was labelled with IgG1-FITC antibodies, the second one was labelled with CD86-FITC antibodies and the third one was labelled with CD54-FITC antibodies. Then, just before flow cytometry analysis of CD86 and CD54 expression, all cells were dyed with propidium iodide for viability discrimination. For each run, the mean fluorescence intensity (MFI) obtained for each test sample was corrected by the isotype control IgG1 MFI value to obtain the corrected MFI. The corrected MFI value from the corresponding vehicle control was set to 100% CD54 and CD86 expression by default. Then, corrected MFI values from each test sample were compared to the corresponding vehicle control to obtain the Relative Fluorescence Index for CD86 and CD54 expression for each tested concentration (RFI CD86 and RFI CD54).

2.5 | In vivo skin sensitization: local lymph node assay (OECD TG 429)

The local lymph node assay (LLNA) test method, based on the OECD TG 429 '*In vivo* Skin sensitization: Local Lymph Node Assay (LLNA)', adopted in July 2010.

2.5.1 | Selection of vehicle

The solubility of the test item was examined in a short preliminary compatibility test. The following standard OECD vehicles were assessed: acetone:olive oil 4:1 (vol/vol) mixture, DMF, methyl ethyl ketone, propylene glycol, dimethyl sulfoxide and 1% aqueous Pluronic PE9200. The best vehicle taking into account the test item characteristics and the requirements of the relevant OECD guideline was considered to be DMF.

2.5.2 | Selection of doses tested for main LLNA study

A pre-screen test was conducted under conditions identical to the main LLNA study, except there was no assessment of lymph node proliferation and fewer animals per dose group have been used. Consecutive doses are normally selected from an appropriate concentration series such as 100%, 50%, 25%, 10%, 5%, 2.5%, 1% and 0.5%. The 5% (wt/vol) formulation was the highest concentration suitable for the test. The 5% (wt/vol) formulation and all the diluted formulations appeared to be solutions by visual examination.

2.5.3 | Test animals (welfare, species, housing conditions, food, water)

During the study, the care and use of animals were conducted in accordance with the relevant principles currently used, reviewed and approved by the Institutional Animal care and use Committee (IACUC) with regard to animal welfare. Charles River Hungry, facility is AAALAC accredited. Mice of CBA/CaOlaHsd (Source: Envigo,

Hygienic level: SPF at arrival; standard housing conditions during the study, acclimatization time:28 days) were used. Females nulliparous, non-pregnant, 9 weeks old at starting (age-matched, within 1 week), body weight at starting: 19.1–21.7 g ± 20% of the mean weight were used because the existing database is predominantly based on females.

Animals were housed in group caging (Type II, polypropylene/polycarbonate). Mice were provided with glass tunnel tubes. Bedding and certified nest building material was available to animals during the study. Room was lighted 12 h daily, from 6.00 AM to 6.00 PM, temperature was 17.0–24.9°C, relative humidity was 31%–80%, ventilation was 15–20 air exchanges/h. The temperature and relative humidity were recorded twice every day during the acclimatization and experimental phases.

Animals were fed with ssniff SM Rat/Mouse—Breeding and Maintenance, 15 mm, autoclavable 'Complete feed for Rats and Mice—Breeding and Maintenance' (Batch numbers: 88329966 and 840 33675), the food was considered not to contain any contaminants that could reasonably be expected to affect the purpose or integrity of the study. Animals received tap water from the municipal supply in 500-ml bottles, ad libitum.

The animals were randomized and allocated to the experimental groups. The randomization was checked by computer software using the body weight to verify the homogeneity and variability between the groups.

2.5.4 | Administration/exposure

Animals assigned to the negative control group were treated with the vehicle only concurrent to the test item-treated groups. Based on the result of the preliminary compatibility test, DMF was selected for vehicle of the study. Animals assigned to the positive control group were treated with 25% (wt/vol) α-hexylcinnamaldehyde solution (dissolved in DMF) concurrent to the test item-treated groups.

In the main study, the treatments were applied on Day 1 (predose), Day 3 (approximately 48 h after the first dose) and Day 6 on the back of each ear of the animal: 25 μl of a suitable dilution of the test substance, of the vehicle alone or of the positive control. Based on the results of the pre-screened study, concentrations of 5%, 0.5%, 0.05% and 0.005% (wt/vol) in DMF, 5% (wt/vol) dose was selected as top dose for the main test. Then the other tested doses of 2.5% and 1% were chosen according to guideline 429. Acetophenone azine was applied at 5%, 2.5% and 1% (wt/vol) formulated in DMF on 20 female CBA/CaOlaHsd mice which were allocated to five groups, each group comprised four animals. Three groups of animals received acetophenone azine (formulated in DMF). A negative control group received the vehicle (DMF) only. A positive control group received 25% (wt/vol) α-hexylcinnamaldehyde HCA (dissolved in DMF). The test item solutions were applied to the dorsal surface of the ears of the experimental animals (25 μl/ear) for three consecutive days (Days 1, 2 and 3) and then maintained on study for an additional 3 days. Cell proliferation in the (local) lymph nodes was assessed by measuring disintegrations per minutes after the incorporation of tritiated methyl thymidine (3HTdR) into the lymph nodes and the values obtained were used to calculate stimulation indices (SI) in comparison with the control group. The test item was weighed and formulations were prepared daily on a weight:volume basis (as % [wt/vol]).

The proliferation indices were compared between the mean proliferation of each test group and the mean proliferation of the control group treated with the vehicle. The results obtained for each treatment group were expressed by an average stimulation index (SI). This SI is obtained by dividing the average BrdU score of each group by the average BrdU score of the solvent-treated control group. The decision process regards a result as positive when SI ≥3. Clinical signs and irritation at the site of application should also be observed and reported as they may indicate systemic toxicity.

3 | RESULTS

3.1 | Stability of acetophenone azine

The stability of acetophenone azine was examined in artificial sweat for 5 days at 37°C. In the first 8 h, no major changes were detected. After 24 h, 30%–40% of the initial acetophenone azine amount was hydrolysed. The decrease was detectable both in the UV and the EIC chromatograms. After 72 h, approximately 95% of the test item has reacted and after 120 h only traces can be detected in the second and the third samples while in Sample 1, no more acetophenone azine was detected. Based on this, it can be stated that the test item completely hydrolyses within 5 days. The hydrolysis product could not be identified by mass spectrometry, but according to the basic organic chemical principles, the production of hydrazine and acetophenone was expected. Hydrazine has a too low mass—and no chromophores—to be detected while acetophenone cannot be ionized by APCI. However, the injection of acetophenone standard at the same concentration level resulted in the appearance of a peak at the same retention time showing approximately the same intensity. Therefore, the hydrolysis product could be considered to be acetophenone (Figure 2: the UV trace of acetophenone and the 72 h sample of the test item solution). The only peak detected appeared in the UV chromatogram at 13.8 min. Based on the retention times, it can be identified as acetophenone. The reason why this peak appears neither in the total ion nor in the extracted ion chromatograms is that acetophenone cannot be ionized by LC/APCI-MS techniques.

3.2 | In vitro skin sensitization: ARE-Nrf2 luciferase test method (KeratinoSens assay)

At the tested concentrations: slight to strong test item precipitates were observed in treated wells at concentrations ≥62.5 μM in the first run and ≥31.3 in the second run. A strong decrease in cell viability (i.e., cell viability <70%) was noted at concentrations ≥125 μM in the first run and ≥250 μM in the second run. The corresponding IC_{30} and IC_{50} were calculated to be 97.68 and 163.11 μM and 152.77 and

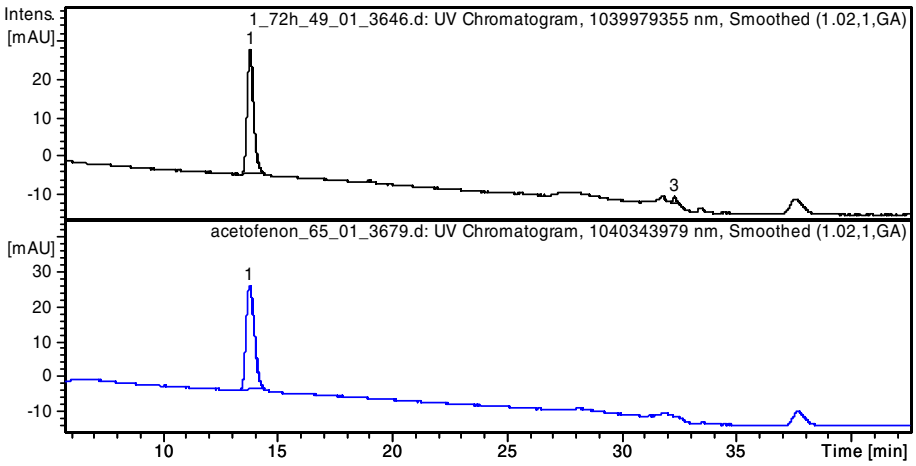

FIGURE 2 The UV trace of acetophenone and the 72-h sample of the test item solution

238.11 μM, in the first and second runs, respectively. Statistically significant change in gene induction above the threshold of 1.5 was noted in comparison to the negative control at several successive concentrations in both runs (from 0.98 to 15.6 μM in the first run and from 0.49 to 31.3 μM in the second run). Moreover, an apparent dose–response relationship was also noted, followed by a decrease of induction related to the appearance of cytotoxicity (i.e., from 62.5 μM in both runs). The I_{max} values were 2.14 and 3.31 and the calculated $EC_{1.5}$ were 0.63 and estimated <0.49 μM in the first and second runs, respectively. The geometric means IC_{30} and IC_{50} of the two validated runs were calculated to be 122.16 and 197.07 μM, for the first and second runs, respectively. The evaluation criteria for a positive response are met in both runs, the final outcome is therefore positive. This positive result can be used to support the discrimination between skin sensitizers and non-sensitizers. Under the experimental conditions of this study, acetophenone azine was positive in the KeratinoSens assay and therefore was considered to activate the Nrf2 transcription factor.

3.3 | In vitro skin sensitization: h-CLAT

The test item was found soluble in DMSO at 250 mg/ml. During both assays, no decrease in cell viability (i.e., cell viability <75%) was noted in test item treated wells and the highest tested concentration retained for the main test was 500 μg/ml. In the main test, all acceptance criteria were reached in each run except for the Run B, where the cell viability of the positive control $NiSO_4$ was <50% (i.e., 45.3%). Therefore, this run was invalidated. For Run A, strong test item precipitate was noted in treated wells from the lowest concentration of 139.54 μg/ml. The relative fluorescence intensity (RFI) of CD86 and CD54 (RFI CD86 and RFI CD54) did not exceed the positivity thresholds at any tested concentration. The Run A was therefore considered negative. For Run C, moderate to strong test item precipitate was noted in treated wells from the lowest concentration of 139.54 μg/ml, RFI CD86 did not exceed the positivity thresholds at any tested concentration. RFI CD54 exceeded the positivity threshold from 139.54 to 241.13 μg/ml. The Run C was therefore considered positive for RFI CD54. For Run D, moderate to strong test item precipitate was noted in treated wells from the lowest concentration of 139.54 μg/ml, RFI CD86 did not exceed the positivity thresholds at any tested concentration. RFI CD54 reached or exceeded the positivity threshold at the concentrations of 167.45, 241.13, 289.35, 347.22 and 500.00 μg/ml (i.e., 210, 200, 214, 200 and 241, respectively). The Run D was therefore considered positive for RFI CD54. Under the experimental conditions of this study, acetophenone azine, was found to be positive in the h-CLAT test method.

3.4 | In vivo skin sensitization: LLNA

No mortality or signs of systemic toxicity was observed during the study. No test item residue was noted on the ears of the animals in any groups. No marked body weight losses (≥5%) were observed in any groups. The results showed the lymph nodes were normal in the negative control group and in the 5%, 2.5% and 1% (wt/vol) test item treated dose groups. The SI values were 0.7, 0.4 and 0.5 at concentrations of 5%, 2.5% and 1% (wt/vol), respectively. Larger than normal lymph nodes were observed in the positive control group. The result of the positive control substance HCA dissolved in the same vehicle was used to demonstrate the appropriate performance of the assay. The positive control substance was examined at a concentration of 25% (wt/vol) in the relevant vehicle (DMF) using CBA/CaOlaHsd mice. No mortality, cutaneous reactions or signs of toxicity were observed for the positive control substance in the study. A lymphoproliferative response in line with historical positive control data (SI value of 3.7) was noted for HCA in the Main Assay. This value was considered to confirm the appropriate performance of the assay. Furthermore, the DPN values observed for the vehicle and positive control substance in this experiment were within the historical control range. Since there were no confounding effects of irritation or systemic toxicity at the applied concentrations, the proliferation values obtained are considered to reflect the real potential of acetophenone azine to cause lymphoproliferation in the LLNA. The resulting stimulation indices observed under these test conditions could be considered evidence that acetophenone azine is a non-sensitizer in this specific

study design. The size of lymph nodes was in good correlation with this conclusion. In conclusion, under the conditions of the present assay, acetophenone azine, tested in *N*,*N*-dimethylformamide, did not show a sensitization potential (non-sensitizer) in the LLNA. In the LLNA acetophenone azine, applied at 5%, 2.5% and 1% (wt/vol), is negative under the experimental conditions. However, it should be noticed that the maximum tested concentrations are still low and therefore if standing alone, these results could not be taken to demonstrate that acetophenone azine is a true non-sensitizer.

4 | DISCUSSION

In 2021, the American Contact Dermatitis Society chose acetophenone azine (AA) as the Allergen of the Year. Acetophenone azine has been shown to be a skin sensitizer in 12 documented case reports on partly severe allergic contact dermatitis in children and adults from Europe (11) and North America (1) (reviewed in Raison-Peyron and Sasseville[13]). Initially and before these experimental studies were performed, four cases of allergic contact dermatitis were described in France (publications from 2016 and 2017). After completion of the studies, eight more cases from Canada, UK, Belgium and France were published in three additional publications (2019–2020). All these cases were included in the CLH report published by ECHA in response to the proposal submitted by ANSES in which these results were included as key evidence to consider acetophenone azine as a skin sensitizer. Koumaki et al.[5] reported the case of a 17-year-old hockey player with allergic contact dermatitis of the shins caused by acetophenone azine present in his shin pads. Besner Morin et al.[6] described a case of acetophenone azine-induced shin pad and sports shoes dermatitis in a 6-year-old soccer player from North America. The child reacted positively to acetophenone azine in petrolatum at concentrations of 1% and 0.1%. Darrigade et al.[16] published a case series of six boys aged 7–14 years, all non-atopic except for one, observed in France or Belgium between January 2018 and July 2019. All patients presented long-standing shin dermatitis related to the wearing of shin pads. Four patients also had secondary episodes of plantar vesicular and/or hyperkeratotic, fissured dermatitis, related to the shoes they were wearing. All six children reacted positively to acetophenone azine petrolatum at concentrations of 0.1% and/or acetone on Day 3 or 4. These human data are summarized in Table S1.

Little information is available on the uses of acetophenone azine because the substance is not yet registered. Acetophenone azine can be used as a synthetic intermediate in the chemical industry. In addition, this substance may result from the reaction of hydrazine (EC 206-114-9 CAS no. 30201-2) with acetophenone (EC 202-708-7, CAS no. 98-86-2).

Acetophenone azine is considered a skin sensitizer. A skin sensitizer refers to a substance that will lead to an allergic response following repeated skin contact as defined by the United Nations Globally Harmonized System of Classification and Labelling of Chemicals.[10]The evidence to consider acetophenone azine as a skin sensitizer is based on several recent case reports of children and adults showing allergic skin reactions following skin contact with sport equipment such as shin pads and shoes. In total, up to now, a causal relationship between skin exposure to acetophenone azine in sensitizing articles has been reported in several case reports involving children and adults.[2–6] Even if a few number of cases were reported, the severity of allergic contact dermatitis caused by acetophenone azine may suggest that this substance is a strong sensitizer. It is indeed important to note that incidences of sensitization are likely to be underestimated because of underdiagnosis, underreporting and lack of registration for milder cases of dermatitis.

It is also difficult to estimate the prevalence of textile allergic dermatitis in the general population in the EU based on available data. The risk of skin sensitization of the general population related to textile and leather articles such as clothing and footwear is of increasing concern in Europe.[11,12] The number of people sensitized to chemicals in textiles and leather is estimated at around 4 to 5 million people in Europe, which corresponds to 0.8%–1% of the population of the European Economic Area 31 (EEA 31). Between 45 000 and 180 000 new cases per year of sensitization (incidence) are estimated, corresponding to 0.01%–0.04% of the population of the EEA. It is also plausible that cases of allergic contact dermatitis would have been missed and labelled irritant contact dermatitis or dyshidrosis.[13]

Skin absorption is also an important consideration when assessing a skin sensitizer. The prediction of the physicochemical properties and the behaviour of the substance in contact with the skin are essential to assess the skin-sensitizing potential of a substance. Indeed, to induce a sensitization, the substance must first cross the skin barrier. The most important factors in the dermal bioavailability of a substance are the molecular weight and lipophilicity that can respectively be evaluated by the molar weight (MW) and the partition coefficient between octanol and water (log *P*). Other factors may also influence bioavailability such as volatility, melting point, contact time and the total exposure dose. The SCCS 2021 notes of guidance estimates that a substance with a molar weight greater than 500 g mol^{-1} and a log $P < -1$ or >4 has a low dermal absorption (about 10%).[14] The ability of the substance to induce sensitization will be therefore limited. However, it is important to note that low exposure may still induce sensitization. With a molecular weight of 236 g mol^{-1} and a log $P = 3.7$, acetophenone azine has a dermal absorption potential and can have the ability to induce skin sensitization.[15]

Additional support to the assessment of the skin-sensitizing potential of acetophenone azine is provided in this publication by two positive in vitro human studies (h-CLAT method and in vitro KeratinoSens) for key events in the AOP for skin sensitization, and by alerts for skin sensitization potential from QSAR modelling. Both in vitro h-CLAT method and KeratinoSens were found positive with acetophenone azine. Based on the prediction model for in vitro skin sensitization testing, two out of three tests have to be concordant to obtain a final classification regarding the skin sensitization potential of a given test substance.[9] Since concordant positive results were observed in KeratinoSens and h-CLAT assays, testing the substance in the direct peptide reactivity assay (DPRA) test detecting the covalent binding of the molecule to two nucleophilic peptides was considered not

necessary because it will not help in the global assessment of the substance. In accordance with the prediction model, the substance is considered to have a skin-sensitizing potential, and the LLNA was performed to allow subcategorization.

However, results from the LLNA test in mice were negative which raises the question of the overall level of evidence to consider acetophenone azine as a skin sensitizer.

An in silico evaluation of acetophenone azine was performed by ANSES using two software packages, the DEREK Nexus 5.0.2 software and the VEGA 2.1.9 platform (including CAESAR 2.1.6 software) and identified structure alerts for skin sensitization and the DEREK Nexus software predicted an EC3 value of 0.15%, for the LLNA thus classifying the substance as a strong sensitizer, whereas based on the VEGA prediction it would be classified as a weak sensitizer. The In silico tools are to evaluate some key events involved in the AOP presented in Figure 2 and as the currently available methods for skin sensitization address a single step of the AOP, they should therefore often be combined in testing strategies.

At the molecular level, to induce an immune reaction at the cellular level, the suspected allergen should have the ability to bind (usually by covalent binding) with the proteins of the skin. This binding leads to the formation of a hapten-protein complex that is responsible for the immune and inflammatory responses. This mechanism corresponds to the initiating event of the AOP and can to be investigated by the OECD TG 442C (DPRA). For the prediction of protein binding, different mechanisms exist and are integrated in the in silico tools to determine, according to the structure of the substance, if protein binding is likely to occur.

At a cellular level, the second key event of the AOP which corresponds to the keratinocytes activation can be evaluated experimentally with the OECD TG 442D (test method ARE-Nrf2 luciferase, KeratinoSens). By assessing with the help of luciferase, the Nrf2-mediated activation of antioxidant response element (ARE)-dependent genes. Under the experimental conditions of this method, acetophenone azine, tested at concentrations: 0.49, 0.98, 1.95, 3.91, 7.81, 15.6, 31.3, 62.5, 125, 250, 500 and 1000 μM, was positive assay and therefore was considered to activate the Nrf2 transcription factor.

Still at a cellular level, activation of dendritic cells via the expression of markers of surface-specific chemokines and cytokines is the third key event of the AOP and can be evaluated experimentally in vitro using the OECD TG 442E. Under the experimental conditions of this study, the test item, acetophenone azine, was concluded to be positive in the h-CLAT test method.

Finally, at the organ level, T-cell proliferation via activation of T cells and histocompatibility complex presentation by dendritic cells corresponds to the fourth key event of the AOP and can be evaluated experimentally in vivo using the OECD TG 429 (LLNA). Under the conditions of the LLNA, acetophenone azine, tested at 5%, 2.5% and 1% (wt/vol) formulated in DMF, did not show a sensitization potential in mice. However, some questions were raised regarding results of negative and positive controls. A major limitation of the test is the low maximum dose treatment of up to only 5%, which is linked to the poor solubility of the test substance in the chosen solvent.

No firm conclusion can then be drawn with regard to the possibility of false negative result due to the rather high DPN readings from the negative control samples and a positive control response out the historical control data range. Nevertheless, the above limitations lower the weight of this negative LLNA test in the overall assessment of acetophenone azine.

5 | CONCLUSION

Overall, considering whole the available data, and in particular, the human case reports, the classification for skin sensitization of acetophenone azine is justified. Considering the low exposure required to be sensitized and the severity of the responses, acetophenone azine has been recognized by ECHA as meeting the criteria for classification as Skin Sens. 1 according to the CLP regulation. However, the limited data (low number of cases reported until now) available did not allow for a sub-categorization. This classification was recently adopted by the Committee for Risk Assessment (RAC) of the European Chemical Agency (ECHA) following a proposal by Anses on behalf of France.[17]

ACKNOWLEDGEMENTS

This study was funded by the French Agency for Food, Environmental and Occupational Health and Safety (ANSES) in the framework of a Research and Development Cooperation Agreement 2017_CRD-08 P6474-H CiToxLAB with Charles River (CiTox Lab, Budapest, Hungary) and in the framework of a Scientific Cooperation Agreement ANSES/CTC 2015-CRD-22 with the technical centre on leather (Centre Technique du Cuir [CTC] Lyon). The authors would like to acknowledge technical supports from Charles River laboratory (including Adam Appl study director from Hungary Ltd.) for performing Local Lymph Node Assay and the experts from the ANSES expert committee for consumer products.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Isabelle Manière https://orcid.org/0000-0002-0425-7220
Jean-Pierre Lepoittevin https://orcid.org/0000-0002-9507-0700

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Manière I, Aubert A, Dubois C, Solal C, Lepoittevin J-P, Rousselle C. Sensitization properties of acetophenone azine, a new skin sensitizer identified in textile. *Contact Dermatitis*. 2023;88(1):35-42. doi:10.1111/cod.14216