The Skin Allergenic Properties of Chemicals May Depend on Contaminants – Evidence from Studies on Coumarin

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\textbf{Key Words}
Contact dermatitis · Coumarin · Local lymph node assay · Perfume allergy

\textbf{Abstract}

\textbf{Background/Aims:} Positive patch tests are considered representative of a contact allergy to the tested chemical. However, contaminants and derivatives rather than the suspected chemical itself could be responsible for the allergic skin reactions. Here, we tested the importance of contaminants in the sensitizing and allergenic properties of coumarin in mice and humans. Coumarin, an ingredient in cosmetics and fragrances, was chosen as the reference chemical since conflicting results have been obtained regarding its ability to induce contact allergy. In some chemical preparations, this could be explained by the presence of coumarin derivatives endowed with allergenic properties. \textbf{Methods:} In mice, three different coumarin preparations were tested in the local lymph node assay. In humans, we assessed the irritant and allergenic properties of highly pure coumarin in nonallergic and fragrance-allergic patients. \textbf{Results:} Pure coumarin did not exhibit irritant or sensitizing properties in the local lymph node assay. In contrast, two other commercially available coumarins and three contaminants that were detected in these coumarin preparations were identified as weak and moderate sensitizers, respectively. In humans, pure coumarin was extremely well tolerated since only 1 out of 512 patients exhibited a positive patch test to the chemical. \textbf{Conclusions:} These results indicate that coumarin cannot be considered as a common contact allergen and further emphasize that purity of chemicals is mandatory for the assessment of their allergenicity.

\textbf{Introduction}

Allergic contact dermatitis (ACD) is a public health concern in industrialized countries and one of the commonest occupational diseases [1–3]. ACD, also referred to as contact hypersensitivity, is a T-cell-mediated skin inflammatory reaction, expressed as erythema, vesicles and oozing, following repeated contact of the skin with nonprotein chemicals called haptens [1, 4–6]. The diagnosis of ACD relies on patch testing using pure chemical preparations. Positive patch tests to a contact allergen are considered representative of a contact allergy to the chem-
ical [7]. However, previous observations have shown that contaminants and derivatives but not the chemical itself could be responsible for the positive patch tests. In this respect, 3-dimethylaminopropylamine is the sensitizing compound of the contact allergy to cocamidopropyl betaine, a tensioactive substance found in several industrialized products. 3-Dimethylaminopropylamine is an intermediate synthesis substance and remains as a quantitatively detectable impurity in all tensioactives employing it in their synthesis [8].

The aim of our study is to test the importance of purity in the skin allergenic properties of a chemical. Coumarin, an ingredient in cosmetics and fragrances, was chosen as the reference chemical, since there is a controversy as to whether coumarin is a contact allergen involved in fragrance allergy. Indeed, coumarin allergy, defined by positive skin patch tests, was observed in up to 6.8% of patients with ACD [9–13]. Moreover, it was shown that coumarin was endowed with irritant properties at the concentration of 8% [13]. However, in a recent study on the concentration of 8% [13]. However, in a recent study on the skin reactivity to 14 frequently used chemicals, Frosch et al. [14] observed positive patch tests to coumarin in less than 0.3% of the 1,855 ACD patients tested.

The main pitfall of the studies demonstrating the allergenicity of coumarin was the lack of information concerning the purity of the coumarin used for patch testing. In most of the studies, coumarin was used at the concentration of 5% in petrolatum and was said to be 95–99% pure. However, coumarins comprise a large variety of natural or synthetic compounds, in which some chemicals are known as moderate (5,7-dihydroxycoumarin, 5,7-dihydroxy-4-methyl-coumarin) or weak (scoparone, isoscoparol and 4-hydroxycoumarin) contact allergens [15]. These chemicals are different from coumarin but are often confused with it. Since the chemical process of coumarin is critical for the purity of the molecule and since coumarin derivatives and contaminants are endowed with irritant and allergenic properties, the postulate that coumarin is a hapten could be questioned.

In this study, pure coumarin (>99.9% purity) was compared with two other commercially available coumarins for their sensitizing properties using the local lymph node assay (LLNA). Three of the main impurities detected in these coumarin preparations were also tested in the LLNA. Additionally, human studies were conducted in suspected ACD patients who received patch tests using the European standard battery and 1 and 10% pure coumarin. This study was followed by two Good Clinical Practice (GCP) studies, one with 101 ACD patients positive to fragrance mix (FM+) patch test and the second with 30 ACD patients with positive patch tests to their own perfume.

Results indicate that the coumarin chemical is extremely well tolerated. In contrast, derivatives contaminating some coumarin preparations are responsible for both the irritant and sensitizing properties previously attributed to coumarin.

Materials and Methods

Mice

Female CBA/j and BALB/c mouse strains were purchased from Charles River Laboratories (L’Arbresle, France). Animals were left to acclimate before entering the study and were provided with food and water ad libitum. All mice used were between 7 and 12 weeks old.

Test Material

Pure coumarin (Rhodiascent extra pure®, Rhodia Organics, Lyon, France) was synthesized according to a controlled Perkin process from pure salicylaldehyde. Two other coumarin samples, coumarin A and coumarin B, produced from α-cresol (2-methylphenol), were purchased. Two of the main impurities that were detected in coumarins A and B were synthesized in Rhodia research laboratories: 6-chlorocoumarin (purity >98%) and 6,12-epoxy-6H,12H-dibenzo[b,f][1,5]dioxocin (dibenzodioxocin). 3,4-Dihydrocoumarin (DHC) was purchased from Aldrich (purity >99%).

LLNA Studies

The LLNA was conducted according to the design validated by the Interagency Coordination Committee on the Validation of Alternative Methods [16] and the Organization for Economic Cooperation and Development guideline No. 429 [17], as originally described by Kimber and Dearman [18]. α-Hexylcinnamaldehyde (HCA; Sigma), routinely used in LLNAs as positive control [19], was included in the study. All the chemicals were dissolved in N,N-dimethylformamide (DMF, Sigma), one of the recommended vehicles in the LLNA [16]. The limited solubility of coumarin in DMF was 50% and higher than in acetone/olive oil (4:1) mixture (25%). The coumarin preparations were tested at 10, 25 and 50%, while 6-chlorocoumarin (whose maximal soluble concentration in DMF was 10%), 3,4-DHC and dibenzodioxocin were tested at 2.5, 5 and 10%.

Two strains of mice, CBA/j and BALB/c, were used for this study. Briefly, groups of mice (n = 4) were painted topically by 25 μl of various concentrations of test chemicals, 25% HCA solution or vehicle alone, on the dorsum of both ears daily for 3 consecutive days (days 1–3). Five days after the initial application (day 6), 20 μCi 3H-thymidine (specific activity 2 Ci/mmol; Amersham Biosciences, Bucks, UK) was injected intravenously through the orbital vein. After 5 h, the auricular draining lymph nodes (LN) were excised and pooled for each experimental group. Cell suspensions were prepared by gentle mechanical disaggregation through a 100-μm nylon cell strainer. Pooled LN cells were extensively washed twice in PBS and precipitated with 3 ml of trichloroacetic acid (5% w/v) overnight at 4°C. The precipitates were centrifuged and resuspended in 1 ml of 5% trichloroacetic acid and added to...
10 ml of scintillation fluid (Insta-Gel Plus, Packard, Groningen, The Netherlands). Incorporation of \(^{3}H\)-thymidine was then assessed on a \(b\)-scintillation counter. The data were expressed as disintegrations per minute (dpm) per node for each experimental group. Stimulation indices (SI) were also calculated according to the following simple formula: SI = dpm coumarin (or HCA)-treated group/dpm vehicle-treated group.

The local irritation was evaluated by ear thickness measurements on the days of painting up to the day of sacrifice. The irritation level was determined as negative if the percentage of increase in ear thickness between day 1 and day 3 was <10%. If it was above 10%, the chemical was considered as irritant.

**Preparation of Coumarin in Petrolatum for the Clinical Study**

Pure coumarin was dispersed in liquid petrolatum (at 45°C) at concentrations ranging from 1 to 10%. Homogeneity was checked by UV analysis against petrolatum. Control of the presence of coumarin and of the homogeneity was done at the beginning and at the end of the studies.

**Patients**

Three clinical studies approved by the local ethical committee were performed according to GCP guidelines.

**Clinical Study 1.** Three hundred and seventy-nine patients referred to the hospital for diagnosis of contact dermatitis were included in the study. The first 100 patients comprised 40 men and 60 women, with ages ranging from 20 to 49 years for men and from 50 to 79 years for women. Twelve patients had a history of atopic dermatitis. Only 2 patients had a clinical history of contact allergy to fragrances. These 100 patients received coumarin patch tests at 1 and 10%. The next 279 patients (124 males and 155 females, ages ranging from 15 to 74 years) received a single coumarin patch test at 2%.

**Clinical Study 2.** One hundred and one FM-allergic ACD patients were recruited during 14 months in a multicenter study involving 16 dermatology departments. The inclusion criterion was the presence of a relevant positive patch test to FM. Seventy females and 31 males with a mean age of 45 years (70% between 30 and 60 years) were included. A history of atopic dermatitis, allergic rhinitis and asthma was found in 11, 8 and 3 patients, respectively.

**Clinical Study 3.** Thirty perfume-allergic patients were recruited in 12 months in a multicenter study involving 7 dermatology departments. The inclusion criterion was the presence of a relevant positive patch test to their own perfumed product. Twenty-seven females and 3 males with a mean age of 30 years were included. Five patients had a clinical history of atopic dermatitis.

**Patch Testing**

All patients underwent patch testing. Patch testing was done on the skin on the back using Finn Chambers on Scanpor (dc 8 mm). Two hundred and fifty-two patients of study 1 received a patch test of 2% coumarin only, while 100 other patients received patch tests of 1 and 10% and the European standard allergen series (Chemotechnique Diagnostics, Malmö, Sweden). The patients from studies 2 and 3 received patch tests of 2% coumarin and the first 8 allergens of the fragrance series. Readings were done after 48/72 h and results were scored using the International Contact Dermatitis Research Group criteria [7]: – = negative; ? = doubtful; + = weak reaction (no vesicle); ++ = strong reaction (edema and vesicles);

+++ = extreme reaction (ulceration, bullies); IR = irritant reaction; NT = not tested.

**Results**

**Chemical Analysis of Different Coumarin Preparations**

The purity of the coumarin preparation was >99.9% for pure coumarin and >99.5% for coumarin A and coumarin B. No chlorinated derivatives were detected for pure coumarin. In contrast, 240 and 27 ppm of total chlorine were recorded for coumarin A and coumarin B, respectively.

Gas chromatography/flame ignition detection analysis showed the presence of 19 impurities (>1 ppm) in coumarin A and coumarin B, which were identified by gas chromatography/mass spectrometry. 6-Chlorocoumarin and DHC were the main impurities of coumarin A (>1,100 ppm) and coumarin B (>2,400 ppm), respectively. The two other major contaminants were 3-methylcoumarin (<180 ppm) and a benzochromene-like structure (230 ppm). Fifteen other impurities were found from trace to 60 ppm. Of note, 6-chlorocoumarin did not cover the total chlorine level, and other chlorinated impurities were detected, including 4,5,7- and 8-monochlorocoumarin (<20 ppm) and 6-chloro-DHC, as well as 2-chlorobenzyl-acetate and 1-chlorostilbene. The benzochromene-like structure found in both coumarins A and B was finally identified as 6,12-epoxy-6H,12H-dibenzo[b,f][1,5]dioxocin.

**Pure Coumarin Is Not a Contact Sensitizer in the LLNA**

The sensitizing activities of the three coumarin preparations (pure, A and B) were assessed in the LLNA. Three impurities, detected in coumarins A and B, were also tested: 6-chlorocoumarin, DHC and dibenzodioxocin (fig. 1).

The chemicals were dissolved in DMF at the maximum concentration of 50%. The study was conducted on CBA/j and BALB/c mice. CBA/j are reference mice in the LLNA, and BALB/c mice have been reported to be suitable for the test [20]. Controls included LLNA studies following skin painting with the vehicle alone (DMF, negative control) and with HCA, a fragrance ingredient endowed with skin-sensitizing properties (positive control). HCA induced a strong proliferation of LN cells at the concentration of 25% in all the performed experiments (SI range 6.38–15.9).
As shown in Table 1, pure coumarin was the only compound tested in the LLNA which did not induce a significant LN cell proliferation, in as much as SI derived from these proliferation data never reached the threshold value of 3 (2.4 at 50%). Coumarin A gave positive LLNA responses in two out of four experiments (SI = 3.2) at 50% concentration. Coumarin B also induced positive responses in one out of four experiments (SI = 3.7) at 25%.

Substantial increase in LN cell proliferation was noted in two out of four experiments for 6-chlorocoumarin (SI = 4.95) at 5% concentration. At the maximal soluble concentration of 10%, a weaker proliferation was obtained (SI = 3), probably due to the recrystallization of the molecule that occurred immediately after its ear application. Animals sensitized with DHC or dibenzodioxocin also exhibited positive LLNA response at 5 and 10% concentration (SI = 5.1 and 3.4), respectively.

Of note, LN cell proliferation induced by the different coumarin preparations and impurities were obtained in the two strains of mice.

According to the classification of the allergenic potential of chemicals in the LLNA [21, 22], these results indicate that (1) pure coumarin cannot be classified as a contact sensitizer as it does not induce LN cell proliferation in the LLNA, (2) coumarins A and B are sensitzers in the

![Fig. 1. Structure of coumarin and the three impurities tested in the LLNA.](image)

**Table 1.** Assessment of the sensitizing activity of different coumarins and impurities in the mouse LLNA

<table>
<thead>
<tr>
<th>Exposure concentration</th>
<th>Pure coumarin</th>
<th>Coumarin A</th>
<th>Coumarin B</th>
<th>6-Chlorocoumarin</th>
<th>DHC</th>
<th>Dibenzodioxocin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>dpm</td>
<td>SI</td>
<td>dpm</td>
<td>SI</td>
<td>dpm</td>
<td>SI</td>
</tr>
<tr>
<td>0%</td>
<td>no</td>
<td>140</td>
<td>no</td>
<td>260</td>
<td>no</td>
<td>127</td>
</tr>
<tr>
<td>2.5%</td>
<td>no</td>
<td>251</td>
<td>no</td>
<td>241</td>
<td>no</td>
<td>246</td>
</tr>
<tr>
<td>5%</td>
<td>no</td>
<td>251</td>
<td>no</td>
<td>241</td>
<td>no</td>
<td>246</td>
</tr>
<tr>
<td>10%</td>
<td>no</td>
<td>251</td>
<td>no</td>
<td>241</td>
<td>no</td>
<td>246</td>
</tr>
<tr>
<td>25%</td>
<td>no</td>
<td>251</td>
<td>no</td>
<td>241</td>
<td>no</td>
<td>246</td>
</tr>
<tr>
<td>50%</td>
<td>no</td>
<td>251</td>
<td>no</td>
<td>241</td>
<td>no</td>
<td>246</td>
</tr>
<tr>
<td>HCA 25%</td>
<td>no</td>
<td>251</td>
<td>no</td>
<td>241</td>
<td>no</td>
<td>246</td>
</tr>
</tbody>
</table>

Experiments were conducted according to the standard LLNA protocol. Only one representative experiment for each chemical is shown.

1. All the chemicals were dissolved in DMF.
2. Local irritation measured by the increase in ear thickness by topical application of chemicals.
3. Incorporation of 3H-thymidine in the draining lymph node cells.
LLNA which categorize them as weak potency allergens, and (3) coumarin impurities 6-chlorocoumarin, DHC and dibenzodioxocin behave as moderate sensitizers.

Allergenic Potential of Pure Coumarin in Humans

Results of the homogeneity of pure coumarin preparations used for patch tests are shown in table 2. The homogeneity was stable for 1 year at 4°C.

Three GCP studies were conducted in humans to test the irritant and allergenic properties of pure coumarin used as patch test.

Study 1. In the first study, 379 patients, suffering from skin inflammatory diseases and referred to the allergology clinic for patch testing, received coumarin at 2% on the skin on the back. One hundred of these patients also received coumarin at 10%. Coumarin was well tolerated at both concentrations and did not induce skin inflammatory reactions at 48/72 h, except for 1 patient with highly sensitive skin who reacted to several chemicals including coumarin at 2% and sodium lauryl sulfate at 0.25%. Interestingly, the highest concentration of coumarin, i.e. 10%, previously reported to induce irritancy [13], was as well tolerated as the 2% concentration in the 100 patients who received this concentration. Results of patch testing with the European standard battery showed that 16/100 patients were allergic to one of the 23 contact allergens. Only 3 of the 16 positive patients were allergic to FM and 2 to fragrances (table 3). Four patients were allergic to Peru balsam and positive reactions were noted to other allergens including chromium, cobalt, nickel, colophony, lanolin and cocoamidopropyl betaine. The remaining 84 patients did not show contact allergy to any of the common contact allergens tested.

Study 2. In the second study, coumarin was patch tested on the skin on the back of 101 patients suffering from ACD and allergic to FM (positive FM patch test). Only 1 patient, a 41-year-old man who developed ACD to deodorant involving armpits, elbows and neck, was positive to coumarin (++). He was also positive to other perfume ingredients: three of the FM series (isoeugenol, geraniol, oak moss) and jasmine-lavender-narcissus absolutes and rose oil (++ for all). Unfortunately, this patient could not be retested with coumarin alone, and no immunological

<table>
<thead>
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<th>Table 2. Homogeneity of coumarin preparations</th>
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<tbody>
<tr>
<td><strong>Theoretical</strong></td>
</tr>
<tr>
<td>1.0</td>
</tr>
<tr>
<td>3.0</td>
</tr>
<tr>
<td>5.0</td>
</tr>
<tr>
<td>8.0</td>
</tr>
<tr>
<td>10.0</td>
</tr>
</tbody>
</table>

Values are expressed as percentages. Three samples were taken at different sites of the preparation in order to assess homogeneity by UV analysis against petrolatum. The 10% concentration reached homogeneity; 8.7% was used as ‘10%’ in the clinical study.

<table>
<thead>
<tr>
<th>Table 3. Clinical studies of the allergenicity of pure coumarin</th>
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<tr>
<td><strong>Studies</strong></td>
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<tr>
<td>-------------</td>
</tr>
<tr>
<td>Study 1 contact dermatitis</td>
</tr>
<tr>
<td>Study 2 FM allergic</td>
</tr>
<tr>
<td>Study 3 perfume allergic</td>
</tr>
</tbody>
</table>

ND = Not determined.
1 Clinically relevant positive patch test to FM.
2 Patients with a personal history of contact allergy to fragrances.
3 Patients with a personal history of atopic diseases.
4 Number of patients with a positive patch test to coumarin in the tested population.
investigations on the positive patch test could be done to formally confirm the allergic nature of the test. Consequently, the relationship between the fragrance allergy and coumarin has been classified as likely for the investigator.

Study 3. At last, we focused on perfume-allergic patients with positive patch tests to their own perfumed product. We were able to include only 30 patients in this part of the study with the help of 7 active ACD clinics, suggesting that fragrance allergy defined by a positive patch test to FM probably overestimates the real frequency of perfume allergy. Patients received coumarin at 2% on the back, and 19 patients received the first 8 allergens of the fragrance series. No patient showed a positive allergic reaction to coumarin. Conversely, a high proportion of patients did react to hydroxycitronellal (35%), isoeugenol (30%), oak moss absolute (30%), cinnamic alcohol, cinnamic aldehyde, eugenol, geraniol (20%) and amylcinnamaldehyde (15%).

This study shows that coumarin is not a common allergen in fragrance-allergic patients.

Discussion

In this study, using coumarin as a model, we demonstrated that the purity of a chemical is a major parameter for its safety. We showed that coumarin is extremely well tolerated when in contact with the skin. The chemical has very low irritant properties and does not behave as a contact allergen. The standard test for skin hazard identification LLNA failed to classify pure coumarin as an allergen, and clinical studies performed with a large panel of allergic patients (n = 510) did not confirm the suspected allergenicity of coumarin.

Experimental LLNA Studies

The LLNA studies show that purity of the coumarin preparation is a major factor in its lack of allergenicity. Pure coumarin was unable to induce LN cell proliferation. In contrast, the two other commercially available coumarins that contained up to 19 impurities (determined by gas and liquid chromatography and mass spectroscopy) were endowed with proinflammatory properties and led to significant LN cell activation. Hence, the presence of impurities in a coumarin preparation seems to directly influence its allergenic potency. Three of the impurities detected in coumarins A and B, i.e. DHC, 6-chlorocoumarin and dibenzodioxocin, were identified as moderate contact sensitizers in the LLNA. They certainly participate to the proinflammatory properties of coumarins A and B. However, it is noteworthy that these contaminants are present at very low levels in coumarins A and B, suggesting that other derivatives may exert synergistic effects with these contaminants to induce sensitization.

The observation that contaminants or degradation products, but not the parent compound, are responsible for skin allergenicity has already been reported for other families of chemicals [8, 23–25]. In perfume products, d-limonene and linalool are unreactive molecules devoid of sensitizing properties. However, upon air exposure, d-limonene and linalool give rise to oxidation products which behave as contact sensitizers [24] and can induce ACD.

Clinical Studies Using Patch Tests with Pure Coumarin

In our study, patch tests using coumarin at 10% concentration did not show any irritant effect on 100 consecutive dermatitis patients, except in 1 patient with highly sensitive skin. We also showed that only 1 out of 510 patients (<0.2%) developed a positive reaction to the coumarin patch test. These results are in agreement with the recent study of Frosch et al. [14] who reported positive patch tests to coumarin in less than 0.3% of the 1,855 ACD patients tested. Furthermore, only one well-documented, clinically relevant case of fragrance allergy to coumarin was reported [26], suggesting that contact allergy to coumarin is an exceptional event. In this respect,
it is noteworthy that there is an opposite evolution during the last 20 years in the frequency of allergy to FM (steadily increasing) [27–29] and that of allergy to coumarin (regularly decreasing) (table 4), suggesting that coumarin cannot be considered as a major fragrance allergen.

The discrepancy between our results and those previously published, demonstrating the allergenic potential of coumarin, probably depends upon the level of purity of coumarin incorporated in petrolatum for the patch tests. Although in all these studies coumarin was said to be 95–99% pure, no information was given on the technical process used in creating the coumarin preparation, the detailed composition of the chemicals and the amount of the impurities found in coumarin. Therefore, we postulate that allergic impurities, which are present in the coumarin preparations, are responsible for the positive allergic reaction attributed to coumarin. Another parameter to consider is the homogeneity of the coumarin preparation itself. We observed that coumarin is not easily mixed homogeneously in petrolatum. Hence, it is possible that the coumarin-induced skin reactions which have been reported before occurred because of the lack of coumarin homogeneity resulting in much higher chemical concentrations than expected at sites of skin testing.

**Conclusion**

The purity of a chemical is a major parameter for its sensitizing and allergenic properties. Therefore, manufacturers should precisely check the purity of the chemical they propose to introduce in preparations used for either patch testing or for cosmetics in order to prevent risks of skin sensitization and allergy.

**Appendix: List of Investigators for the Clinical Studies**

**Study 1**: G. Chabeau, J.F. Nicolas (Pierre Bénite), C. Goujon, J.F. Nicolas (Pierre Bénite), H. Assier-Bonnet ( Créteil), A. Barbaud (Nancy), A. Bircher (Basel), J.L. Bourrain (Grenoble), M. Castelain (Marseille), G. Ducombs (Bordeaux), M.L. Fléchet (Paris), F. Giordano-Labadie (Toulouse), G. Jelen (Saverne), Ch. Le Coz (Strasbourg), A. Nassif (Paris), A. Pons-Guiraud (Paris), D. Tennestedt (Bruxelles), M. Vigan (Besançon).


Apart from the last investigator (G.M.), all are members of GERDA (Groupe d’Etudes et de Recherche en Dermato-Allergologie) or of REVIDAL (Réseau de Vigilance en Dermato-Allergologie) GERDA.

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We will be delighted to provide samples of pure coumarin as well as information on the origin of the other coumarin samples used in these studies to any person who will address a demand to Arielle Gard (arielle.gard@eu.rhodia.com). For deontological and commercial reasons, no information on the source of coumarins A and B is given here.

**References**


The Skin Allergenic Properties of Chemicals May Depend on Contaminants
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