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INVESTIGATIVE REPORT

Structural Analogues Inhibit the Sensitizing Capacity of Carvone

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The aim of the study was to investigate the effect of non-allergenic structural analogues on the sensitizing potential of carvone, a fragrance allergen. The possibility that one molecule might inhibit the allergenic activity of another molecule has been debated for 25 years. The Research Institute for Fragrance Materials states that the sensitizing activity from certain fragrance aldehydes is "quenched" by the addition of other specific chemicals. However, other studies do not confirm the results, although several attempts have been made. We used a guinea pig method designed to study the sensitizing capacity of fragrance allergens. Induction was performed with either carvone alone or with a mixture of carvone and one of two analogues. A significant difference in the response rates ($p<0.001$) was observed between the animals induced with carvone alone and those induced with any of the mixtures. Our investigation shows that by using selected molecules it is possible to significantly reduce the sensitizing effect of a fragrance allergen. ***Key words: experimental sensitization; guinea pigs; quenching; skin.***

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Interest and investigations regarding the possibility that one molecule might inhibit or reduce the allergenic activity of another have focused on three fragrance aldehydes and their "quenching" chemicals. This was first reported in a publication from the Research Institute for Fragrance Materials (RIFM) 25 years ago (1). The term "quenching" was employed to describe the inhibition of the sensitizing effect of the selected fragrance aldehydes by the presence of certain other fragrance chemicals at defined ratios. Based on these results, the International Fragrance Industries Association (IFRA) developed guidelines to limit the sensitizing potential of fragrance materials containing any of the three fragrances studied. It was stated that: citral was quenched by the addition of 25% *d*-limonene or mixed citrus terpenes or α-pinene; cinnamic aldehyde should be used along with equal parts of eugenol or *d*-limonene; and phenylacetaldehyde should be used with equal parts of phenylethyl alcohol or dipropenyl glycol (2).

During the years, observations regarding the inhibition of the sensitizing effect of the fragrance aldehydes have been reinvestigated in different ways (3). The most thorough investigation was performed using different guinea pig methods for predictive testing of the sensitizing potential of a chemical (4). In not one case in more than 20 individual tests could a reduction in the allergenic activity using combinations of cinnamic aldehyde/eugenol (1:1) or citral/limonene (4:1) be demonstrated. Neither was any reduction demonstrated in the murine local lymph node assay (LLNA) (5). The Scientific Committee on Cosmetic Products and Non-Food Products (SCCNFP) intended for consumers within the European Union presented a position paper on the phenomenon of quenching in February 2000 based on the material published (6). This paper concludes that on the balance of evidence then available, the existence of quenching of certain fragrance allergens by other specific fragrance components should be regarded as a hypothesis only. The paper also states that convincing clinical data of the efficacy of quenching are lacking.

The aim of the present study was to investigate the effects of specially selected non-allergenic structural analogues on the sensitizing potential of a known fragrance chemical, carvone (Fig. 1) using a guinea pig method for predictive testing of fragrance allergens.

MATERIAL AND METHODS

Chemicals

(*R*)-(-)-Carvone (98%) was purchased from Aldrich Chemical (Stockholm, Sweden). (2*R*, 5*R*)-5-isopropenyl-2-methyl cyclohexanone (2*R*, 5*R*-dihydrocarvone) and (5*R*)-2,3-dimethyl-5-isopropenyl-2-cyclohexene-1-one (*R*-methylcarvone) were synthesized as described previously (7–9). Both structures have been demonstrated to be non-sensitizers. Only one of 15 exposed animals showed a positive response to the highest test concentration (3.3×10^{-4} mol/g) of 2*R*, 5*R*-dihydrocarvone and *R*-methylcarvone, respectively (9).

Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA) were obtained from Difco (Detroit, MI, USA). White petrolatum was obtained from Apoteket AB, Stockholm, Sweden. Other chemicals used were of pharmaceutical or analytical grade.

Studies on the inhibitory effect of the structural analogues in guinea pigs

The studies were approved by the local ethics committee in Stockholm. Female, outbred, albino Dunkin Hartley guinea pigs weighing 250–300 g were purchased from Bio Jet service, Uppsala, Sweden. The animals were housed in Macrolon cages, kept on a guinea pig standard diet and water *ad lib*.

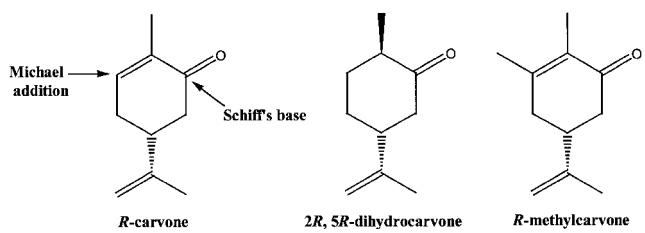

Fig. 1. Structures of compounds studied. The reactive sites for antigen formation by a Michael addition at C3 or Schiff's base formation at C1 are indicated.

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Effect of 2R, 5R-dihydrocarvone on R-carvone sensitization

The Freund's complete adjuvant test (FCAT) (10) on guinea pigs was used. On days 0, 6 and 10 the animals received one intradermal injection (0.1 ml) on the upper back for induction. At the second and third inductions the animals received the test material in a Freund's incomplete adjuvant FIA/H_2O emulsion as opposed to the first injection with FCA/H_2O emulsion. The modification was performed according to recommendations from the ethics committee. Three groups of 15 animals in each were used. The first group was injected with *R*-carvone 3.3×10^{-4} mol/g (5% w/w) in FCA(FIA)/H_2O emulsion (1:1). The second group was injected with *R*-carvone and 2*R*, 5*R*-dihydrocarvone in concentrations 1:2 (3.3×10^{-4} mol/g: 6.6×10^{-4} mol/g) in FCA(FIA)/H_2O emulsion. The third group was a control group sham-treated with an FCA(FIA)/H_2O emulsion. Closed challenge testing was performed on day 21 (11). The test material (0.015 g) was applied on the shaved flanks of the animals for 24 h using alumina test chambers (Finn Chambers®, i.d. 8 mm; Epitest Tuusla, Finland). The reactions were assessed at 48 h and 72 h after application. The minimum criterion for a positive reaction was a confluent erythema. The test concentrations of *R*-carvone were chosen according to earlier experiences (12) and the concentrations of the synthesized analogue were chosen to be 2× those of carvone. The compounds in the concentrations used were shown to be non-irritating in pretests on FCA-treated guinea pigs. All animals were challenged with *R*-carvone 6.6×10^{-5}, 3.3×10^{-5} and 1.3×10^{-5} mol/g (1, 0.5, 0.2%), with *R*-carvone+ 2*R*, 5*R*-dihydrocarvone $6.6\times10^{-5}+13.2\times10^{-5}$ mol/g, $3.3\times10^{-5}+6.6\times10^{-5}$ mol/g and $1.3\times10^{-5}+2.6\times10^{-5}$ mol/g (1+2, 0.5+1, 0.2+0.4%), with 2*R*, 5*R*-dihydrocarvone 6.6×10^{-5} mol/g (1%) and with a vehicle control. Petrolatum was used as vehicle for all test materials.

Rechallenge. On day 49, all animals were retested with *R*-carvone 3.3×10^{-5}, 1.3×10^{-5}, 0.66×10^{-5}, 0.33×10^{-5}, 0.13×10^{-5} mol/g (0.5, 0.2, 0.1, 0.05, 0.02%) and with petrolatum.

Effect of R-methylcarvone on R-carvone sensitization

The experiment was performed according to the method described above. Three groups of 15 animals in each were used. The first group was injected with *R*-carvone 3.3×10^{-4} mol/g in FCA(FIA)/H_2O emulsion (1:1). The second group was injected with *R*-carvone and *R*-methylcarvone in concentrations 1:2 (3.3×10^{-4} mol/g: 6.6×10^{-4} mol/g) in FCA(FIA)/H_2O emulsion. The third group was a control group sham-treated with an FCA(FIA)/H_2O emulsion. All animals were challenged with *R*-carvone 3.3×10^{-5}, 0.66×10^{-5}, 0.33×10^{-5}, 0.66×10^{-6} mol/g (0.5, 0.1, 0.05, 0.01%), with *R*-carvone+ *R*-methylcarvone $0.66\times10^{-5}+1.3\times10^{-5}$, $0.33\times10^{-5}+0.66\times10^{-5}$ mol/g (0.1+0.22%, 0.05+0.11%), with *R*-methylcarvone 6.6×10^{-5} mol/g (1.1%) and with petrolatum as vehicle control. The compounds in the actual concentrations were shown to be non-irritating in pretests on FCA-treated guinea pigs.

Rechallenge. On day 55, all animals were retested with *R*-carvone 3.3×10^{-5}, 1.3×10^{-5}, 0.66×10^{-5}, 0.46×10^{-5}, 0.33×10^{-5}, 0.13×10^{-5} mol/g (0.5, 0.2, 0.1, 0.07, 0.05, 0.02%) and with petrolatum.

Statistics

A difference in response between exposed animals and controls was evaluated using the Fisher exact test. The two-factor analysis of variance was used for evaluation of an inhibitory effect.

RESULTS

Effect of 2R, 5R-dihydrocarvone on R-carvone sensitization

The animals in both exposed groups were sensitized (Table I). The animals induced with a mixture of *R*-carvone and 2*R*, 5*R*-dihydrocarvone showed a significantly lower response than those induced with *R*-carvone only when challenge-tested with *R*-carvone. The addition of 2*R*, 5*R*-dihydrocarvone to *R*-carvone at challenge-testing did not influence the response (Table I). In the control animals, one reaction was found to carvone 6.6×10^{-5} mol/g and one to carvone in mixture with dihydrocarvone $1.3\times10^{-5}+2.6\times10^{-5}$ mol/g at the 72 h reading.

Rechallenge confirmed the results (Fig. 2a). A significant difference ($p<0.001$) was found between the response rate of group 1 induced with *R*-carvone only, compared to that of

Table I. *The inhibitory effect of 2R, 5R-dihydrocarvone on R-carvone sensitization*

Test material in challenge concentration (mol/g)		1[a] ($n=15$)	2[b] ($n=15$)	Significance 1 vs. 2 $p=$
R-carvone (6.6×10^{-5})	48 h	13[c,d]	7[c,e]	0.022
	72 h	14[d]	9[e]	0.037
R-carvone (3.3×10^{-5})	48 h	14[d]	6[e]	0.0025
	72 h	15[d]	8[e]	0.0032
R-carvone (1.3×10^{-5})	48 h	7[e]	2	0.047
	72 h	12[d]	4[f]	0.0043
R-carvone (6.6×10^{-5})+ 2*R*, 5*R*-dihydrocarvone (13.2×10^{-5})	48 h	14[d]	8[e]	0.016
	72 h	15[d]	9[d]	0.0084
R-carvone (3.3×10^{-5})+ 2*R*, 5*R*-dihydrocarvone (6.6×10^{-5})	48 h	14[d]	5[f]	<0.001
	72 h	13[d]	8[e]	0.047
R-carvone (1.3×10^{-5})+ 2*R*, 5*R*-dihydrocarvone (2.6×10^{-5})	48 h	10[d]	3	0.011
	72 h	15[d]	6[f]	<0.001
2*R*, 5*R*-dihydrocarvone (6.6×10^{-5})	48 h	0	0	
	72 h	0	0	
Petrolatum	48 h	0	0	
	72 h	1	0	

[a]Group 1 is induced with *R*-carvone.
[b]Group 2 is induced with *R*-carvone+ 2*R*, 5*R*-dihydrocarvone.
[c]The results are given as number of animals responding in the exposed groups 1 and 2. In the control animals, one reaction was found to carvone 6.6×10^{-5} mol/g and one to carvone in mixture with dihydrocarvone $1.3\times10^{-5}+2.6\times10^{-5}$ mol/g at the 72 h reading.
[d] p(exposed/controls)<0.001.
[e] p(exposed/controls)<0.01.
[f] p(exposed/controls)<0.05.

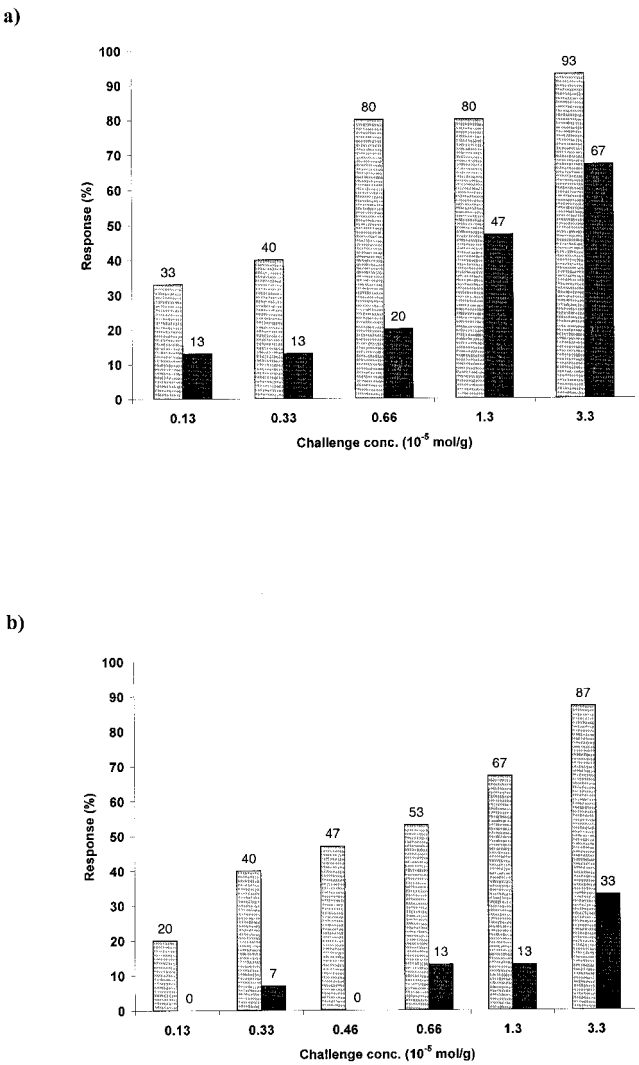

Fig. 2. Results at rechallenge, 72 h reading. Response rates (%) to carvone in animals induced with (a) carvone alone □ and carvone in a mixture with dihydrocarvone 1:2 ■ and with (b) carvone alone □ and carvone in a mixture with methylcarvone 1:2 ■. A significant dose–response relationship ($p<0.001$) was obtained to carvone in all four experiments. A significant difference in the response rates ($p<0.001$) was seen between the animals induced with carvone alone and those induced with mixtures of carvone and either dihydrocarvone or methylcarvone.

group 2 induced with the mixture (Fig. 2a). When compared to the response in the controls, a significant response ($p<0.05$) was seen to all concentrations of carvone in group 1, while in group 2 a significant response ($p<0.05$) was seen only to 3.3×10^{-5} mol/g and 1.3×10^{-5} mol/g. However, a significant dose–response relationship ($p<0.001$) was seen in both experiments (Fig. 2a). No positive reactions were seen in the control animals.

Effect of R-methylcarvone on R-carvone sensitization

The animals in both exposed groups were sensitized (Table II). Those induced with a mixture of *R*-carvone and *R*-methylcarvone showed a significantly lower response than the animals induced with *R*-carvone when tested with carvone 3.3×10^{-5} and 0.66×10^{-5} mol/g and with carvone+methylcarvone ($0.66\times10^{-5}+1.3\times10^{-5}$ mol/g). The addition of *R*-methylcarvone to *R*-carvone at challenge-testing did not influence the response (Table II). No positive reactions were seen in the control animals.

Rechallenge confirmed the results (Fig. 2b). A significant difference ($p<0.001$) was found between the response rate of group 1, induced with *R*-carvone only, compared to that of group 2, induced with the mixture (Fig. 2b). When compared to the response in the controls, a significant response ($p<0.01$) was seen in group 1 to all but the lowest concentration, which gave a non-significant response. In group 2, a significant response ($p<0.05$) was seen only to the highest concentration of carvone (3.3×10^{-5} mol/g). However, a significant dose–response relationship ($p<0.001$) was seen in both experiments (Fig. 2b). No positive reactions were seen in the control animals.

DISCUSSION

For the first time, an inhibitory effect of one molecule on the allergenic effect of another molecule has been demonstrated experimentally.

R-Carvone was chosen as the model compound to investigate a possible inhibitory effect from non-allergenic structural analogues. It is a stable molecule with known allergenic activity (12–14) and a structure that allows synthetic structural changes necessary for the study. In a preceding study (9), an observed difference in chemical reactivity of carvone towards hard and soft nucleophiles indicated that the major path for antigen formation is via a Michael addition and not via Schiff's base formation (Fig. 1). The results were confirmed when no eliciting effect was seen from the synthesized structural analogues dihydrocarvone (Fig. 1) and methylcarvone (Fig. 1) in carvone-sensitized animals and in patients with known contact allergy to carvone (9). The reactive site for a Michael addition at C-3 is absent in dihydrocarvone and sterically hindered in methylcarvone. Furthermore, both analogues were found to be non-sensitizers (9), showing that Schiff's base formation is of no importance for the antigen formation. Since the skin penetration of the analogues should be similar to that of carvone due to small differences in the log P values (9), the two analogues were considered suitable for testing inhibition of carvone allergy.

Carvone is a fragrance component with the same overall structure as other monoterpenes used as fragrances. To investigate a possible inhibitory effect, we used a guinea pig method (10) originally designed for studying the sensitizing capacity of fragrance allergens. The guinea pig models have the advantage compared to the more recently designed mouse model, the Local Lymph Node Assay (LLNA) (15), that they allow investigation of the elicitation effect. This is important, since we also wanted to investigate whether a decrease in the response to carvone could be seen when the analogues were added to the challenge preparations. However, no inhibitory effect was demonstrated on elicitation in carvone-sensitized animals.

The mechanism of the inhibition of sensitization cannot be explained easily. Chemical interaction between carvone and its analogues to change the structure of the hapten is not possible under the conditions in the investigation. To increase the probability of inhibition, we added the carvone analogues

Table II. *The inhibitory effect of R-methylcarvone on R-carvone sensitization*

Test material in challenge concentration (mol/g)		1[a] ($n=14$)	2[b] ($n=15$)	Significance 1 vs. 2 $p=$
R-carvone (3.3×10^{-5})	48 h	11[c,d]	6[c,e]	0.0035
	72 h	12[d]	6[e]	0.013
R-carvone (0.66×10^{-5})	48 h	6[e]	0	0.0063
	72 h	7[e]	1	0.012
R-carvone (0.33×10^{-5})	48 h	2	0	
	72 h	2	0	
R-carvone (0.66×10^{-6})	48 h	0	0	
	72 h	0	0	
R-carvone (0.66×10^{-5} + *R*-methylcarvone 1.3×10^{-5})	48 h	8[d]	0	<0.001
	72 h	9[d]	0	<0.001
R-carvone (0.33×10^{-5} + *R*-methylcarvone 0.66×10^{-5})	48 h	3	0	
	72 h	3	0	
R-methylcarvone (6.6×10^{-5})	48 h	0	0	
	72 h	0	0	
Petrolatum	48 h	0	0	
	72 h	0	0	

[a]Group 1 is induced with *R*-carvone. One animal undressed during the challenge testing.
[b]Group 2 is induced with *R*-carvone+ *R*-methylcarvone.
[c]The results are given as number of animals responding in the exposed groups 1 and 2. No reactions were seen in the control group.
[d] p(exposed/controls)<0.001.
[e] p(exposed/controls)<0.01.

in a ratio of 2:1 compared to the allergen. The use of twice as much of the inhibitors compared to carvone could contribute to the inhibition, but it cannot be the only explanation. If it is just the amount that is of importance towards obtaining a reduction in allergenic activity, any molecule in combination with carvone would do. In such a case, clinical cases of carvone allergy are unlikely to be found, since consumers are exposed to carvone as one of many compounds in natural materials such as spearmint oil and oxidized limonene (13, 14, 16).

A competitive inhibition may cause the quenching, although this competition does not involve a covalent binding of the two carvone analogues to the macromolecule. One could speculate that the formation of antigen takes place directly on endogenous peptides in the antigen-binding groove of HLA-class II molecules on the antigen-presenting cells, as has been postulated for nickel (17, 18). In such a case the analogues could prevent induction by temporary blocking binding sites in the groove without antigen formation due to similarity in structure. However, the blocking is not sufficiently effective to prevent elicitation in already sensitized individuals, since the amount of antigens needed is very low. It should be noted that we observed some response also when the analogues were added at induction.

Quenching has been investigated and debated since it was first proposed. In previous studies on quenching, it seems that no consideration has been given to sensitizing capacity, structural similarity and skin penetration of the allergen and the inhibiting chemicals used. The investigations carried out have included only those chemicals originally claimed to either be quenched or act as quenchers. It is obvious that if a compound is to be of any value in preventing an allergic reaction it cannot have an allergenic effect in itself. However, this was not considered in the quenching studies performed by the industry using for example eugenol and *d*-limonene to try to reduce the allergenic activity of other compounds (1, 3). Eugenol has an allergenic activity in itself, while *d*-limonene readily forms strong allergens upon air oxidation (16). It is also important to consider the effect of skin penetration when deciding the ratio between the allergen and the inhibiting molecule. In the quenching experiments, the sensitizer is applied in higher or similar concentrations compared to the supposed quencher (4). The possibility of inhibition when adding the inhibitor in higher concentrations was not investigated. RIFM has presented a recent study (19) on the quenching effect of the sensitizing potential of two of the fragrance aldehydes, citral and phenylacetaldehyde, using the human maximization test (20, 21) and a human repeated insult patch test (22). In these investigations, no sensitizing activity could be demonstrated when repeatedly applying different mixtures of the sensitizing fragrances and quenchers according to the methods used. It was once more concluded that a quenching effect does exist for citral and phenylacetaldehyde using the stipulated quenchers (19). However, the effects observed when applying citral and phenylacetaldehyde alone are not presented. Furthermore, according to the description of the test method used, the test material was allowed to volatilize for 15–30 min after application on the patch, i.e. before the patch was applied to the skin. Since the fragrances are volatile compounds, there is an obvious risk that the dose of allergen is reduced below the sensitizing level.

The results from our study show that by using selected molecules it is possible to reduce the sensitizing effect of a fragrance allergen. This could be a way of accomplishing a reduction in the effect of some of the most frequent sensitizers in fragrance materials. Since fragrances, besides nickel salt, are the most common causes of contact allergy and allergic contact dermatitis, this would be an important contribution to increased public health. However, further studies are needed to understand the mechanism behind our observations. It is likely that the structural similarity between the synthesized analogues and carvone prevents antigen formation from

occurring. In future studies we will investigate the structural requirements for the inhibitory effect.

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REFERENCES

1. Opdyke DLJ. Inhibition of sensitization reactions induced by certain aldehydes. Fd Cosmet Toxicol 1976; 14: 197–198.
2. International Fragrance Research Association. Code of Practice. IFRA, Geneva, 1980.
3. Basketter D. Quenching: fact or fiction? Contact Dermatitis 2000; 43: 253–358.
4. Basketter DA, Allenby CF. Studies of the quenching phenomenon in delayed contact hypersensitivity reactions. Contact Dermatitis 1991; 25: 160–171.
5. Basketter DA. The value of animal assays and the quenching phenomenon. In: Frosch PJ, Johansen JD, White I, eds. Proceedings of the Fragrance Symposium Heidelberg, Springer Verlag, 1997: 166–174.
6. Opinion concerning the predictive testing of potentially cutaneous sensitizing cosmetic ingredients or mixtures of ingredients adopted by the SCCNFP during the 11th plenary session of 17 February 2000. Health and consumer protection – scientific committee for cosmetic products, and non-food products intended for consumers – outcome of discussion 102 http://europa.eu.int/comm/food/fs/sc/sccp/out112_en.pdf
7. Maestro M A, Castedo L, Mouriño A. A convergent approach to the dihydrotachysterol diene system. Application to the synthesis of dihydrotachysterol$_2$ (DHT_2), 25-hydroxydihydrotachysterol$_2$ (25-OH-DHT_2), 10(*R*), 19-dihydro-(5*E*)-3-epivitamin D_2 and 25-hydroxy-10(*R*), 19-dihydro-(5*E*)-3-epivitamin D_2. J Org Chem 1992; 57: 5208–5213.
8. Fortunato JM, Ganem BJ. Lithium and potassium trialkylborohydrides. Reagents for direct reduction of α, β-unsaturated carbonyl compounds to synthetically versatile enolate anions. J Org Chem 1976; 41: 2194–2200.
9. Nilsson A-M, Gäfvert E, Salvador L, Luthman K, Bruze M, Gruvberger B, Nilsson JLG, Karlberg A-T. Mechanism of the antigen formation of carvone and related α, β-unsaturated ketones. Contact Dermatitis 2001; 44: 347–356.
10. Klecak G. The Freund's complete adjuvant test and the open epicutaneous test. A complementary test procedure for realistic assessment of allergenic potential. In: Andersen KE, Maibach HI, eds. Contact allergy predictive tests in guinea pigs. Curr Probl Dermatol Basel: Kargel, 1985: 152–171.
11. Boman A, Karlberg A-T, Wahlberg JE. Experiences with Freund's complete adjuvant test (FCAT), when screening for contact allergens in colophony. Contact Dermatitis 1988; 18: 25–29.
12. Karlberg A-T, Magnusson K, Nilsson U. Air oxidation of d-limonene (the citrus solvent) creates potent allergens. Contact Dermatitis 1992; 26: 332–340.
13. Andersen K. Contact allergy to toothpaste flavours. Contact Dermatitis 1978; 4: 195–198.
14. Paulsen E, Andersen KE, Carlsen L, Egsgaard H. Carvone: an overlooked contact allergen cross-reacting with sesquiterpene lactones? Contact Dermatitis 1993; 29: 138–143.
15. Gerberick GF, Ryan CA, Kimber I, Dearman RJ, Lea LJ, Basketter DA. Local lymph node assay validation assessment for regulatory purposes. Am J Cont Derm 2000; 11: 13–18.
16. Karlberg A-T, Magnusson K, Nilsson U. Influence of an antioxidant on the formation of allergenic compounds during autoxidation of d-limonene. Ann Occup Hyg 1994; 38: 199–207.
17. Romagnoli P, Labhardt AM, Sinigaglia F. Selective interaction of Ni with an MHC-bound peptide. EMBO J 1991; 10: 1303.
18. van den Broeke LT, Heffler LC, Tengvall Linder M, Nilsson JLG, Karlberg A-T, Scheynius A. Direct Ni^{2+} antigen formation on cultured human dendritic cells. Immunology 1999; 96: 578–585.
19. Api AM. Quenching of citral, cinnamaldehyde and phenylacetaldehyde sensitization in human repeated insult patch tests. Contact Dermatitis 2000; 42: Suppl 2. Abstr, 40.
20. Kligman AM. The identification of contact allergens by human assay. III. The maximization test. A procedure for screening and rating contact sensitizers. J Invest Dermatol 1966; 47: 393–409.
21. Kligman AM, Epstein W. Updating the maximization test for identifying contact allergens. Contact Dermatitis 1975; 1: 231–239.
22. Marzulli FN, Maibach HI. Contact allergy: predictive testing in humans. In: Marzulli FN, Maibach HI, eds. Advances in modern toxicology, dermatoxicology and pharmacology. New York: John Wiley & Sons, 1977: 353–372.