



Citral: Identifying a threshold for induction of dermal sensitization

J. Lalko *, A.M. Api

Human Health, Research Institute for Fragrance Materials, Inc., 50 Tice Boulevard, Woodcliff Lake, NJ 07677, USA

Received 17 July 2007

Abstract

Citral [CAS# 5392-40-5; EINECS# 226-394-6; RIFM # 116; cis- and trans-3,7-dimethyl-2,6-Octadienal] is an important fragrance ingredient appreciated for its powerful lemon-aroma. It is widely used in fragrance formulations and incorporated into numerous consumer products. A comprehensive review of the dermal sensitization data available for citral was undertaken with the goal of identifying a threshold for the induction of dermal sensitization. In 2007, a complete literature search was conducted. On-line databases that were surveyed included Chemical Abstract Services and the National Library of Medicine. In addition, the toxicologic database of the Research Institute for Fragrance materials, Inc. (RIFM) was searched, which includes numerous unpublished reports. Based on a weight of evidence approach, the data from this survey demonstrate that the human NOEL (No Observed Effect Level) for induction of dermal sensitization to citral is 1400 $\mu\text{g}/\text{cm}^2$. The identification of this induction threshold will allow for risk assessments to focus on primary prevention of contact allergy to citral based on a new Quantitative Risk Assessment (QRA) paradigm. This subsequent assessment will form the basis of a risk management approach; specifically a new IFRA (International Fragrance Association) standard on the use of citral in consumer products.

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Keywords: Citral; Dermal sensitization; Threshold; Induction; Review

1. Introduction

Citral [CAS# 5392-40-5; EINECS# 226-394-6; RIFM # 116; cis- and trans-3,7-dimethyl-2,6-Octadienal] is an important fragrance ingredient appreciated for its powerful lemon-aroma and is widely used in fragrance formulations in numerous consumer products (Arctander, 1969). Citral is a common component of many essential oils, such as lemongrass and *litsea cubeba* oils (Lawrence, 2003). It may be produced synthetically or obtained from natural oils (Bedoukian, 1967). Citral is generally recognized as a safe food additive and has been approved by the Food and Drug Administration for use in foods (FDA, GRAS, 21 CFR 182.60). The International Fragrance Association (IFRA) has issued a standard on the use of citral in fragrance formulations based on its allergenic potential (IFRA, 1980). Citral is one of 26 fragrance materials iden-

tified as a suspected cause of allergic contact dermatitis by the European Commissions advisory committee—the Scientific Committee for Cosmetic and Non-Food Products (SCCNFP—currently known as the Scientific Committee on Consumer Products [SCCP]). In 2003 Directive, 2003/15/EC, the 7th amendment of the European Cosmetic Directive 76/768/EEC was published based on the recommendations of the SCCNFP. Within the European Union, the directive requires that citral be listed on the ingredient label of consumer products when present at greater than 10 ppm for leave-on cosmetic products and 100 ppm for rinse-off cosmetics. The Member States and the European Parliament adopted the above concentrations as a pragmatic approach to labeling ingredients. Little is known about the dose–response relationship between induction and elicitation of contact allergy. The labeling requirement will provide a degree of secondary prevention by assisting consumers and physicians in avoidance of materials demonstrated or suspected of eliciting an individual contact dermatitis. There still remains a need to conduct accurate

* Corresponding author. Fax: +1 201 689 8090.

E-mail address: jlalko@rifm.org (J. Lalko).

risk assessments to prevent the induction of sensitization to these materials—primary prevention.

A review of all safety data on citral was first published in 1979 by Opdyke. Since that time, a significant number of studies investigating the sensitization potential of citral have been conducted. This paper provides a comprehensive review of the dermal sensitization data available on citral. A review is necessary in order to determine the threshold for induction of sensitization in humans. The identification of an induction threshold will allow for risk assessments to focus on primary prevention of contact allergy to citral based on a new Quantitative Risk Assessment (QRA) paradigm. This subsequent assessment [the details of which are outlined in a paper concurrently submitted with this review (Api et al., *this issue*)] will form the basis of a new IFRA standard on the use of citral in consumer products.

2. Methods

In 2007, a complete literature search was conducted on citral. On-line databases that were surveyed included Chemical Abstract Services and the National Library of Medicine. In addition, the toxicologic database of the Research Institute for Fragrance materials, Inc. (RIFM) was searched, which includes numerous unpublished reports. All relevant references are included in this review.

3. Structural analysis

QSAR (Quantitative Structure Activity Relationship) analysis of citral (Fig. 1) reveals structural alerts for potential toxic dermal effects such as sensitization (Ford et al., 2000). Application of the rule based DEREK (Deductive Estimation of Risk from Existing Knowledge) system also identifies citral as a potential contact allergen (Hostynek et al., 1998). In order for a chemical to act as a contact allergen, it must first diffuse from the skin surface to the viable epidermis where it may react with skin proteins to form an immunogen. Citral has a molecular weight of 152.24 Da and a calculated octanol/water partition coefficient, $\log K_{O/W}$, of 3.45 suggesting that it would permeate fairly readily through human skin. As a volatile organic, it would be expected that (under non-occlusive conditions)

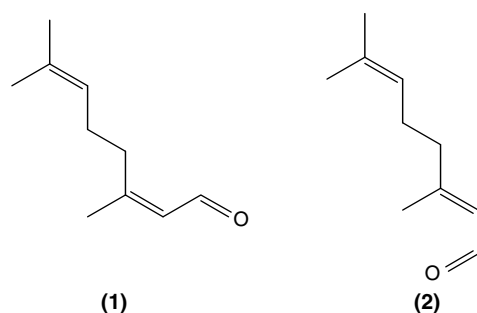


Fig. 1. Citral [CAS# 5392-40-5; EINECS# 226-394-6] is a mixture of two isomers (1) cis- and (2) trans-3,7-dimethyl-2,6-Octadienal typically present at proportions of 1/3 and 2/3, respectively.

evaporative loss of citral would compete with dermal absorption and result in an exposure less than the applied dose. There is little quantitative information available on the dermal absorption of citral; however, what data is available suggests that citral does penetrate readily through both human and animal skin (Mutalik and Udupa, 2003; Barbier and Benezra, 1983; Meyer and Meyer, 1959). Citral is an α,β -unsaturated carbonyl and, as such, may react with skin proteins via a 1,4 Michael addition or Schiff base formation to create an immunogen (see Fig. 2) (Smith and Hotchkiss, 2001). Studies investigating peptide reactivity have identified citral as being protein reactive (Gerberick et al., 2004).

4. Available data

4.1. Predictive tests in animals

Citral has been evaluated in a total of 16 guinea pig sensitization studies and 11 murine local lymph node assays (LLNAs). The weight of evidence from these animal predictive assay shows that citral is a weak to moderate sensitizer.

4.1.1. Guinea pig studies (Table 1)

Guinea pig maximization tests according to the Magnusson and Kligman (1969) method were conducted on citral. Induction consisted of a series of 6 intradermal injections with and without FCA of the test material followed 6–8 days later by a 48-h occluded patch. The animals were challenged 12–14 days later by an occluded 24 h patch application. Reactions were read 24 and 48 h after patch removal. The following results were obtained.

Induction was conducted with 0.4% for the intradermal injection and 1% for the occluded patch. Challenge application was conducted with 0.25%. No vehicle was reported. Sensitization reactions were observed in 40% (4/10) of the animals (Goodwin and Johnson, 1985).

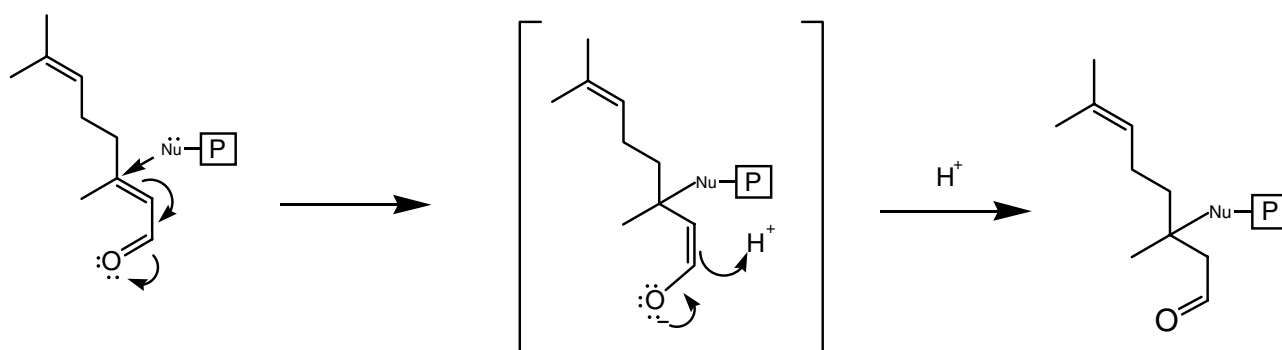
Induction was conducted with 0.2% in 4:1 acetone:olive oil for the intradermal injection and 5% in 4:1 acetone:olive oil for the occluded patch. Challenge application was conducted with 0.5% in 4:1 acetone:olive oil. Sensitization reactions were observed in 60% (6/10) of the animals (Basketter et al., 1991).

Induction was conducted with 0.2% in 0.9% saline solution for the intradermal injection and 5% in 70:30 acetone/PEG 400 for the occluded patch. Challenge application was conducted with 0.5% in 70:30 acetone/PEG 400. Sensitization reactions were observed in 50% of the animals (Basketter and Scholes, 1992).

Ishihara et al. (1986a) reported that in a maximization test using 10% citral throughout the induction and challenge period resulted in sensitization effects being observed (no further details were provided).

Induction was conducted with 5% for the intradermal injection and 25% in petrolatum for the occluded patch.

(A) 1,4 Michael addition



(B) Schiff Base formation with primary amine

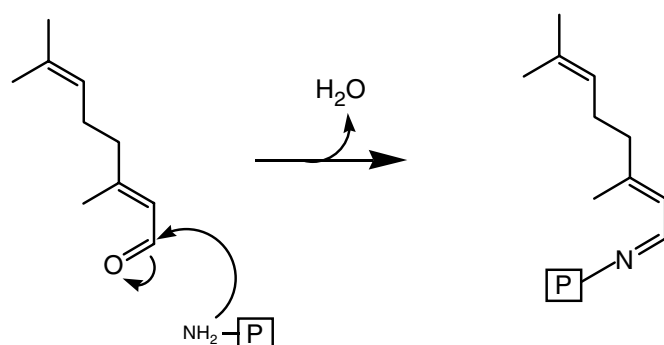


Fig. 2. Probable mechanisms of citral protein reactivity. (A) 1,4 Michael addition. (B) Schiff Base formation with primary amine.

Challenge application was conducted with a subirritant concentration in petrolatum. Sensitization reactions were observed (No further details were provided) (Klecak et al., 1977).

Citral was tested in a Modified Buehler Delayed Hypersensitivity Test in guinea pigs (Buehler, 1965; Ritz and Buehler, 1980). Induction consisted of three 6-h closed patch applications to the same clipped site on the dorsal surface with 20% citral in petrolatum. Induction applications were made once a week for 3 weeks. Following a 10–14 day rest, the guinea pigs were challenged with 20% citral in petrolatum. Challenge application was a 6-h occluded patch at a naive skin site. Control animals were challenged at the same time in an identical manner. Reactions were read 24 and 48 h after patch removal. Sensitization was observed in 5/5 animals (RIFM, 1973). Under the same conditions, samples of citral were tested to determine if changes in sample storage conditions would affect the sensitization potential. Sensitization reactions (5/5 animals per test) were observed to samples of citral that had been stored under nitrogen, stored with the addition of (butylated hydroxyanisole) BHA and after oxygen saturation (RIFM, 1973).

Modified Draize sensitization tests were conducted on citral. In these studies conducted by Sharp (1978), induction was via four simultaneous intradermal injections with 0.25% citral. Challenge was conducted 14 days later by an

intradermal injection of 0.1% citral into one flank and a topical open application of 20% citral on the contralateral flank. The vehicle utilized throughout the study was not reported. Reactions were scored 24 h after challenge. A second challenge was carried out 7 days later. If no sensitization reactions were observed, the test was repeated. No reactions were observed in 0/10 animals (Sharp, 1978). A second assay was conducted under the same conditions utilizing 1% citral during intradermal induction with 0.4% (intradermal) and 20% (topical) challenge concentrations. Sensitization effects were observed (no further details were reported) (Sharp, 1978).

Klecak et al. (1977) reported on a modified Draize test conducted with 10 intradermal induction applications on alternate days and an intradermal challenge conducted on days 35 and 49. Induction and challenge treatments were made with 0.1% citral in saline. Sensitization effects were observed (no further details were reported).

Citral was tested in a Maguire Delayed Hypersensitivity Test in guinea pigs. Induction consisted of open applications of 8% citral in petrolatum to abraded skin on days 0, 2, 4 and 7 with an intradermal injection of Freund's Complete Adjuvant (FCA) on day 4. Following a 14-day rest period, open challenge applications of 8% citral in petrolatum were made to the naive, contralateral flank. Reactions to challenge were read at 24, 48 and 72 h. Sensitization was observed in 4/5 animals (RIFM,

Table 1
Citral studies in guinea pigs

Method	Induction concentration	Challenge concentration	Results	References
Maximization	0.4% (intradermal; VNR) 1% (topical; VNR)	0.25% (VNR)	Sensitization observed	Goodwin and Johnson (1985)
Maximization	0.2% in 4:1 acetone:olive oil (intradermal) 5% in acetone:olive oil (topical)	0.5% in 4:1 acetone:olive oil	Sensitization observed	Basketter et al. (1991)
Maximization	0.2% in 0.9% saline solution (intradermal) 5% in 70:30 acetone/PEG 400 (topical)	0.5% in in 70:30 acetone/PEG 400	Sensitization observed	Basketter and Scholes (1992)
Maximization	10% for both intradermal and dermal (VNR)	10% (VNR)	Sensitization observed	Ishihara et al. (1986a)
Maximization	5% (intradermal; VNR) 25% in petrolatum (topical)	Dose reported as a subirritant concentration (VNR)	Sensitization observed	Klecak et al. (1977)
Buehler	20% in petrolatum	20% in petrolatum	Sensitization observed ^b	RIFM (1973)
Draize	0.25% (VNR)	0.1% (intradermal injection, VNR) 20% (Topical, VNR)	Sensitization observed	Sharp (1978)
Draize	1% (VNR)	0.4% (intradermal injection, VNR) 20% (Topical, VNR)	Sensitization observed	Sharp (1978)
Draize	0.1% in saline	0.1% in saline	Sensitization observed	Klecak et al. (1977)
Maguire	8% in petrolatum	8% in petrolatum	Sensitization observed	RIFM (1972a)
Maguire	8% in petrolatum	8% in petrolatum	Sensitization observed	RIFM (1974a)
FCAT	50% citral in FCA	Dose reported as a subirritant concentration in petrolatum	Sensitization observed	Klecak et al. (1977)
OET	10% citral (VNR)	1% (VNR)	Sensitization observed	Klecak et al. (1977)
CET	3% citral (VNR)	1% Citral (VNR)	Sensitization observed	Ishihara et al. (1986a)
SIAT	0.4% in FCA	0.5% (VNR)	Sensitization observed ^a	Goodwin and Johnson (1985)
SIAT	0.4% in saline with FCA	0.5% in acetone/PEG	Sensitization observed	RIFM (1982)

Abbreviations: Buehler, Buehler delayed hypersensitivity test; CET, Closed Epicutaneous Test; Draize, modified Draize test; FCA, Freund's complete adjuvant; FCAT, Freund's complete adjuvant test; maximization, Magnusson and Kligman guinea pig maximization test; Maguire, modified Maguire delayed hypersensitivity test; OET, Open Epicutaneous Test; PEG, polyethylene glycol; SIAT, Single Injection Adjuvant Test; VNR, vehicle not reported.

^a Goodwin and Johnson (1985) also reported on a modified SIAT using two, three and four induction applications. Positive responses were reported for all induction regimes.

^b The same results were obtained when four separate samples of citral were tested. Each was stored under different conditions.

1972a). In a repeat study, on a separate sample of citral, reactions were observed to 8% citral in petrolatum in 6/8 animals (RIFM, 1974a).

A Freund's Complete Adjuvant Test (FCAT) in guinea pigs was conducted with citral. Induction was via intradermal injection of 0.1 ml of a 50:50 mix of citral and FCA on days 0, 2, 4, 7 and 9. Challenge was made with a 24-h closed patch with a subirritant concentration of citral in petrolatum on days 21 and 35. Sensitization effects were observed (No further details were provided) (Klecak et al., 1977).

The sensitization potential of citral was evaluated in an Open Epicutaneous Test (OET) in guinea pigs (Klecak et al., 1977). Induction applications were made via 21 daily open applications to an 8 cm² area on the clipped flank with 10% citral. Challenge was conducted on days 21 and 35 via open application to the contralateral flank with 1% citral. Induction and challenge vehicles were not reported. Reactions were read at 24, 48 and 72 h. Sensitization reactions (1/6) were observed.

Citral was evaluated in a Closed Epicutaneous Test (CET) in guinea pigs. Induction was made via 48 h occluded patches three times a week for 2 weeks with 3% citral. Challenge was conducted 14 days later by a 48-h occluded patch with 1% citral. Reactions were read at patch removal and again 24 and 48 h after patch removal.

Sensitization reactions (1/6) were observed (Ishihara et al., 1986a).

Goodwin and Johnson (1985) reported on the results of citral when tested in the Single Injection Adjuvant Test (SIAT) along with the results of a modified SIAT. The SIAT was conducted in guinea pigs. Induction was made via a single intradermal injection with 0.4% citral in Freund's complete adjuvant. Twelve days later a single 6-h occluded challenge application of 0.5% citral (vehicle not reported) was made to a clipped, shaved flank. A second occluded challenge application was made to the opposite flank 1 week later. Reactions were read 22 and 46 h after patch removal. Sensitization reactions (3/10) were observed.

A modified SIAT was conducted as above with the addition of two, three and four intradermal induction applications. Sensitization reactions were reported for each of the modifications—2/10 following two induction applications, 1/10 following three, and 5/10 following four (Goodwin and Johnson, 1985).

In another SIAT, induction consisted of a single intradermal injection of 0.4% in saline with Freund's complete adjuvant followed by a challenge with 0.5% citral in 0.5% acetone/PEG. Up to three rechallenges were also conducted. Sensitization reactions were observed (RIFM, 1982).

4.1.2. Local lymph node assays (Table 2)

Citral has been evaluated in the LLNA following the method of Kimber and Basketter (1992, 1994) as formalized in OECD Guideline 429 and OPPTS Guideline 870.26 (OECD, 2002; NIH, 1999).

Briefly, groups of mice are dosed topically on the dorsum of both ears with test material or vehicle control. Each group receives up to one of five test concentrations. Dosing occurs daily for three consecutive days. On the sixth day after the first application, all mice are injected intravenously via the tail vein with radiolabeled methyl thymidine ($^3\text{HTdR}$). Five hours later, the mice are euthanized and the draining auricular lymph nodes are excised and pooled for each experimental group or by individual animal. Suspensions of the lymph node cells are prepared by mechanical disaggregation and precipitated overnight. The samples are then pelleted by centrifugation and transferred to scintillation vials. The incorporation of $^3\text{HTdR}$ is measured by β -scintillation counting and expressed as disintegrations per minute (dpm) per lymph node for each experimental group or individual animal (which are then averaged by

dose group). For each concentration of test material, a stimulation index (SI) relative to the concurrent vehicle-treated control is calculated. A material is considered a sensitizer if at least one concentration of the test material is observed to have an SI value of 3 or more. The EC3 value, a measure of relative potency, can then be derived from the dose–response curve. The EC3 value is the estimated concentration that is required to elicit an SI value of 3 or more.

The LLNAs reported on citral utilized the Ethanol:DEP vehicle system or 4:1 acetone:olive oil as a vehicle. EC3 values in the range of 1.2% to 13% have been reported in these studies. The weighted mean, based on vehicle, of the reported EC3 values for this data set is 5.7% (1414 $\mu\text{g}/\text{cm}^2$). Utilizing the potency estimations of Gerberick et al. (2001) citral is classified as a weak sensitizer in the LLNA.

4.2. Non-diagnostic patch test data in humans (Table 3)

The dermal sensitization potential of citral has been evaluated in humans in both the Human Repeated Insult Patch Test (HRIPT) and the Human Maximization Test (HMT). The weight of evidence from these studies show that a No Observed Effect Level (NOEL) of 1400 $\mu\text{g}/\text{cm}^2$ exists in the HRIPT when citral was tested in an ethanol vehicle. In all but one HMT, citral induced sensitization in subjects exposed to 4–8% (2759–5517 $\mu\text{g}/\text{cm}^2$) citral in petrolatum. The lowest observed effect level (LOEL) was 2759 $\mu\text{g}/\text{cm}^2$ citral in petrolatum in the HMT.

The HRIPT is generally performed utilizing a total of nine 24-h occluded applications over 3-weeks with test material and appropriate controls followed by a 2-week rest period. A single 24-h challenge application is then made to a naïve site with the same materials. Observations at challenge coupled with the patterns of reactivity observed during induction provide the basis for an interpretation of contact allergy (Marzulli and Maibach, 1977; McNamee et al., this issue). Citral has been tested in the HRIPT over a range of concentrations. The following results were obtained.

An HRIPT was conducted on 8 female volunteers with 5% citral in alcohol SDA39C. The patches consisted of a 1 in² webril pad with 0.5 ml of test material; which resulted in a dose of 3876 $\mu\text{g}/\text{cm}^2$. Sensitization reactions were observed in 5/8 subjects. Four subjects, who reacted during the initial study, were rechallenged approximately 7 months later with both a patch and a single open application behind one ear. Two subjects (2/4) reacted to the patch at rechallenge, no reactions (0/4) were observed following open application (RIFM, 1964a).

No reactions were observed to 1400 $\mu\text{g}/\text{cm}^2$ citral in an HRIPT conducted on 101 subjects (30 male and 71 female). The patches consisted of a 25 mm Hill Top Chamber[®], corresponding to a dosing area of 2.54 cm², with 0.3 ml of 1.2% citral in 3:1 DEP:EtOH (RIFM, 2004b).

When 1240 $\mu\text{g}/\text{cm}^2$ citral was tested in an HRIPT, no reactions were observed in 50 subjects. The patches con-

Table 2
Local lymph node assay results for citral

LLNA potency estimation		Vehicle ^b	References ^d
EC3 values (%)	EC3 value ($\mu\text{g}/\text{cm}^2$) ^a		
1.2	300	EtOH:DEP [1:3]	RIFM (2004a)
1.5	375	EtOH:DEP	RIFM (2003)
2.1	525	[3:1] + 0.1%Toc	
		EtOH:DEP	RIFM (2003)
3.7	925	[3:1] + AO Mix	
		EtOH:DEP	RIFM (2003)
4.6	1150	[3:1] + 0.1%TrIC	
		EtOH:DEP [3:1]	RIFM (2003)
4.6	1150	EtOH:DEP	RIFM (2003)
		[3:1] + AO Mix	
5.3	1325	EtOH:DEP [3:1]	RIFM (2003)
5.8	1400	EtOH:DEP	RIFM (2003)
		[3:1] + 0.1%TrIC	
6.3	1575	EtOH:DEP [1:3]	Lalko and Api (2006)
6.8	1700	EtOH:DEP	RIFM (2003)
		[3:1] + 0.1%Toc	
13.0	3250	AOO	Basketter et al. (2002a)
Wt. mean ^c = 5.7		Wt. mean ^c = 1414	

^a Percent (%) EC3 values were converted to their dose per unit area ($\mu\text{g}/\text{cm}^2$) equivalents assuming an application area of 1 cm² and a dose volume of 25 μl .

^b Abbreviations: AOO, acetone:olive oil (4:1); EtOH, ethanol; DEP, diethyl phthalate; Toc, α -tocopherol; TrIC, Trolox C; AO Mix, antioxidant mix of 0.3% BHT/tocopherol/eugenol.

^c Weighted mean EC3 value based on the vehicle utilized.

^d Two LLNAs were excluded from the table due to an uncharacteristic dose–response that appeared to be the result of irritation from the specific test sample. These were conducted on citral in 1:3 EtOH:DEP at a concentration of 0.5–10% and 5.0% to 100%. The assays resulted in a negative response and a positive response with an EC3 value of 26.7% (6675 $\mu\text{g}/\text{cm}^2$), respectively (RIFM, 2005a,b). Removal of these outliers resulted in a more conservative mean EC3 value.

Table 3
Human non-diagnostic patch tests with citral^a

Induction dose ($\mu\text{g}/\text{cm}^2$)	Test method ^{a,b}	Dose volume/Patch area	Test material concentration and vehicle	Incidence of positive responses	References
3876	HRIPT	0.5 ml/6.45 cm ²	5% in SDA39C	5/8	RIFM (1964a)
1400	HRIPT	0.3 ml/2.54 cm ²	1.2% in 3:1 DEP:EtOH	0/101	RIFM (2004b)
1240	HRIPT	0.2 ml/6.45 cm ²	4% in petrolatum	0/50	RIFM (1971a)
775	HRIPT	0.5 ml/6.45 cm ²	1.0% in SDA39C	0/40	RIFM (1965)
388	HRIPT	0.5 ml/6.45 cm ²	0.5% in SDA39C	0/41	RIFM (1964b)
5517	HMT	1 ml/14.5 cm ²	8% in petrolatum	8/24	RIFM (1971b)
3448	HMT	1 ml/14.5 cm ²	5% in petrolatum	16/25	RIFM (1974a)
3448	HMT	1 ml/14.5 cm ²	5% in petrolatum	14/25	RIFM (1974c)
3448	HMT	1 ml/14.5 cm ²	5% in petrolatum	12/25	RIFM (1974c)
3448	HMT	1 ml/14.5 cm ²	5% in petrolatum	8/25	RIFM (1974c)
3448	HMT	1 ml/14.5 cm ²	5% in petrolatum	11/24	RIFM (1974d)
3448	HMT	1 ml/14.5 cm ²	5% in butylene glycol	0/25	RIFM (1974e)
2759	HMT	1 ml/14.5 cm ²	4% in petrolatum	3/25	RIFM (1972c)
2759	HMT	1 ml/14.5 cm ²	4% in petrolatum	3/25	RIFM (1972b)
2759	HMT	1 ml/14.5 cm ²	4% in petrolatum	9/25	RIFM (1971c)
2759	HMT	1 ml/14.5 cm ²	4% in petrolatum	5/25	RIFM (1972c)
2759	HMT	1 ml/14.5 cm ²	4% in petrolatum	4/25	RIFM (1971c)
2759	HMT	1 ml/14.5 cm ²	4% in petrolatum	5/25	RIFM (1971c)
1379	HMT	1 ml/14.5 cm ²	2% in petrolatum	2/24	RIFM (1972d)

Abbreviations: DEP, diethyl phthalate; EtOH, ethanol; HMT, Human Maximization Test; HRIPT, Human Repeated Insult Patch Test; SDA39C, alcohol SDA39C.

^a Excludes those studies in which insufficient detail was provided describing the patch conditions resulting in the inability to convert to the dose applied ($\mu\text{g}/\text{cm}^2$).

^b HRIPTs were generally conducted according to the methods discussed by Marzulli and Maibach (1977) and McNamee et al. (this issue). HMTs were generally conducted according to the method described by Kligman and Epstein (1975).

sisted of a 1 in² webril pad with 0.2 ml of 4% citral in petrolatum (RIFM, 1971a).

No reactions were observed to 755 $\mu\text{g}/\text{cm}^2$ citral in an HRIPT conducted on 40 subjects (11 males and 29 females). The patches consisted of a 1 in² webril pad with 0.5 ml of 1% citral in alcohol SDA39C (RIFM, 1965).

An HRIPT was conducted on 12 male and 29 female volunteers with 0.5% citral in alcohol SDA39C. The patches consisted of a 1 in² webril pad with 0.5 ml of test material; which resulted in a dose of 388 $\mu\text{g}/\text{cm}^2$. No sensitization reactions (0/41) were observed (RIFM, 1964b).

Steltenkamp et al. (1980) reported on the positive results of HRIPTs conducted with 0.5%, 1% and 5% citral in EtOH. However, little detail regarding the methods employed was provided and the information did not allow for the conversion of the reported concentration to their dose per unit area equivalents.

The HMT is typically conducted on 25 human subjects by utilizing 5 alternate day 48-h occluded induction applications of test material and appropriate controls. Following a 10 to 14-day rest period 48-hour challenge applications are made to naïve sites. Patches may be made with and without pretreatment of sodium lauryl sulfate depending upon the inherent irritancy of the test material. Observations at challenge coupled with the patterns of reactivity observed during induction provide the basis for an interpretation of contact allergy (Kligman and Epstein, 1975). Citral has been tested in the HMT over a range of concentrations. The patches utilized for

each of the reported studies consisted of a 14.5 cm² webril pad with 0.5 ml of test material. The following results were obtained.

An HMT was conducted with 8% (5517 $\mu\text{g}/\text{cm}^2$) citral in petrolatum on 24 males volunteers. Sensitization reactions were observed in 8/24 subjects (RIFM, 1971b).

In an HMT conducted with 5% (3448 $\mu\text{g}/\text{cm}^2$) citral in petrolatum on 16 male and 9 female volunteers. Sensitization reactions (16/25) were observed (RIFM, 1974b).

Samples of citral, which had been treated and stored under differing conditions, were tested in the HMT at 5% (3448 $\mu\text{g}/\text{cm}^2$) in petrolatum on male and female volunteers. Citral stored under nitrogen both with and without the addition of BHA added resulted in sensitization reactions in 14/25 and 12/25 subjects, respectively (RIFM, 1974c). Citral stored under oxygen resulted in sensitization reactions in 8/25 volunteers (RIFM, 1974c).

A sample of citral was stored under nitrogen with the addition of BHA and tested in the HMT at 5% (3448 $\mu\text{g}/\text{cm}^2$) in DEP on 24 male volunteers. Sensitization reactions were observed in 11/24 subjects (RIFM, 1974d).

A sample of citral was stored under ambient air with the addition of BHA and tested in the HMT at 5% (3448 $\mu\text{g}/\text{cm}^2$) in butylene glycol on 16 male and 9 female volunteers. No sensitization reactions (0/25) were observed (RIFM, 1974e).

In an HMT conducted with 4% (2759 $\mu\text{g}/\text{cm}^2$) citral in petrolatum on 25 male volunteers. Sensitization reactions (3/25) were observed (RIFM, 1972c).

A series of five HMTs were conducted on samples of citral derived from different processes. Each HMT was conducted with 4% (2759 $\mu\text{g}/\text{cm}^2$) citral in petrolatum on male subjects. A sample of citral derived from lemongrass oil resulted in sensitization reactions in 3/25 subjects (RIFM, 1972b). Two samples of citral, described as citral refined, resulted in sensitization reactions of 9/25 (sample# 71-4-3) and 5/25 (sample# DL-4-10R) (RIFM, 1971c, 1972c). A sample described as citral synthetic (sample# 714-2) resulted in sensitization reactions in 4/25 subjects (RIFM, 1971c). A sample identified as citral natural (sample#744-1) resulted in sensitization reactions in 5/25 subjects (RIFM, 1971c).

In an HMT conducted with 2% (1379 $\mu\text{g}/\text{cm}^2$) citral in petrolatum on 24 male volunteers. Sensitization reactions (2/24) were observed (RIFM, 1972d).

4.3. Clinical diagnostic patch tests on patients (Table 4)

Routine clinical diagnostic patch tests with citral have been reported in the literature. The results of studies reported in greater than 100 consecutive patients with varying dermatitis showed a range of positive patch tests reactions to citral in these populations.

Ishihara et al. (1981) reported on the results of patch tests with 5% citral. Reactions were observed in cosmetic dermatitis patients (4/155) and eczema/dermatitis patients (5/159). No reactions were observed in control subjects (0/48).

Patch tests were conducted between the years 1978–1986 in dermatologic patients (Itoh et al., 1986, 1988; Nishimura et al., 1984). When citral was tested at 5% (vehicle not reported), reactions were observed in cosmetic dermatitis patients (8/310), non-cosmetic dermatitis patients (9/408) and in one control subject (1/122). When citral was tested at 2% (vehicle not reported), reactions were observed in

cosmetic dermatitis patients (1/240) and non-cosmetic dermatitis patients (2/584). No reactions were observed in control subjects (0/105).

In a series of patch tests conducted in six dermatological centers in Europe, 1701 contact dermatitis patients were patch tested for 48 h using Finn Chambers on Scanpor tape (Frosch et al., 2004, 2005). The patients were tested concurrently with a fragrance mix (FM) and the six individual materials comprising the mixture, which included citral. When patients were patched with the FM at 28% and citral at 2.0%, reactions were observed to citral in (12/1701) patients with doubtful/irritant reactions being reported (40/1701). When patients were patched with the FM at 14% and citral at 1%, reactions were observed to citral in (6/1701) patients with doubtful/irritant reactions being reported (14/1701).

Heydorn et al. (2002) reported on the results of patch tests with 2% citral in petrolatum on 315 patients with hand eczema. Positive reactions were observed in 2% of the patients. In an update by Heydorn et al. (2003a), 2% positive reactions were reported to 2% citral when patch tests were conducted on 586 patients. Additionally Heydorn et al. (2003b) reported that 28 positive reactions were observed to 2% citral in 658 hand eczema patients.

In a multicenter study conducted from September 1998 to April 1999, 1825 patients were patch tested with nine fragrance materials, including citral. Reactions (19/1825) were observed with 2.0% citral in petrolatum (DeGroot et al., 2000).

In a multi center study with 1825 patients, it was reported that 2% citral resulted in 25 doubtful reactions and 21 positive reactions (Frosch et al., 2002).

Mitchell et al. (1982) reported that patch tests conducted on 228 eczema patients resulted in 1% positive reactions.

Frosch et al. (1995) reported on the results of patch tests conducted with 0.1% and 1.0% citral in petrolatum. The

Table 4
Diagnostic patch tests with citral^a

Concentration/Vehicle	Results	References
5% in petrolatum	4/155 cosmetic dermatitis patients 5/159 eczema/dermatitis patients 0/48 control subjects	Ishihara et al. (1981)
5% VNR	8/310 cosmetic dermatitis patients 9/408 non-cosmetic patients 1/122 control subjects	Itoh et al. (1986, 1988), Nishimura et al. (1984)
2% VNR	21/1825 patients	Frosch et al. (2002)
2% in petrolatum	19/1825 patients	DeGroot et al. (2000)
2% VNR	12/1701 patients	Frosch et al. (2004, 2005)
2% VNR	28/658 patients	Heydorn et al. (2002, 2003a,b)
2% VNR	1/240 cosmetic dermatitis patients 2/584 non-cosmetic patients 0/105 control subjects	Itoh et al. (1986, 1988), Nishimura et al. (1984)
1% VNR	6/1701 patients	Frosch et al. (2004, 2005)
1% VNR	4/228 patients	Mitchell et al. (1982)
1% in petrolatum	8/192 patients	Frosch et al. (1995)
0.1% VNR	1/192 (reaction was questionable) patients	Frosch et al. (1995)

Abbreviations: VNR, vehicle not reported.

^a The table is restricted to the literature reports of clinical diagnostic patch tests to citral in greater than 100 consecutive patients.

test material was applied to the back for 2 days using Finn chambers[®] on Scanpore[®]. Reactions were assessed per International Contact Dermatitis Research Group (ICDRG) guidelines on days 2 and 3 or on days 2 and 4. Eight reactions (8/192) were observed to 1% citral. One questionable reaction (1/92) was observed to 0.1% citral.

4.4. 'Quenching' studies (Tables 5 and 6)

Studies on the inhibition of the induction of sensitization to citral by another material, 'the quenching phenomenon' (see below for details), have been reported in both human and animal studies. The 'quenching' of citral by various materials has been demonstrated in humans. However, 'quenching' of citral sensitization has not been demonstrated in animal models of dermal sensitization.

Opdyke (1976) reported that citral was shown to be a sensitizer in the HMT, however essential oils that contained significant amounts of citral did not induce sensitization. It was theorized that some other component(s) of the essential oil inhibited the induction of sensitization. As a test of this hypothesis, several terpenes and alcohols found along with citral in the oils were combined. Opdyke (1976) showed that in the HMT citral produced sensitization reactions when applied alone but produced no reactions when applied in the simple mixtures. This inhibition of sensitization was termed the 'quenching' phenomenon and was incorporated into risk management strategies for contact allergy to citral in addition to two other aldehydes—cinnamic aldehyde and phenylacetaldehyde (Opdyke, 1976). Later work showed that while this 'quenching' phenomenon could not be duplicated in an HRIPT for cinnamic aldehyde and phenylacetaldehyde, for citral it was demonstrated in both the HMT and HRIPT (Cocchiara et al., 2004; Api and Isola, 2000; Api, 2000). No reactions were observed in HMTs conducted as described above on two samples of 5% lemongrass, 4% citral + 1% citrus terpene, 4% citral + 1% α -pinene, 4% and citral + 1% D-limonene (Opdyke, 1976; Api and Isola, 2000; Api, 2000). No reactions (0/118) were observed in an HRIPT conducted as described above on 4% citral + 1% D-limonene in DEP (Api, 2000; Api and Isola, 2000). The

results of the human non-diagnostic patch test studies conducted on 'quenched' citral are summarized in Table 5.

The dermal sensitization potential of 'quenched' citral has been evaluated in guinea pig assays and the LLNA (Table 6).

A guinea pig maximization test was conducted as described above on a mixture of 4:1 citral:eugenol. Induction was conducted with 0.2% in 4:1 acetone:olive oil for intradermal injection and 5% in 4:1 acetone:olive oil for the occluded patch. Challenge application was conducted with 0.5% in 4:1 acetone:olive oil. Sensitization reactions were observed in 70% (7/10) of the animals (Basketter and Allenby, 1991). When a mixture of 4:1 citral:D-limonene was tested under the same condition, sensitization reactions (7/10) were observed with 2/10 questionable reactions (Basketter and Allenby, 1991; Basketter, 1998).

Hanau et al. (1983) reported on a series of guinea pig assays conducted to evaluate the sensitization potential of a 1:1 solution of citral:D-limonene. Induction applications followed a modified FCAT method. Challenge applications were made both open and with the use of an occluded patch. No significant difference between the mixture of 1:1 citral:D-limonene and citral alone was observed following visual scoring. Histological examination of the closed challenge application sites showed increased total infiltrate and lymphocyte reactions to citral when compared to 1:1 citral:D-limonene. In a follow up to this study, Barbier and Benezra (1983) reported on experiments with radiolabeled citral + D-limonene. When the mixture was applied an increase in binding of citral to insoluble protein was observed, which the authors concluded may play a role in the 'quenching' of citral.

No difference in sensitization potential was observed between citral and 4:1 citral + D-limonene when each was tested in the LLNA; both materials resulted in an EC3 value of 13% (Basketter, 1998; Basketter et al., 2000).

A series of LLNAs conducted on 'quenched' citral were reported by Lalko and Api (2004). A sample of citral + D-limonene at a constant ratio of 4:1 was tested along with citral alone as a control. No significant difference was observed between the calculated EC3 value for each material—1.2% for citral and 0.8% for 4:1 citral:D-limonene.

Table 5
Citral 'quenching' studies—human non-diagnostic patch tests

Method ^a	Test material	Results	References
HMT	5% Lemongrass (with a 4% citral content) in petrolatum	0/25	Opdyke (1976), Api and Isola (2000), Api (2000)
HMT	5% Lemongrass (with a 3.2% citral content) in petrolatum	0/25	
HMT	4% citral + 1% citrus terpenes in petrolatum	0/25	
HMT	4% citral + 1% α -pinene in petrolatum	0/25	
HMT	4% citral + 1% D-limonene in petrolatum	0/25	
		0/24	
HRIPT	4% citral + 1% D-limonene in DEP	0/118	Api and Isola (2000), Api (2000)

Abbreviations: DEP, Diethyl phthalate; HMT, Human Maximization Test; HRIPT, Human Repeated Insult Patch Test.

^a HMTs were generally conducted according to the method described by Kligman and Epstein (1975). HRIPTs were generally conducted according to the methods discussed by Marzulli and Maibach (1977) and McNamee et al. (this issue).

LLNAs were conducted on two essential oils that contained significant amounts of citral—lemongrass and *litsea cubeba* oil—to determine if any difference in the sensitization potential of citral could be observed based on its exposure in a mixture (Lalko and Api, 2006). Lemongrass oil, which contained 68.8% citral, resulted in an EC3 value of 6.5%. *Litsea cubeba* oil, which contained 85.7% citral, resulted in an EC3 value of 8.4%. Adjusting the EC3 value to account for the amount of citral in the oil showed that the response observed was strongly driven by citral and no evidence of the ‘quenching’ phenomenon could be observed.

4.5. Conclusions

The chemical structure and properties of citral suggest that it would have the potential to act as a contact sensitizer. Indeed, hazard studies conducted in guinea pigs utilizing a variety of methods and in the mouse using the LLNA identify citral as a contact allergen. The available guinea pig studies (Table 1) do not provide sufficient dose response data, making the determination of a NOEL for the induction of dermal sensitization impossible in the guinea pig models (though with an adequate design, it would be possible to extract such information from a future study). The guinea pig sensitization data can be used to classify the potency of citral. Based on those studies that provide sufficient data, citral can be classified as a weak to moderate sensitizer in the guinea pig models (ECETOC, 2003; Kimber et al., 2003). There are several reports on the positive results of citral in the LLNA (Table 2). In addition to its application as a method to identify potential contact

allergens, the LLNA provides an objective and quantitative measure of relative skin sensitizing potency—the EC3 value (Basketter et al., 1999, 2000). EC3 values in the range of 1.2% (300 $\mu\text{g}/\text{cm}^2$) in 1:3 EtOH:DEP to 13% (3250 $\mu\text{g}/\text{cm}^2$) in 4:1 acetone:olive oil have been reported. The weighted mean EC3 value, based on vehicle utilized, for citral is 5.7% (1414 $\mu\text{g}/\text{cm}^2$) showing that citral is a weak allergen in the LLNA.

In humans, the available data provide for the determination of a NOEL and LOEL (Table 3). In the HRIPT, the NOEL is 1400 $\mu\text{g}/\text{cm}^2$ for the induction of dermal sensitization to citral when tested in an ethanol vehicle. There is no identifiable NOEL in the HMT over the range of concentrations tested (Table 3). However, the HMT data exhibit a general dose-dependent trend toward decreasing activity with decreasing dose. In the HMT the LOEL for citral is 2759 $\mu\text{g}/\text{cm}^2$ when tested in petrolatum. The HMT protocol was designed to take advantage of the ease with which compromised skin maybe sensitized with the goal of maximizing the tests ability to identify allergens (Kligman, 1985). This procedure is a harsh method that employs pretreatment with the anionic surfactant sodium lauryl sulfate (SLS) and extended (often 48 h) occlusive patches (Kligman and Epstein, 1975). Human patch testing methodology has evolved over more than 50 years; the current confirmatory safety test most typically conducted on fragrance ingredients in humans is the HRIPT (Api, 2002). The HRIPT protocol utilizes 24 h patches over a 3-week period without the use of SLS (Marzulli and Maibach, 1977; McNamee et al., this issue). The HRIPT was designed and refined to exaggerate normal, realistic use conditions with the goal of confirming safe use levels.

Table 6
Citral ‘quenching’ studies in animals

Method	Test material	Results	References
GPMT	4:1 citral:eugenol	Sensitization reactions observed in 7/10 animals	Basketter and Allenby (1991), Basketter (1998)
GPMT	4:1 citral:D-limonene	Sensitization reactions observed in 7/10 animals with 2 questionable reactions	Basketter and Allenby (1991), Basketter (1998)
FCAT	1:1 citral:D-limonene	No difference in sensitization reactions were reported between the mixture and citral alone Histological results showed increased infiltrate and lymphocyte reactions to citral alone when compared to the mixture In a follow, citral was found to bind preferentially to insoluble protein when applied in the mixture. The authors considered this evidence of a quenching effect	Hanau et al. (1983) Barbier and Benezra (1983)
LLNA	4:1 citral:D-limonene	Both the mixture and citral alone resulted in an EC3 value of 13%	Basketter (1998), Basketter (2000)
LLNA	4:1 citral:D-limonene	No significant difference in EC3 value was observed between the mixture and citral alone	Lalko and Api (2004)
LLNA	Lemongrass oil (containing 68.8% citral)	Positive with an EC3 value of 6.5%	Lalko and Api (2006)
LLNA	<i>Litsea cubeba</i> oil (containing 85.7% citral)	Positive with an EC3 value of 8.4%	Lalko and Api (2006)

Abbreviations: FCAT, Freund’s complete adjuvant test; GPMT, guinea pig maximization test; LLNA, murine local lymph node assay.

The HRIPT used today is a confirmatory assay for the predicted NOEL for induction of sensitization in a population of healthy volunteers (Api, 2002; McNamee et al., [this issue](#)). This focus on exaggerated realistic exposure conditions makes the HRIPT more relevant, when compared to the HMT, to the identification of a threshold for induction under foreseeable usage scenarios.

Historically, risk management focusing on primary prevention of contact allergy to citral from fragrance use has been based on the ‘quenching’ phenomenon—the inhibition of the induction of sensitization to one material by the presence of another as described by Opdyke (1976) (see Section 4.4). The use of citral in fragrance formulations is currently restricted by an IFRA Standard requiring that “it be used in conjunction with substances preventing sensitization [quenching agents], as for example 25% D-limonene, mixed citrus terpenes or α -pinene” (IFRA, 1980). The ‘quenching’ of the induction of dermal sensitization to citral by various materials has been demonstrated in humans (Table 5). However, ‘quenching’ of citral sensitization has not been demonstrated in animal models of contact allergy (Table 6). Similar contradictory results can be found in the literature with respect to the theory of ‘quenching’ for other fragrance ingredients; data to support and refute the phenomenon have been reported (Karlberg et al., 2000; Nilsson et al., 2002; Basketter, 2000). Since there is conflicting data and no adequate mechanism to explain the phenomenon, it is recently being replaced as a basis for risk management of contact allergy to certain fragrance ingredients—e.g., the IFRA Standard for cinnamic aldehyde has been replaced to limit its use based on the NOEL for induction to the discreet material (IFRA, 2003).

In conclusion, the data reported here show by a weight of evidence that the human NOEL for induction of sensitization to citral is 1400 $\mu\text{g}/\text{cm}^2$. The available guinea pig studies identify citral as a weak to moderate dermal sensitizer. Studies in the LLNA resulted in positive responses with a vehicle weighted average EC3 value of 1414 $\mu\text{g}/\text{cm}^2$. Based on this calculated EC3 value citral is classified as a weak sensitizer in the LLNA. The EC3 value has been demonstrated to closely correlate to experimentally determined human NOELs for induction (Basketter et al., 2005; Gerberick et al., 2001; Lalko and Api, 2005). The EC3 value reported here is remarkably close to the human NOEL of 1400 $\mu\text{g}/\text{cm}^2$ identified for citral in the HRIPT. In the HMT, the LOEL for citral is 2759 $\mu\text{g}/\text{cm}^2$ when tested in petrolatum. Positive reactions to citral have been reported in dermatologic patients. The goal of primary prevention is to avoid inducing sensitization altogether, thereby avoiding the manifestation of contact dermatitis. Currently risk management strategies for the use of citral as a fragrance ingredient are based on the ‘quenching phenomenon’. Due to the discordance between the human and animal studies on quenching and no mechanistic data to support the observations, ‘quenching’ is currently being discontinued by the fragrance industry as a basis for risk

management. The identified NOEL for the induction of sensitization to citral can be applied to risk assessment paradigms such as the QRA procedure outlined in a paper concurrently submitted with this review (Api et al., [this issue](#)) and Gerberick et al. (2001); with the aim of refining the acceptable use level of citral in various consumer product types.

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