P31 - Potency of Citral in the Local Lymph Node Assay

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ABSTRACT #144

Citral is an important fragrance ingredient appreciated for its lemon aroma. The use of citral is restricted by an IFRA Standard requiring that it be used with substances preventing sensitization such as a 4:1 mixture of citral and *d*-limonene. The Standard was based on the "quenching phenomenon," a term used to describe the inhibition of the induction of sensitization by the presence of another fragrance material. The quenching of sensitization to citral by the addition of *d*-limonene has been demonstrated in humans. However, the mechanism of action has remained elusive. Further, the phenomenon has not been clearly demonstrated in the traditional animal models of sensitization. In the more contemporary Murine Local Lymph Node Assay (LLNA), we investigated the sensitization potential of citral both alone and together in several mixtures based on the quenching phenomenon. To investigate the sensitization potential of classically quenched citral assays were conducted in 1:3 Ethanol:Diethyl phthalate (EtOH:DEP) on citral and citral + *d*-limonene at a 4:1 ratio. In each case, a positive response was observed resulting in EC3 values of 1.2% and 0.8% respectively. To study the sensitization potential resulting from exposure to citral in essential oils, we conducted a series of assays on two essential oils that contain significant amounts of citral—Litsea cubeba and lemon grass oils. Positive responses to each essential oil in 1:3 EtOH:DEP were observed resulting in EC3 values of 6.5% and 8.4% respectively. In this series of LLNAs, citral would be considered a weak to moderate sensitizer. This classification is consistent with the results of other studies conducted in both humans and animal models of sensitization. The results show that the quenching of citral cannot be demonstrated in the LLNA. It is interesting to note that in humans a NOEL of ~0.5% or 1400μg/cm² exists for the induction of sensitization to citral. In the future, this NOEL could be applied to a quantitative risk assessment for dermal sensitization with the aim of refining the acceptable use level of citral in various consumer product types.

INTRODUCTION

Citral is an important fragrance ingredient appreciated for its powerful lemon-aroma and is widely used in fragrance formulations in numerous consumer products (Arctander, 1969). The use of citral is currently restricted by an IFRA Standard requiring that it be used in conjunction with substances preventing sensitization such as a 4:1 mixture of citral and *d*-limonene (IFRA, 1980). The standard was based on the quenching phenomenon first discussed by Opdyke (1976). The term "quenching" was used to describe the inhibition of the induction of sensitization by the presence of another fragrance material. Opdyke (1976) based this hypothesis on the observation that citral was shown to be a sensitizer, but the individual essential oils in which it naturally occurred did not induce sensitization. This hypothesis was further bolstered by studies showing that exposure to citral along with several terpenes and alcohols, which are present in the natural compositions, inhibited the induction of sensitization in humans (Api and Isola, 2000). A brief review of the available human data is given in Table 1. Though this phenomenon has been demonstrated in humans, the mechanism of action has remained elusive. Further, the phenomenon has not been clearly demonstrated in the traditional animal models of sensitization, such as the guinea pig maximization test (Basketter, 1998). In the more contemporary Murine Local Lymph Node Assay (LLNA), we investigated the sensitization potential of citral both alone and together in several mixtures based on the quenching phenomenon. The LLNA takes as its endpoint a measure of the induction of sensitization; therefore it is a useful tool to study effects that have been observed to act at induction such as the quenching of citral.

Table 1. Review of the available human data on the quenching of citral

Test	Test Material	Results
HMT	5% Lemongrass oil (4% citral) in petrolatum	0/25
HMT	4% Lemongrass oil (3.2% citral) 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
HRIPT	4% Citral + *d*-limonene in DEP	0/118
Numerous study types	Neat citral at concentrations of 8% - 0.5% in a variety of vehicles	Positive results with a NOEL in ethanol of ~ 0.5% or 1400 ì g/cm²

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

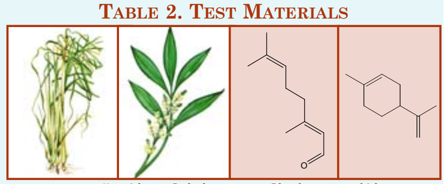
To study the sensitization potential resulting from exposure to citral in essential oils, we conducted a series of assays on two essential oils that contain significant amounts of citral—Litsea cubeba and lemon grass oils. To investigate the sensitization of classically quenched citral we tested citral in a 4:1 mixture with *d*-limonene. In each case LLNAs were conducted concurrently on citral alone to allow for comparison of relative potency.

METHODS AND MATERIALS

TEST MATERIALS

The materials included in this study are given in Table 2. The individual components of the essential oils were characterized by GC/MS. All materials were tested for peroxide content according to the FMA-IASC method a potassium iodide titration (FMA, 2002).

Table 2. Test Materials



Lemongrass Oil	Litsea Cubeba	Citral	*d*-Limonene
CAS# 8007-02-1	CAS# 68855-99-2	CAS# 5392-40-5	CAS# 5989-27-5

LOCAL LYMPH NODE ASSAYS

The LLNAs were conducted according to the method of Kimber *et al.* as formalized in OECD Guideline 429 (Kimber and Basketter, 1992; Kimber *et al.*, 1994; OECD, 2002). Groups of female CBA/CA mice (n=4) were dosed topically on the dorsum of each ear with 25μL of test material in 1:3 ethanol:diethyl phthalate (Lalko *et al.*, 2004). Each group received one of five test concentrations. To study the sensitization potential resulting from exposure to citral in essential oils assays were conducted at concentrations of 2.5%-50%. To investigate the sensitization potential of classically quenched citral, assays were conducted at concentrations of 0.4%-20% on neat citral and citral + *d*-limonene at a 4:1 ratio. A vehicle control group was similarly treated with 1:3 ethanol:diethyl phthalate. Dosing occurred daily for three consecutive days. The animals "rested" for two days and on the sixth day after the first application, all mice were injected intravenously via the tail vein with 250μL of phosphate buffered saline containing 20μCi of radiolabelled methyl thymidine (3HTdR). Five hours later, the mice were euthanized and the draining auricular lymph nodes were excised and pooled for each experimental group. Suspensions of the lymph node cells were prepared by mechanical disaggregation through 200-mesh stainless steel gauze. The cell suspensions were washed three times with phosphate buffered saline and precipitated overnight at 4°C with 5% w/v trichloroacetic acid (TCA). The samples were then pelleted by centrifugation. The cells were resuspended in 1ml of TCA and transferred to scintillation vials containing 10ml of scintillation fluid. The incorporation of 3HTdR was measured by β-scintillation counting and expressed as disintegrations per minute (dpm) per lymph node for each experimental group. For each concentration of test material, a stimulation index (SI) relative to the concurrent vehicle-treated control was calculated. The SI value for each test material was calculated by dividing the dpm at a given dose level by the dpm of the vehicle control group. 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.

MATHEMATICAL ANALYSIS

The EC3 value, a measure of relative potency, was then derived from the dose response curve; it is the estimated concentration that is required to elicit an SI value of 3 or more. The EC3 value was derived by linear interpolation utilizing the following equation described by Basketter, *et al.* (2000):

$$EC3 = c + [(3-d)/b-d)] \ (a-c)$$

Where the data points lying directly above and below the SI value of 3 on the dose-response curve have the coordinates (a,b) and (c,d), respectively. The vehicle treated control values (SI=1) cannot be used for the latter. The concentrations of test material, given as % w/v, lie on the x-axis and the SI values lie on the y-axis.

RESULTS

Lemongrass, Litsea cubeba and neat citral were observed to have SI values greater than 3 and, as such, were considered to be potential sensitizers under the conditions of the test. The results of the individual assays are summarized in Table 3 along with an analysis of the individual components of each essential oil and the peroxide content.

Both neat citral and the 4:1 mixture of citral + *d*-Limonene were observed to have SI values greater than 3 and, as such, were considered to be a potential sensitizer under the conditions of the test. The results of these assays are summarized in Table 4.

Table 3: Local Lymph Node Assay Results
Essential Oils Containing Significant Amounts of Citral

Test material		Concentrations of Test Material (%)	DPM/Lymph Node	Stimulation Index	EC3
Principal Name	Analysis				
Lemongrass Oil (Gutamalen)	Citral 68.8% d-Limonene 6.7% Geraniol 6.1% Geranyl acetate 2.2% Caryophyllene 1.6% Trans-isocitral 1.4% 6-methyl 5-hepten-2-one 1.3% caryophyllene oxide 1.2% 4-nonanone 1.0% Peroxide content: 2 mmol/l	1:3 ethanol/DEP 2.5 5.0 10.0 25.0 50.0	925 861 1941 4736 9537 12145	 0.93 2.10 5.12 10.31 13.13	6.5%
Litsea Cubeba	Citral 85.69% d-Limonene 2.91% Linalool 1.73% Citronellal 1.42% Caryophellene 0.99% Methyl heptanone 0.64% Peroxide content: 1.2 mmol/l	1:3 ethanol/DEP 2.5 5.0 10.0 25.0 50.0	608 1268 1429 2011 4842 9147	 2.09 2.35 3.31 7.96 16.03	8.4%
Citral	99.5% by GC/MS 20.03 mmol/l	1:3 ethanol/DEP 2.5 5.0 10.0 25.0 50.0	355 982 815 1800 4050 7864	 2.77 2.3 5.07 11.41 22.15	6.3%

Table 4: Local Lymph Node Assay Results
The Traditional Quenching Mixture

Test Materials (%)		EC3
Citral at 0.4, 2.0, 4.0, 8.0 and 20		1.2%
Citral	*d*-Limonene	0.8%
0.4	0.1	
2.0	0.5	
4.0	1.0	
8.0	2.0	
20.0	5.0	

Vehicle for all assays - 1:3 EtOH:DEP

DISCUSSION

Both citral and its related essential oils were observed to have the potential to induce skin sensitization. In general, the potency of each essential oil (6.5% for lemon grass oil and 8.4% for Litsea cubeba) did not differ significantly from the potency observed for citral alone (6.3%). Due to the nature of the LLNA, it is not possible to determine which components of the essential oils may have been responsible for inducing sensitization. The assay takes as a terminal endpoint induction; therefore cross challenges cannot be performed to determine elicitation potential. It is reasonable to suspect that citral, which is a major component of each of the oils, would contribute to the overall sensitization reactions observed.

The results of the assays conducted on classically quenched citral show that there was little difference in potency between citral and citral + *d*-limonene (EC3 values of 1.2% and 0.8% respectively). The EC3 value obtained with the quenched material was slightly lower than that of the discreet material. The autoxidation products of *d*-limonene have been observed to act as skin sensitizers and experience has shown *d*-limonene to have a mild irritant potential (Karlberg and Lindell, 1993). In the current series of assays, the peroxide content of d-limonene was very low (0.2 meq/kg) and the sample included an antioxidant making oxidation products an unlikely cause for the increased potency. However, the skin's response to allergens has been observed to be augmented by the presence of an irritant (Pedersen *et al.*, 2004). The combination of citral's inherent ability to act as a skin sensitizer and *d*-limonene's irritant potential would be expected to produce this slight increase in potency if quenching was not demonstrable in the LLNA.

Lastly, it has been hypothesized that the quenching phenomenon may be due to an antioxidant effect of the quenching agent. We previously reported on a series of studies to investigate the effect of autoxidation on sensitization potential and the protective effects of antioxidants (Lalko, 2002). A series of assays on both freshly distilled and air exposed citral with and without the addition of antioxidants was conducted. In general, it was observed that the autoxidation of citral and the addition of antioxidants did not affect its sensitization potential. EC3 values in the range of 4%-5% were observed for each test material in 3:1 EtOH:DEP (Lalko, 2002).

In these series of LLNAs, citral would be considered a weak to moderate sensitizer based on the potency classifications of Gerberick *et al.* (2001). This classification is consistent with the results of other studies conducted in both humans and animal models of sensitization (Basketter, 1998). Further, the results show that the quenching of citral cannot be demonstrated in the LLNA. It is interesting to note that in humans a NOEL of ~0.5% or 1400 μg/cm² exists for the induction of sensitization to citral. In the future, this NOEL could be applied to a quantitative risk assessment for dermal sensitization using the methods proposed by Gerberick et al. (2001), with the aim of refining the acceptable use level of citral in various consumer product types.

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