Amino Acid-Catalyzed Conversion of Citral: *cis*−*trans* Isomerization and Its Conversion into 6-Methyl-5-hepten-2-one and Acetaldehyde

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Under alkaline conditions, amino acids or proteins catalyze the deacetylation of citral, a major aroma component, resulting in methylheptenone and acetaldehyde formation. 3-Hydroxycitronellal is an intermediate in this reaction. Amino acids also catalyze the *cis*−*trans* isomerization of the pure isomers of citral, geranial, and neral. Most likely the amino acids are involved in stabilizing intermediates of the isomerization and deacetylation reaction of citral. On the basis of the findings presented, some consequences for the application of citral, or its isomers, in food are discussed.

Keywords: *Citral; geranial; neral; methylheptenone; amino acids; cis*−*trans isomerization; deacetylation*

INTRODUCTION

Citral is widely used in the flavor and fragrance industry; its application ranges from meat products to hard candy. The amounts used in the products differ greatly, amounting to as little as 0.20 ppm in cheese and as many as 429.8 ppm in chewing gum. Citral has a strong, lemon-like odor and a characteristic bittersweet taste (Burdock, 1995). It was originally reported in lemongrass, accounting for up to 75% of the oil. Citral is also found in several other plant oils, and it is present in lemon and lime oils. Commercial citral is obtained by isolation from citral-containing essential oils or by chemical synthesis from β-pinene or isoprene (Burdock, 1995). With an annual world consumption of 1200 tons (in 1996) it is one of the most applied flavor compounds (Somogyi, 1996). Moreover, citral has antimicrobial (Onawunmi, 1989) and pheromone activities (Kuwahara et al., 1983; Robacker and Hendry, 1977) and is used in the production of vitamin A and ionones (Shadab et al., 1992).

The linear monoterpene citral is a mixture of the *cis* and *trans* isomers of 3,7-dimethyl-2,6-octadien-1-al, referred to as neral and geranial, respectively. Commercial citral typically contains 60% geranial and 40% neral. The *cis*−*trans* isomers geranial and neral have different characteristics. They differ in odor threshold (neral, 8.8 ng/L of air; geranial, 12.0 ng/L of air) (Schieberle and Grosch, 1988). Also, the aroma profiles of the two citral isomers differ; neral has a somewhat harsh and grassy odor, whereas geranial is milder and more lemon-like (Clark and Chamblee, 1992). Furthermore, neral is an alarm pheromone in some types of mites, whereas geranial is not (Kuwahara et al., 1983).

In this paper we report on the amino acid-catalyzed deacetylation and isomerization of geranial and neral, under neutral and alkaline conditions.

MATERIALS AND METHODS

Materials. Citral and methylheptenone were purchased from Fluka (Buchs, Switzerland). Acetaldehyde was purchased from Merck (Darmstadt, Germany). A reference sample of 3-hydroxycitronellal was prepared as described by Fkyerat and Tabacchi (1997). Geranial (containing 95% geranial and 5% neral) and neral (containing 11% geranial and 89% neral) were obtained by separating commercial citral using preparative GC. Of a 10% citral solution in acetone, 30 μL was injected on a packed column (2.0 mm × 4 mm) filled with 8.6 g of Chromsorb 100−120 containing 9.8% Carbowax. GC was performed on a Varian model 3700 GC equipped with a thermoconductor detector. The detector and filament temperatures were 130 and 160 °C, respectively. The injector and oven temperatures were 200 and 110 °C, respectively, and the flow of the carrier gas (H_2) was 30 mL min^{-1}. The purified isomers were dissolved in hexane and stored at 4 °C.

***cis*−*trans* Isomerization.** Experiments were carried out in 4 mL vials fitted with Teflon Mininert valves (Supelco, Zwijndrecht, The Netherlands). Two hundred microliters of a geranial or neral solution in hexane (equaling 1 mM final concentration in solution) was added to the vials, and subsequently the hexane was evaporated. After the addition of 1 mL of reaction mixture, the vials were vigorously shaken for 30 s, to obtain a homogeneous mixture, and placed in a shaking water bath (150 rpm, 25 °C). The reaction mixture was an aqueous solution containing 0.1 M amino acid and buffer [typically a 50 mM phosphate buffer (pH 7.0), or others (0.1 M) when the pH dependence was tested]. The reaction was followed by analyzing samples (volume = 100 μL), taken from the headspace, by GC. At the end of each experiment the reaction mixture was extracted with 1 mL of ethyl acetate to determine nonvolatile intermediates and products, which could not be detected in the headspace samples. The vials were vigorously shaken to quantitatively extract the terpenes. The ethyl acetate phase was separated from the aqueous phase by centrifuging the mixture in a 2 mL microcentrifuge tube (1 min, 13000 rpm). Subsequently, 5 μL of the ethyl acetate phase was analyzed by GC.

Conversion of Citral into Methylheptenone. The glycine-catalyzed conversion of citral was determined in 15 mL

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vials, fitted with Teflon Mininert valves. Reaction mixtures (1 mL) contained 1 mM citral and 0.1 M glycine/NaOH buffer (pH 10.0). At different times, 66.6 μL samples were taken and analyzed immediately for acetaldehyde using an acetaldehyde test kit (Boehringer, Mannheim, Germany). The remaining 0.933 mL sample was extracted with an equal amount of ethyl acetate and analyzed by GC.

The pH dependence of the transformation of citral into methylheptenone catalyzed by different amino acids was monitored in reaction mixtures (1 mL) containing 0.1 M amino acid and 0.25 M buffer. The reaction was started by the addition of 1 μL of citral (5.66 mM final concentration). After 20 h in a shaking water bath (300 rpm, 25 °C), the terpenes were extracted with 1 mL of ethyl acetate and analyzed by GC.

Identification of Products. Geranial, neral, 3-hydroxycitronellal, and methylheptenone were identified by comparing GC retention times and MS spectra with those of authentic samples. The MS spectra of geranial, neral, and methylheptenone were identical to those in the NIST spectral database. The MS spectrum of 3-hydroxycitronellal was as follows: *m*/*z* (relative intensity) 152 (17), 137 (18), 94 (89), 82 (83), 69 (100), 55 (40), 43 (92). Acetaldehyde was identified by comparing the GC retention time with that of authentic acetaldehyde and enzymatically using an acetaldehyde test kit.

Analytical Methods. All terpenes were analyzed by GC using a fused silica cyclodextrin capillary column (type α-DEX 120; length = 30 m; inside diameter = 0.25 mm; film thickness = 0.25 μm; Supelco, Zwijndrecht, The Netherlands). GC was performed with a Hewlett-Packard 6890 GC, equipped with a flame ionization detector with N_2 as the carrier gas. The detector and injector temperatures were 250 and 200 °C, respectively, and the split ratio was 1:50. The samples were analyzed isocratically at 140 °C. Acetaldehyde was determined by analyzing the headspace (100 μL) using a Hewlett-Packard 6890 GC equipped with a CP-sil 19CB wood fused silica column (30 m × 0.32 mm). The oven temperature was held at 30 °C for 10 min and subsequently raised to 80 °C (10 °C min^{-1}). The carrier gas was N_2 (flow = 1 mL min^{-1}). GC-MS analyses were carried out on a model Hewlett-Packard 5970 MSD GC equipped with a fused silica capillary column (type HP-5MS; length = 30 m; inside diameter = 0.25 mm; film thickness = 0.25 μm). The flow rate of the carrier gas, He, was 1.0 mL min^{-1}. The injector temperature was 220 °C, and the oven temperature was increased from 70 to 175 °C at 7 °C min^{-1}. The injection volume was 1 μL, and the split ratio was 1:50. Electron impact MS data were obtained at 70 eV.

RESULTS

Amino Acid-Catalyzed Isomerization of Geranial and Neral. When the bioconversion of the pure isomers of citral was studied, a spontaneous "chemical" isomerization was observed in samples containing boiled cell extracts. This spontaneous isomerization was also observed in samples that contained only glycine/NaOH buffer, whereas phosphate and Tris buffers did not result in isomerization. Because the amino acid-catalyzed isomerization of geranial or neral was not reported before, this reaction was studied in more detail.

The glycine-catalyzed isomerization of geranial is a relatively fast process (Figure 1A). After 80 min, an equilibrium was reached with 60% of the citral in the geranial form and 40% in the neral form (Figure 2). This is the same composition of isomers as present in commercial citral. Starting with neral, the same equilibrium was reached (Figure 1B).

Also, several other amino acids catalyzed the isomerization of geranial, albeit at different rates (Figure 3). DL-Asparagine was the most efficient catalyst, whereas DL-aspartic acid was the least efficient at a 3.5 times lower rate. Also, the protein bovine serum albumin

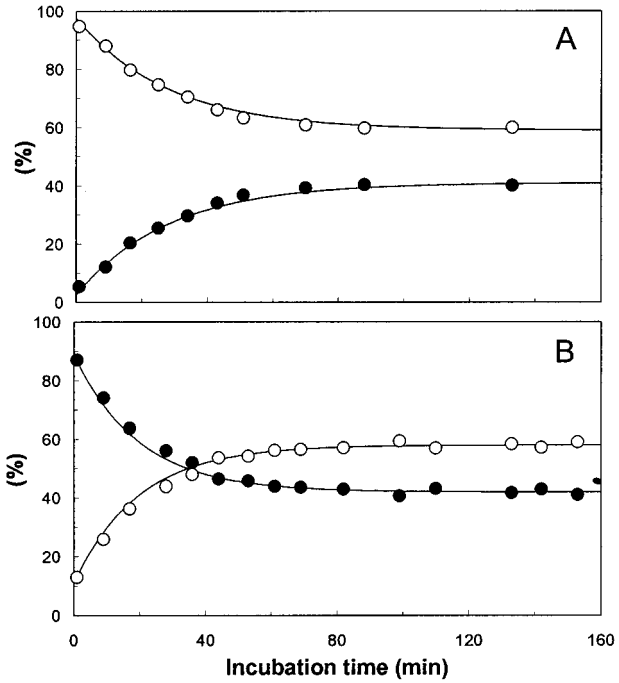

Figure 1. Amino acid-catalyzed isomerization of geranial (A) and neral (B). Each reaction mixture (25 °C) contained 0.1 M glycine, 50 mM potassium phosphate buffer (pH 7.0), and 1 mM substrate. Geranial and neral are expressed as a percentage of total citral. Symbols: (●) neral; (○) geranial.

(BSA) (10 mg mL^{-1} equaling 0.14 mM protein or 88 mM of the individual amino acids) catalyzed the isomerization of geranial to neral (Figure 3). In the absence of amino acids, no isomerization of geranial and neral was observed (not shown). Experiments were performed to determine whether the effect was due to the presence of a positively charged nitrogen (i.e., ammonium) or the carboxyl group (i.e., acetate) as present in amino acids. Ammonium sulfate, acetate, and a combination of both did not catalyze the isomerization of geranial. This indicates that the presence of an amino and a carboxyl group in one molecule is essential for catalysis.

Becuase most chemical reactions of citral involve oxidative or free radical mechanisms (Clark and Chamblee, 1992; Grein et al., 1994), it was determined whether this was also true for the amino acid-catalyzed isomerization reaction. The isomerization catalyzed by glycine at neutral pH was performed in the absence of oxygen (N_2 atmosphere) or in the presence of 25 mM vitamin C, a well-known radical scavenger, respectively. Neither the absence of oxygen nor the presence of vitamin C affected the isomerization rate (not shown).

The pH dependence of the isomerization was tested using glycine as the catalyst (Figure 4). The isomerization rate was clearly pH dependent, displaying very low activity at pH 4.0 and increasingly higher rates at higher pH.

Amino Acid-Catalyzed Conversion of Citral into Methylheptenone and Acetaldehyde. During the glycine-catalyzed isomerization of geranial (**1a**) and neral (**1b**) some other reaction products were detected. Of these products the three most dominant were identified as 3-hydroxycitronellal (**3**), methylheptenone (**6**), and acetaldehyde (**5**) (Figure 2).

The glycine-catalyzed deacetylation of citral was followed in time (Figure 5). Both geranial and neral were converted at similar rates. Methylheptenone and

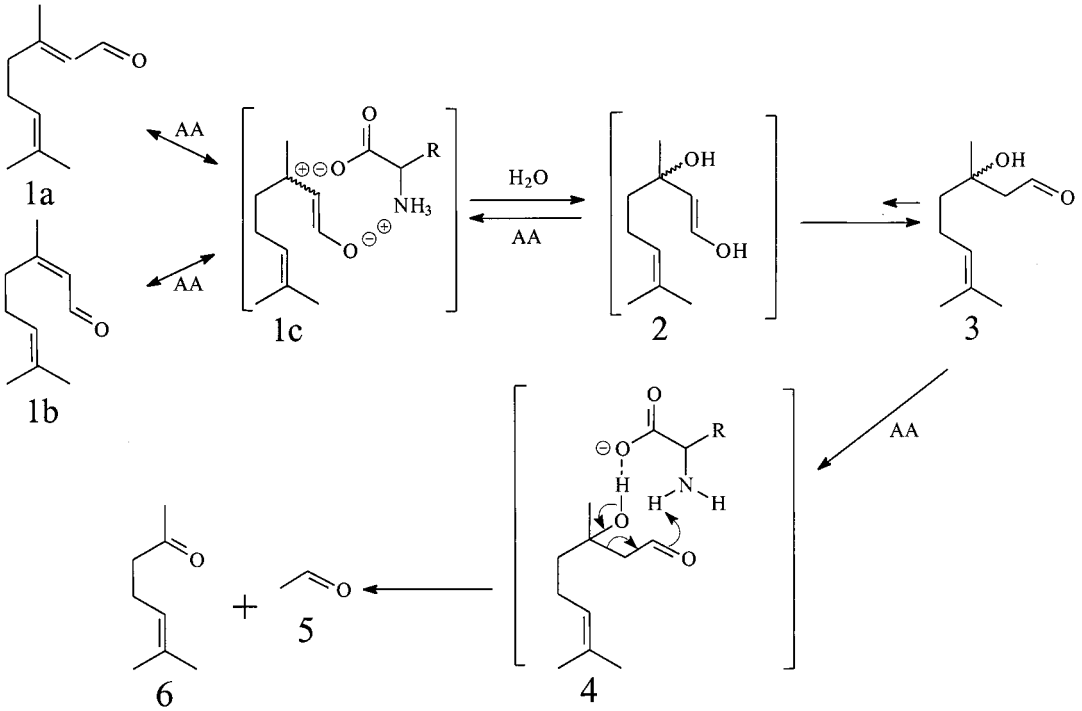

Figure 2. Proposed pathway for the isomerization of geranial (**1a**) and neral (**1b**) and their conversion via 3-hydroxycitronellal (**3**) into methyheptenone (**6**) and acetaldehyde (**5**), catalyzed by amino acids (AA), in alkaline aqueous solution.

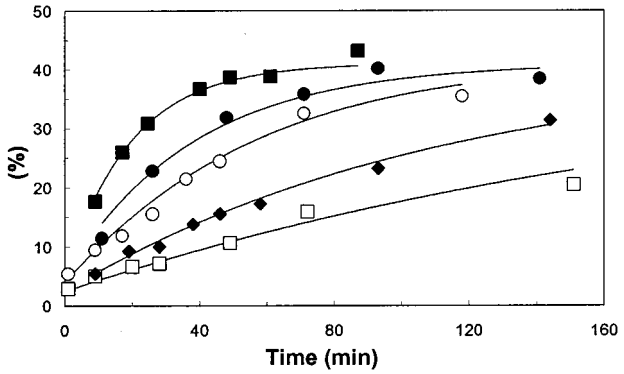

Figure 3. Effect of different amino acids and BSA on the isomerization of geranial. Each reaction mixture (25 °C) contained 0.1 M amino acid or 10 mg mL^{-1} BSA, 50 mM potassium phosphate buffer (pH 7.0), and 1 mM substrate. The neral formed from geranial is expressed as a percentage of the total amount of neral and geranial. Symbols: (■) DL-asparagine; (●) L-glutamic acid; (○) BSA; (◆) β-alanine; (□) DL-aspartic acid.

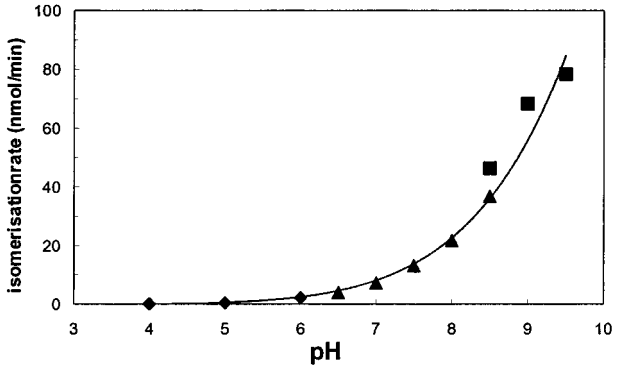

Figure 4. pH dependence of the glycine-catalyzed isomerization of geranial. Each reaction mixture (1 mL, 25 °C) contained 0.1 M glycine, 0.1 M buffer, and 1 mM substrate. The isomerization rate is expressed as nanomoles of neral formed per minute. Symbols: (◆) acetate buffer; (▲) phosphate buffer; (■) borax buffer.

acetaldehyde were formed in equimolar amounts. As with the amino acid-catalyzed isomerization, also the formation of these products was catalyzed by other amino acids (i.e., L-glutamic acid, DL-aspartic acid, L-arginine, L-glutamine, L-methionine, and DL-asparagine). Of these amino acids L-glutamic acid was the most effective catalyst (0.8 mM methylheptenone formed in 20 h), whereas DL-asparagine was the least effective, with a 12 times lower methylheptenone formation rate (not shown). No formation of methylheptenone was found using ammonium sulfate as catalyst or when only buffer was used.

The pH dependence of the amino acid-catalyzed conversion of citral into methylheptenone was studied in more detail (Figure 6A). As for the isomerization reaction, the deacetylation was fastest at high pH. Especially with glycine, a very clear pH dependence was observed.

Amino Acid-Catalyzed Formation and Conversion of 3-Hydroxycitronellal. During the conversion of citral, also 3-hydroxycitronellal was formed, with an initial rate higher than that of methylheptenone and acetaldehyde formation (Figure 5). The 3-hydroxycitronellal concentration remained constant after the first 3 h. After 24 h, the substrate was completely converted and also 3-hydroxycitronellal was no longer detected, indicating that it was converted into methylheptenone and acetaldehyde (not shown), suggesting that it is a reaction intermediate.

The formation of 3-hydroxycitronellal from citral was not affected by the addition of 25 mM vitamin C or by flushing with N_2. The 3-hydroxycitronellal formation was shown to be pH dependent, although there was no clear trend (Figure 6B). Besides being pH dependent, 3-hydroxycitronellal formation was also dependent on the buffer used; in samples containing borax buffer the formation rate was much lower than in phosphate buffer (Figure 6B). 3-Hydroxycitronellal formation also de-

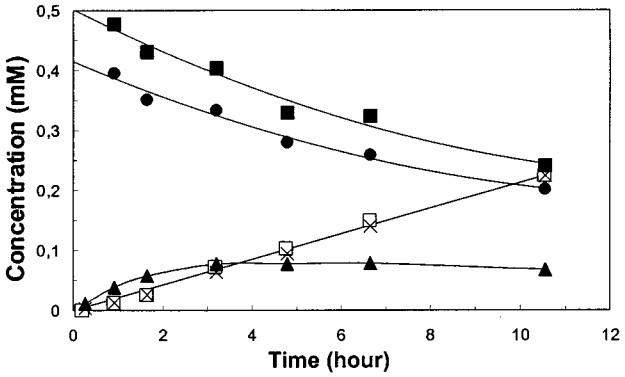

Figure 5. Glycine-catalyzed transformation of citral into methylheptenone, acetaldehyde, and 3-hydroxycitronellal. Each reaction mixture (25 °C) contained 1 mM citral and 0.1 M glycine/NaOH buffer (pH 10.0). Symbols: (■) geranial; (●) neral; (×) methylheptenone; (□) acetaldehyde; (▲) 3-hydroxycitronellal.

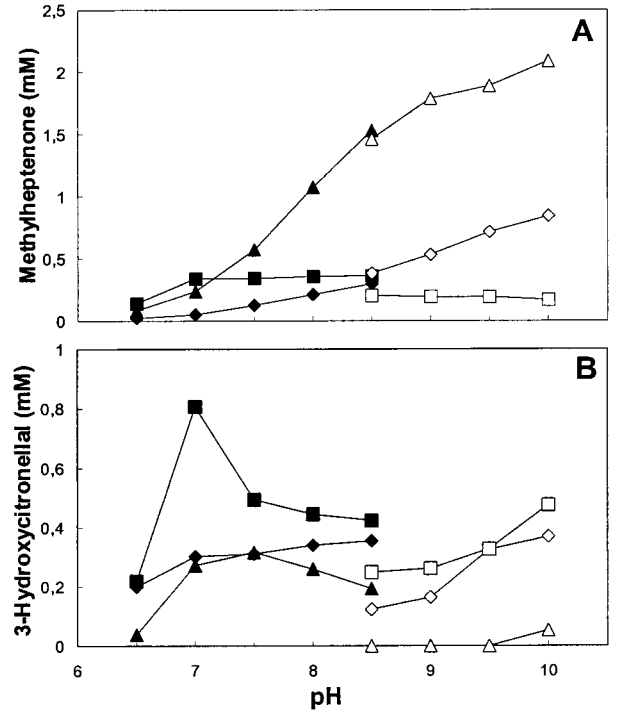

Figure 6. pH dependence of the amino acid-catalyzed transformation of citral into methylheptenone and 3-hydroxycitronellal. Each reaction mixture (25 °C) contained 0.1 M amino acid, 5.66 mM citral, and 0.25 M phosphate buffer (solid symbols) or borax buffer (open symbols). The concentrations of methylheptenone (A) and 3-hydroxycitronellal (B) formed were determined after 20 h. Symbols: (▲) glycine; (◆) DL-aspartic acid; (■) DL-asparagine.

pended on the amino acid used as the catalyst; with DL-asparagine a 2 times higher 3-hydroxycitronellal concentration was observed than with glycine (Figure 6B).

Experiments using 3-hydroxycitronellal as the substrate showed that 3-hydroxycitronellal was converted much more rapidly into citral than into methylheptenone. The rate at which 3-hydroxycitronellal was converted to citral, in the presence of 0.1 M glycine (pH 10.0), was determined to be 2.5 nmol min^{-1} mL^{-1}. This conversion rate is 6 times higher than the rate at which citral is converted into methylheptenone under the same conditions. After 96 h, 3-hydroxycitronellal was converted completely into methylheptenone.

DISCUSSION

This paper describes the amino acid-catalyzed conversion of both citral isomers. In the presence of amino acids, and especially under alkaline conditions, the pure isomers undergo *cis*−*trans* isomerization and are subsequently (and more slowly) converted via 3-hydroxycitronellal into methylheptenone and acetaldehyde (Figure 2). Citral is converted quantitatively into equimolar amounts of methylheptenone and acetaldehyde. The deacetylation is accompanied by the transient accumulation of 3-hydroxycitronellal, suggesting that this is an intermediate in the reaction.

The proposed mechanism for the isomerization of geranial and neral and the formation of methylheptenone is shown in Figure 2. Geranial (**1a**) and neral (**1b**) are transformed into the resonant state **1c**; amino acids, being zwitterions, stabilize **1c**. When addition of water to **1c** occurs, intermediate **2** is formed, which is subsequently transformed into the more stable 3-hydroxycitronellal (**3**) by keto−enol tautomerization. Under alkaline conditions amino acids will form a hydrogen bridge with the hydroxyl group of 3-hydroxycitronellal (**3**), resulting in intermediate **4**, which yields methylheptenone (**6**) and acetaldehyde (**5**) after rearrangement. When the addition of water to intermediate **1c** does not occur, **1c** will be converted back into either geranial (**1a**) or neral (**1b**), resulting in isomerization.

The isomerization of geranial and neral was previously reported by Kuwahara et al. (1983). They described the isomerization of the citral isomers, by bodies of mites, into an equilibrium mixture of 40% neral and 60% geranial. These authors suggest that the reaction was enzymatic or at least protein-catalyzed, but they did not study the reaction in more detail. Isomerization of geranial was also described by Kimura et al. (1982) in citric acid aqueous solutions. They report the production of cyclic compounds directly from neral rather than from geranial, so isomerization of geranial into neral is the first step in the cyclization of geranial.

3-Hydroxycitronellal was also described as an intermediate in the formation of a volatile cyclic compound from citral under oxygen atmosphere in aqueous acidic solution (Grein et al., 1994). They suggest the involvement of oxygen in the formation of 3-hydroxycitronellal from citral. This is in contrast to our results; we show that the amino acid-dependent 3-hydroxycitronellal formation is oxygen independent.

The conversion of citral into methylheptenone and acetaldehyde has only been described under a more extreme reaction condition. Boiling citral with alkaline (K_2CO_3) gives a retro-aldol condensation resulting in methylheptenone and acetaldehyde (Karrer, 1963). The deacetylation of several other α,β-unsaturated aldehydes was reported by Grein et al. (1993).

Remarkably, asparagine, the best catalyst for the isomerization of geranial, is relatively ineffective in catalyzing the conversion to methylheptenone. In addition, the amount of 3-hydroxycitronellal produced is highest when asparagine is used as catalyst. On the other hand, glycine, which is a comparably effective catalyst for the isomerization reaction, is a much more effective catalyst for the conversion of citral into methylheptenone. This indicates that the function of the amino acid is different in the isomerization and deacetylation reactions.

Citral is widely applied as a flavor compound in foods. Most foods have pH values of ≤7, and the conversion of

citral into methylheptenone will not be a major problem; however, upon longer storage of foods the deacetylation of citral might occur, resulting in an alteration of the flavor of the food. However, the application of citral in protein-rich, alkaline foods such as West African iru, Japanese natto, and Indian kinema (Steinkraus, 1997), obtained by alkaline fermentation using bacilli, will be limited. The conditions of these products (high pH and high concentration of amino acids) will quickly result in the transformation of citral into methylheptenone and acetaldehyde and, as a consequence, in an alteration of the flavor profile. Citral is also used in nonfoods such as soaps, detergents, creams, lotions, and perfumes (Opdyke, 1979). Application of the pure isomers geranial and neral for fragrance differentiation is useful in only some of these products, dependent on their pH and the presence of amino acids or protein.

In conclusion, the deacetylation of citral and consequent alteration of the flavor profile is not expected to be a major problem in many foods, except upon longer storage. The application of pure geranial or neral, however, should be limited to neutral or slightly acidic products or products not containing amino acids or proteins.

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