JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Model Study of the Enzymatic Modification of Natural Extracts: Peroxidase-Based Removal of Eugenol from Rose Essential Oil

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Supporting Information

ABSTRACT: A protocol based on the use of horseradish peroxidase (HRP) is proposed for the removal of allergenic eugenol from rose essential oil without loss of the organoleptic quality and with a good conservation of the chemical composition. For the first time, an enzyme-based strategy is proposed for essential oils treatment and opens new opportunities in the detoxification of natural extracts used in perfumery and cosmetics. Our results on eugenol in rose essential oil constitute a first step toward the development of efficient and mild processes for the removal of more toxic compounds of natural extracts.

KEYWORDS: *HRP, enzymatic remediation, natural extracts, fragrances, allergens*

INTRODUCTION

Natural extracts were the first type of material used in perfumery with incenses of ancient oriental civilizations and are still of paramount importance in modern fragrances formulas. Precious materials such as essential oils from rose, sandalwood, patchouli, and vetiver among others are used in almost every fine fragrance.[1,2]

These extracts are typically composed of several dozens of individual compounds with a great qualitative and quantitative variability, most of them being of terpenoid origin.[3]

In spite of an ancestral use for many of these, the need of consumers and authorities of Western countries for absolutely benign products on the market could in a near future be a threat on a fair number of natural extracts. To date, the presence of allergens belonging to the list of 26 has to be specified on the final manufactured products when they exceed minimum levels. A series of putative carcinogens such as methyleugenol,[4,5] safrole,[6,7] skin sensitizers such as atranol derivatives (contained by tree moss extracts which are listed among the 26),[8,9] or hepatotoxic suspected agents such as estragole are also under close surveillance.[10]

To anticipate the plausible ban of certain natural materials containing these compounds, research efforts are currently done in many laboratories, both in academia and industry, to find efficient processes allowing for the selective removal of these suspected toxic compounds. A critical issue for such processes is to avoid the alteration of the organoleptic quality of the material treated. The main type of process for this purpose is distillation, which suffers from weak selectivity and high energy demand. Others solutions have been evaluated such as laser photolysis,[11] or trapping on specific solid supports such as molecular imprinted polymers,[12] but have not yet been applied to such processes so far.

In such context, biocatalysis could offer both the specificity, the most famous asset of enzyme activity, and the mildness required to handle sensitive materials such as essential oils. In addition to these practical advantages, biocatalysis could also provide more sustainable chemical processes, compared to the conventional time- and energy-consuming distillation strategies, operating with selectivity issues. In this paper is presented a model study of the enzymatic removal of eugenol from rose essential oil, one of the most precious materials of fine perfumery. This study is the first example of enzyme-assisted modification of essential oils aimed at removing a single compound and constitutes a proof-of-concept series of experiments demonstrating that such strategy could be viable in the future for more challenging toxic compounds.

MATERIALS AND METHODS

Chemicals. Solvents (Et_2O, CH_2Cl_2), salts (NaH_2PO_4, Na_2HPO_4), and eugenol were purchased from Aldrich and used as received. Horseradish peroxidase (HRP) was purchased from Sigma, and catalase was from Fluka, stored at −18 °C, and let warm to room temperature prior to use. A rose essential oil communelle (blending of oils of various origins) was used in this study. Samples of dieugenol[13] and diacetyldieugenol[14] could be prepared following procedures from the literature.

Apparatus. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker AC 200. ^{1}H NMR spectra are described as follows: chemical shift (δ) in ppm relative to TMS at 0 ppm, multiplicity (s = singlet, d = doublet, m = multiplet), coupling constants (Hz), and integration. ^{13}C NMR spectra chemical shifts are reported in ppm (δ) relative to $CDCl_3$ at 77.16 ppm. Fast-GC/MS analyses were performed for routine analyses with a Shimadzu QP2010S-MS chromatograph (EI, 70 eV) equipped with an SLB-5 ms capillary column (thickness, 0.10 μm; length, 15 m; inside diameter, 0.10 mm). Temperature program: 80 °C then 18°/min to 200 °C and maintained at this temperature for 30 min. Analytical thin-layer chromatography (TLC) was performed on 0.2 mm precoated plate silica gel 60 F254 (Merck).

Quantitative Gas Chromatography Analysis. GC analysis was carried out using an Agilent 6890N gas chromatograph, under the following operation conditions: vector gas, helium; injector and detector temperatures, 250 °C; injected volume, 1 μL; split ration 1/

Received: August 10, 2011
Revised: December 27, 2011
Accepted: December 30, 2011
Published: December 30, 2011

100; HP1 column (J&W Scientific), poly(dimethylsiloxane) (50 m × 0.20 mm i.d., film thickness 0.33 μm; constant flow 1 mL/min) and INNOWAX (poly(ethylene glycol), (50 m × 0.20 mm i.d. × film thickness 0.4 μm; Interchim, Montluçon, France). Temperature program 45−250 at 2 °C/min and 250 °C for 60 min. Retention indices were determined with C_5−C_{24} alkane standards as reference. Relative amounts of individual components are based on peak areas obtained without flame ionization detector (FID) response factor correction. Three replicates were performed for each sample. The average of these three values and the standard deviation were determined for each component identified.

Gas Chromatography/Mass Spectrometry Analysis. Gas chromatography/mass spectrometry (GC/MS) analysis was carried out using an Agilent 6890N coupled to an Agilent 5973 MS (Agilent, Massy, France). Samples were analyzed on a fused-silica capillary column HP-1 (poly(dimethylsiloxane), 50 m × 0.20 mm i.d. × film thickness 0.33 μm; Interchim, Montluçon, France) and INNOWAX (poly(ethylene glycol), 50 m × 0.20 mm i.d. × film thickness 0.4 μm; Interchim, Montluçon, France). Carrier gas, helium, constant flow of 1 mL/min; injector temperature, 250 °C; split ratio, 1:100; temperature program, 45−250 °C or 230 °C, at 2 °C/min then held isothermal (20 min) at 250 °C (apolar column) or 230 °C (polar column); ion source temperature, 230 °C; transfer line temperature, 250 °C (apolar column) or 230 °C (polar column); ionization energy, 70 eV; electron ionization mass spectra were acquired over the mass range of 35−400 amu.

Identification of the Components. Identification of the components was based on computer matching against commercial libraries (Wiley, MassFinder 2.1 Library, NIST98), laboratory mass spectra libraries built up from pure substances, and MS literature data[15−20] combined with comparison of GC retention indices (RI) on apolar and polar column. RIs were calculated with the help of a series of linear alkanes C8−C24 on apolar and polar columns (HP-1 and HP-INNOWAX). Compounds available in the laboratory were confirmed by external standard compound coinjection.

HRP-Catalyzed Oxidation of Eugenol. HRP (124 U/mg, 5 mg) was dissolved in 250 mL of pH 7 phosphate buffer (20 mM) containing eugenol (500 mg). The reaction flask was covered with an aluminum foil to avoid peroxide decomposition. Reaction was initiated by the slow addition of a 35% aqueous H_2O_2 solution at 0.1 mL/h to ensure the final addition of 2 equiv of hydrogen hydroperoxide with respect to the phenolic substrate. After completion, monitored by GC-FID analysis of aliquots withdrawn from the reaction medium, the precipitated product was recovered by filtration. Extraction of the aqueous filtrate by Et_2O allowed the final recovery of the dieugenol product in 94% yield after solvent removal in vacuo.

Dieugenol (3,3′-Dimethoxy-5,5′-di-(2-propen-1-yl)-1,1′-biphenyl-2,2′-diol) [4433-08-3]: yield 94%; ^{1}H NMR (200 MHz, $CDCl_3$, 20 °C): 3.30 (d, J = 12 Hz, 4H); 3.80 (s, 6H); 4.90−5.05 (m, 4H); 5.70−6.00 (m, 2H); 6.70−6.50 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$, 20 °C): 39.92; 56.04; 110.63; 115.65; 123.04; 124.36; 131.85; 137.59; 140.85; 147.17.

Acetylation of Dieugenol. Since dieugenol could not be analyzed by GC/MS, its diacetylated derivative was prepared. Dieugenol (100 mg; 0.31 mmol) was dissolved in CH_2Cl_2 (1 mL). Triethylamine was then added (853 μL; 11 mmol) followed by Ac_2O (576 μL; 6 mmol) under an inert atmosphere. After completion of the reaction, monitored by TLC (SiO_2, cyclohexane/ethyl acetate 1:1, $Rf_{dieugenol}$ = 0.55, $Rf_{diacetyldieugenol}$ = 0.73), the crude reaction mixture was evaporated in vacuo. The solid obtained was dissolved in CH_2Cl_2 and washed with water. After drying of the organic layer with $MgSO_4$ and evaporation, diacetyl dieugenol was quantitatively obtained as a white solid.

Diacetyldieugenol (3,3′-Dimethoxy-5,5′-di-(2-propen-1-yl)-1,1′-biphenyl-2,2′-diacetoxy): yield 99%; ^{1}H NMR (200 MHz, $CDCl_3$, 20 °C): 3.30 (d, J = 12 Hz, 4H); 3.80 (s, 6H); 4.90−5.05 (m, 4H); 5.70−6.00 (m, 2H); 6.70−6.50 (m, 4H). ^{13}C NMR (75 MHz, $CDCl_3$, 20 °C): 20.30; 39.99; 55.90; 111.90; 116.15; 122.40; 131.15; 135.00; 136.80; 138.00; 151.05; 168.00. MS (EI, 70 eV): 410(0) [M + ·], 206(7), 164(10), 149(27), 133(22), 131(23), 121(14), 104(16), 91(15), 77(18), 55(14).

H_2O_2 Titration by $KMnO_4$. Typical example for a total reaction volume of 25 mL: A sample of 1 mL is withdrawn from the reaction mixture, and aqueous 0.1 M H_2SO_4 solution (1 mL) is added, followed by 1 mL of iced distilled water. An aqueous solution of $KMnO_4$ (1 mM) is added dropwise until persistent coloration is observed.

HRP-Catalyzed Eugenol Removal from Rose Essential Oil. At the milligram scale: HRP (124 U/mg, 2.5 mg) was dissolved in 25 mL of pH 7 phosphate buffer (20 mM). The reaction flask was covered with an aluminum foil, and 35% aqueous H_2O_2 solution (3.6 μL) was added. After 1.5 h, catalase (<2000 U/mg, 2.5 mg) was added and agitation was maintained 1 additional hour. Rose essential oil (50 mg) was then added, and the reaction was run for 4 h. After completion, the reaction mixture was filtered and insoluble solid material was collected. Extraction of the aqueous filtrate by Et_2O allowed the final recovery of the modified essential oil after solvent removal. GC/MS and GC-FID analysis of the product obtained confirmed the disappearance of eugenol.

Optimized procedure for 1.25 g essential oil: HRP (124 U/mg, 20 mg) was dissolved in 1000 mL of pH 7 phosphate buffer (20 mM). The reaction flask was covered with an aluminum foil, and 35% aqueous H_2O_2 solution (20 μL) was added. After 1.5 h, catalase (<2000 U/mg, 20 mg) was added and agitation was maintained 1 additional hour. Rose essential oil (1.25 g) was then added, and the reaction was run for 15 h. After completion, the aqueous phase was exposed to light and saturated with NaCl. Extraction by Et_2O (600 mL) allowed the recovery of the modified essential oil (1.22 g, 98% yield) after drying over magnesium sulfate, filtration, and solvent removal at atmospheric pressure and water bath at 45 °C. Fast-GC/MS analysis was performed on the Et_2O solution of the modified rose essential oil to avoid compounds loss during solvent removal and allow for an accurate analysis of the outcome of the reaction.

Sensory Evaluation by Triangular Testing. A set of three samples, two of these being identical, was prepared with modified and nonmodified rose essential oil in ethanol. A group of 12 nonexpert panelists, performing their evaluation separately, had to identify the sample deemed "different".

The following formula have been used to determine x, the number of correct answers needed to valid that the sample was significantly different, at various levels of confidence, N being the number of panelists:

$$95\%\ \text{confidence:}\quad x1 = 0.77\sqrt{N} + \frac{2N+3}{6} + 0.5$$

$$99\%\ \text{confidence:}\quad x2 = 1.10\sqrt{N} + \frac{2N+3}{6} + 0.6$$

$$99.9\%\ \text{confidence:}\quad x3 = 1.46\sqrt{N} + \frac{2N+3}{6} + 0.8$$

For a panel of 12 persons, eight correct answers are necessary declare the samples different at 95% level of confidence.

Olfactory Evaluation by Trained Perfumers. Samples of HRP-modified rose essential oil were submitted to the evaluation of trained perfumers as 10% v/v ethanolic solutions.

> Perfumer 1: HRP-modified sample was described as "reminding of good quality synthetic oil, lacking some facets, less rich, single note, and close from the idea of rose odor of consumers."
>
> Perfumer 2: HRP-modified sample was described as "less green, artichoke leave, citrus, more litchi-like, with a loukoum note, powdery "and was very much appreciated.

RESULTS

HRP-Catalyzed Oxidation of Eugenol. A prerequisite to the enzymatic removal of eugenol from rose essential oil was to be able to transform pure eugenol. A protocol for the HRP-catalyzed oxidation of pure eugenol was thus established based

on previous related studies.[21,22] The course of the reaction was monitored by GC-FID analysis of the consumption of eugenol. In our hands, the best conditions were the use of 1% (w/w) HRP in pH 7 phosphate buffer (20 mM) for the conversion of eugenol (2 g/L) triggered by the slow addition of 2 equiv of H_2O_2 (0.1 mL/h of a 35% aqueous solution) to form dieugenol with 94% yield (Figure 1).

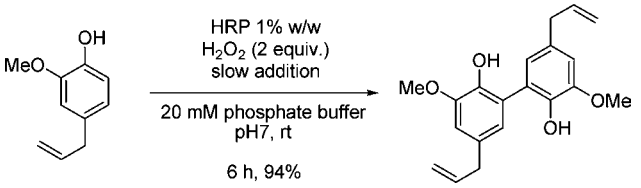

Figure 1. HRP-catalyzed eugenol dimerization.

It is known in peroxidase chemistry that the enzymatic generation of a phenoxyl radical upon phenolic derivative one-electron oxidation is followed by nonenzymatic couplings of the radicals in solution.[23] In the particular case of eugenol, the radical can undergo $H^{\bullet}$ elimination to form a quinone methide, stabilized by extended conjugation on the allyl side chain. Polymeric materials are obtained as final products, regardless of the intermediates formed (Figure 2).[24]

Monitoring of H_2O_2 Consumption and the Role of Catalase. Although efficient when dealing with pure eugenol as starting material, issues of side reactions involving H_2O_2 had to be addressed. Sensitive materials such as essential oils, composed of a variety of terpenoids, were being indeed altered by the presence of hydrogen peroxide. Tentative modifications of the addition rate of H_2O_2 were unsuccessful (data not shown), and we thus envisaged to desynchronize the intervention of H_2O_2 and the substrate and separate the two sequences by the action of catalase to decompose residual H_2O_2 and therefore avoid terpenoids oxidation.

During phase 1, HRP is activated while H_2O_2 concentration decreases, monitored by $KMnO_4$ titration (Figure 3). During phase 2, catalase achieves to consume free H_2O_2, in a fast reaction liberating O_2. During phase 3, eugenol is consumed.

HRP-Catalyzed Removal of Eugenol from Rose Essential Oil. The reaction was performed applying a three-phase sequence. After the action of H_2O_2 (phase 1, 1.5 h), followed by the addition of catalase (phase 2, 1 h), rose essential oil was added to the reaction mixture (phase 3). A biocatalyst loading of 5% (w/w) was used. The reaction was maintained 4 h, and after completion and extraction, no eugenol was detectable in the product analyzed by GC/MS and GC-FID.

The aqueous buffer containing HRP was collected, and fresh essential oil was added (50 mg). The reaction was performed a second time, and again, no eugenol could be found in the final product. The aqueous phase could be reused up to nine times and showed decreased activity during the 10th reaction. An activation cycle could then be performed again using H_2O_2 followed by the addition of catalase to regenerate active HRP.

Gram-Scale Experiment. The reaction was performed at the gram scale (1.25 g of rose essential oil) following slightly modified conditions (see Materials and Methods). HRP modification of the essential oil allowed the total consumption of eugenol and a remarkable conservation of the chemical composition of the modified oil. Besides eugenol, only phenylethanol and geraniol proportion decreased during the enzymatic reaction, as already reported to occur with terpenyl alcohols and HRP.[25] To avoid confusion, GC/MS analysis of the outcome of the enzymatic reaction was performed directly on the ethereal solution obtained after extraction of the aqueous reaction medium, saturated with NaCl, and not on the product obtained after evaporation.

Sensory Evaluation by Triangular Testing. The sensory evaluation could only be performed on the HRP-modified essential oil obtained after diethyl ether evaporation. Two sets of samples were tested. For the modified essential oil obtained in the presence of excess active HRP (milligram-scale experiments), two samples containing pure rose essential oil and one sample containing HRP-modified essential oil were prepared at 0.5% (w/w) in EtOH and submitted to the panel. Although the odor remained floral, roselike, the panel could easily identify the modified essential oil (11/12 panelists). For the modified essential oil obtained in optimized conditions (gram-scale experiment), two samples containing HRP-modified rose essential oil and one sample containing pure essential oil were prepared at 0.3% (w/w) in EtOH and submitted to the panel. The score obtained was 3, 3, and 6,

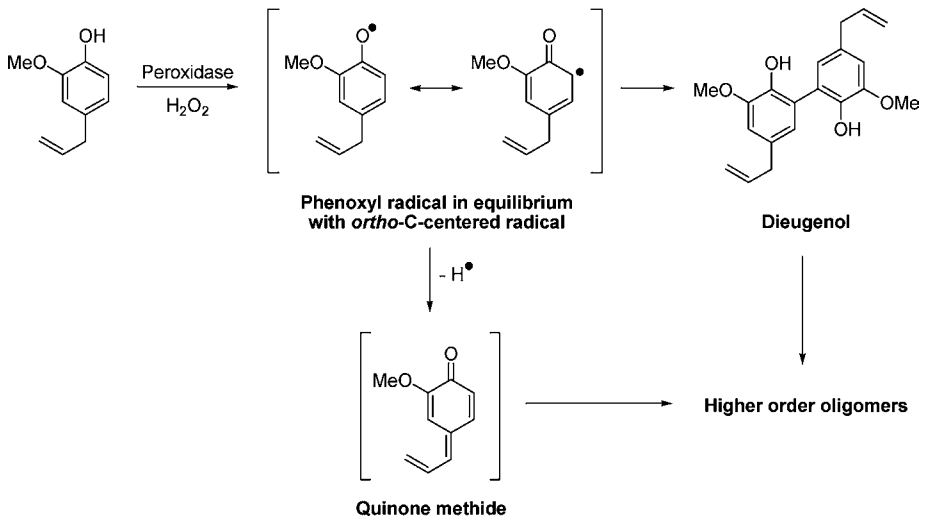

Figure 2. Radicals formed from eugenol upon peroxidase oxidation.

dx.doi.org/10.1021/jf205194v | J. Agric.Food Chem. 2012, 60, 1052–1058

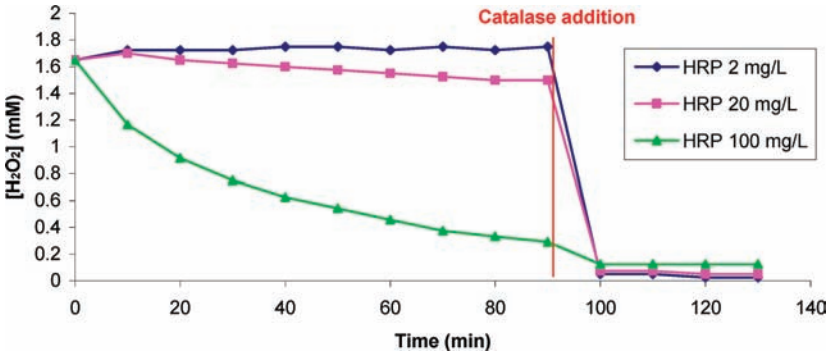

Figure 3. Time-course monitoring of H_2O_2 consumption in the reaction medium in the presence of various concentrations of horseradish peroxidase (HRP) by $KMnO_4$ titration.

respectively, thereby showing that the modified and non-modified samples were not significantly different to nontrained panelists from a sensory point of view.

Samples evaluation by perfumers led to different evaluations. The HRP-modified rose essential oil was different from the nonmodified but presented some interesting notes on the rose variation.

Qualitative and Quantitative GC Analyses. Besides routine fast-GC/MS analysis used for the monitoring of the reaction progress, detailed GC-FID and GC/MS analyses were performed for the qualitative and quantitative characterization of the chemical composition of the pure rose essential oil and the HRP-modified oil obtained after evaporation of diethyl ether used to recover it from the aqueous reaction medium. The results are summarized in Table 1 and show only small quantitative differences, the overall composition being the same. Besides the target compound eugenol, which was entirely removed, phenylethanol and citronellol showed a decrease of their proportion.

DISCUSSION

Eugenol is a ubiquitous phenylpropanoid present in the composition of various natural extracts from fruits, flowers, leaves, and barks. Although listed among the 26 allergens by the European Commission (EC), the compound rather presents a beneficial biological profile and has been used for a long while as an antiseptic in dentistry when mixed with zinc oxide.[26] In contrast, its methylated analogue, methyleugenol, is a suspected carcinogenic agent.[5] Due to its occurrence in many natural extracts, including essential oils used in fine perfumery such as rose essential oil, it will soon be necessary to be able to offer technical solutions to selectively remove those compounds from complex mixtures without modifying the overall properties of the mixtures, i.e., the subtle odor of rose.

In the frame of our project dedicated to the enzymatic modification of essential oils, we started our investigations on a model study of the peroxidase-based removal of eugenol from rose essential oil.

The first milestone of this study was to determine optimized conditions for the HRP-catalyzed eugenol oligomerization in the presence of H_2O_2. As described above, with our conditions, eugenol was totally consumed and the dimer formed by coupling of two eugenyl radicals was obtained in 94% yield, significantly higher than results found in the literature. This high yield was mandatory for the use of such enzymatic system in the detoxification of a complex mixture.

In a second part of the study, we had to manage the use of highly reactive H_2O_2, which could cause nonselective oxidation reactions of unsaturated terpenoids such as limonene, geraniol, nerol, and citronellol, which are numerous in plant natural extracts in general and rose in particular. Since degradation products were observed in many reactions where the order and the mode of addition of reagents were changed (data not shown), we had to design a protocol where H_2O_2 and the organic substrate would not be in contact, which is a paradox because the former is the oxidant and the latter reducing agent of the redox reaction resulting in the phenolic radical generation, catalyzed by HRP. This could be achieved by setting up a "stoichiometric" use of the enzyme. The idea was to initially activate the enzyme by treatment with H_2O_2 in an aqueous buffer, monitored by peroxide titration. After this period of activation, catalase was added to decompose residual H_2O_2 and ensure a nonoxidative medium for the essential oil. The efficiency of HRP activation could be characterized by comparing the consumption of H_2O_2 for various enzyme concentrations (Figure 3). With 100 mg/L of HRP and after 90 min, a consumption of 1.35 mM of H_2O_2 is measured, while it was almost zero with a 2 mg/L loading of enzyme. This value accounted for the concentration of active enzyme in solution, being in its compound I or compound II form, HRP existing under five different forms, two of these having oxidative ability.[27]

Rose essential oil was then added to this active solution of HRP accounting for a loading of 5% (w/w) with respect to the mass of essential oil, and the organic products were recovered after 4 h of shaking at room temperature. GC/MS and GC-FID analysis of the crude product showed the absence of eugenol. The aqueous HRP solution was reused nine times with successful removal of eugenol from fresh essential oil introduced at each cycle. Following fresh H_2O_2 addition, HRP could be regenerated.

To perform the reaction at the gram scale, optimized conditions were determined from the previous experiments taking into account (1) that the estimated active HRP in this reaction conditions, taken from Figure 3, is 100 mg/L of HRP for 1.35 mM H_2O_2 and is a large excess compared to the eugenol contents of the essential oil; (2) the post-treatment involving diethyl ether extraction and evaporation results in a loss of the most volatile compounds; (3) citronellol and other alcohols are possible peroxidase substrates, albeit phenol-derived eugenol would probably be the most reactive, which makes it necessary to optimize the concentration of active HRP to avoid nondesired side reactions with other substrates; (4)

dx.doi.org/10.1021/jf205194v | J. Agric.Food Chem. 2012, 60, 1052−1058

Table 1. Chemical Composition of Rose Essential Oil and HRP-Modified Oil Used in This Study

compds[a]	LRI_{HP1}[b]	LRI_{INNO}[b]	rose EO (% ± SD)[c]	HRP-modified rose EO (% ± SD)[c]	identification methods
hexanol	852	1360	tr	–	LRI, MS, std
phenol	965	1980	tr	–	LRI, MS, std
myrcene	981	1148	tr	tr	LRI, MS, std
limonene	1020	1194	tr	tr	LRI, MS, std
eucalyptol	1022	1188	tr	–	LRI, MS, std
(*Z*)-*β*-ocimene	1032	1235	tr	tr	LRI, MS, std
benzyl alcohol	1035	1880	0.2 ± 0.1	0.1	LRI, MS, std
(*Z*)-linalool oxide furanoid	1071	–	tr	–	LRI, MS
(*E*)-linalool oxide furanoid	1075	1465	tr	tr	LRI, MS
heptanoic acid	1080	1951	tr	tr	LRI, MS, std
nonanal	1082	1365	tr	tr	LRI, MS, std
linalol	1089	1526	0.7 ± 0.1	0.4	LRI, MS, std
phenylethyl alcohol	1094	1897	18.3 ± 0.3	9.7 ± 0.2	LRI, MS, std
rose oxide 1	1099	1327	tr	tr	LRI, MS
rose oxide 2	1114	1340	tr	tr	LRI, MS
nerol oxide	1136	1438	tr	tr	LRI, MS
citonellal	1139	1465	tr	tr	LRI, MS
2-phenylethylformiate	1157	1760	0.2 ± 0.1	tr	LRI, MS
terpinen-4-ol	1163	1561	0.3 ± 0.1	0.2	LRI, MS, std
benzoic acid	1152	2440	tr	–	LRI, MS, std
α-terpineol	1175	1651	0.2	0.2	LRI, MS, std
citronellol	1221	1730	37.0 ± 0.3	31.9 ± 0.6	LRI, MS, std
phenylethyl acetate	1225	1775	tr	tr	LRI, MS, std
neral	1229	1688	0.2	0.1	LRI, MS
geraniol	1254	1814	12.5 ± 0.4	9.9 ± 0.5	LRI, MS, std
geranial	1250	1697	0.4 ± 0.1	0.5 ± 0.2	LRI, MS, std
citronellylformiate	1260	1586	0.3 ± 0.1	0.2 ± 0.1	LRI, MS
geranylformiate	1284	1635	tr	tr	LRI, MS
tridecane	1299	1298	0.1	tr	LRI, MS, std
methyl geraniate	1304	1658	tr	tr	LRI, MS, std
citronellic acid	1313	–	tr	–	LRI, MS
α-terpinyl acetate	1320	1695	0.6 ± 0.1	0.5 ± 0.1	LRI, MS
citronellyl acetate	1332	1660	0.7 ± 0.1	0.7 ± 0.1	LRI, MS
eugenol	1336	2110	0.5 ± 0.1	–	LRI, MS, std
geranic acid	1341	2281	tr	tr	LRI, MS
neryl acetate	1343	1694	0.1	tr	LRI, MS, std
geranyl acetate	1362	1730	0.1	tr	LRI, MS, std
methyl eugenol	1372	1977	1.3 ± 0.1	1.4 ± 0.1	LRI, MS, std
β-bourbonene	1395	1493	0.1	tr	LRI, MS
β-caryophyllene	1430	1650	1.2 ± 0.1	1.2	LRI, MS
α-guaiene	1432	1563	0.3	0.2	LRI, MS, std
α-humulene	1456	1627	0.3	0.4 ± 0.1	LRI, MS, std
γ-muurolene	1476	1663	0.1	0.1	LRI, MS
germacrene D	1487	1675	0.2 ± 0.1	0.1	LRI, MS
α-selinene	1490	1722	0.2	0.5 ± 0.1	LRI, MS
valencene	1493	1697	tr	tr	LRI, MS
pentadecane	1499	1500	0.1	0.1	LRI, MS, std
Δ-guaiene	1501	1710	tr	tr	LRI, MS
δ-cadinene	1514	1701	tr	tr	LRI, MS
elemol	1533	2031	0.1	0.1	LRI, MS
nerolidol	1546	2033	0.1	tr	LRI, MS, std
hexadecene[d]	1572	1689	tr	tr	LRI, MS
caryophyllene oxide	1575	1925	0.2	0.3 ± 0.1	LRI, MS, std
phenylethyl tiglate	1580	2180	0.1	tr	LRI, MS
hexadecane	1599	1600	0.2	0.3	LRI, MS, std
epi-*γ*-eudesmol	1632	2112	0.2 ± 0.1	0.2	LRI, MS
β-eudesmol	1650	2169	0.2	0.2	LRI, MS
α-eudesmol	1655	2161	0.2	0.3 ± 0.1	LRI, MS
heptadecene[d]	1675	1722	0.3 ± 0.1	0.3	LRI, MS
(*Z*,*E*)-farnesol	1692	2322	1.9 ± 0.2	2.8 ± 0.1	LRI, MS
heptadecane	1699	1704	1.8 ± 0.1	1.6 ± 0.1	LRI, MS, std

dx.doi.org/10.1021/jf205194v | J. Agric.Food Chem. 2012, 60, 1052–1058

Table 1. continued

compds[a]	LRI_{HP1}[b]	LRI_{INNO}[b]	rose EO (% ± SD)[c]	HRP-modified rose EO (% ± SD)[c]	identification methods
(*E*,*E*)-farnesol	1710	2307	1.8 ± 0.1	0.2	LRI, MS
(*Z*,*Z*)-farnesol	1715	–	0.2	tr	LRI, MS
(*E*,*E*)-farnesal	1722	2290	0.2	0.2 ± 0.1	LRI, MS
mintsulfide	1727	2093	tr	tr	LRI, MS
benzyl benzoate	1730	2570	0.1	0.1	LRI, MS
ethyl myristate	1778	2035	tr	tr	LRI, MS
octadecane	1798	1795	0.2	0.3 ± 0.1	LRI, MS, std
farnesyle acetate[d]	1815	2228	0.1	0.1	LRI, MS
phenylethyl benzoate	1826	2696	0.1	0.1	LRI, MS
dehydroaromadendrene	1841	2316	tr	tr	LRI, MS
nonadecene[d]	1889	1921	3.1 ± 0.2	4.4 ± 0.1	LRI, MS
nonadecane	1905	1900	9.9 ± 0.2	17.3 ± 0.5	LRI, MS, std
palmitic acid	1945	2910	0.1	0.2	LRI, MS
eicosene[d]	1972	2228	tr	tr	LRI, MS
ethyl palmitate	1980	2250	tr	tr	LRI, MS
eicosane	2001	1999	0.7	1.4 ± 0.1	LRI, MS, std
heneicosene[d]	2068	2125	0.2	0.2	LRI, MS
heneicosene[d]	2084	2132	0.2	0.3	LRI, MS
heineicosane	2109	2101	2.8 ± 0.1	6.1 ± 0.1	LRI, MS, std
docosene[d]	2189	–	0.1	0.2	LRI, MS
docosane	2198	2199	0.2 ± 0.1	0.3 ± 0.1	LRI, MS, std
tricosene[d]	2284	2346	0.2 ± 0.1	0.1	LRI, MS
tricosane	2304	2300	0.5	1.3 ± 0.1	LRI, MS, std
tetracosane	2396	2409	tr	0.1	LRI, MS, std
pentacosane	2499	2497	0.1	0.1	LRI, MS, std
heptacosane	–	–	0.3 ± 0.1	0.4 ± 0.1	LRI, MS, std

[a]Compounds are listed in order of their elution time from an HP-1 column. Compositional values less than 0.1% are denoted as traces (tr). Presence of a compound is indicated by its GC-FID percentage with SD, absence is indicated by "–". [b]RI = retention indices, determined on HP-1 and INNOWAX columns using the homologous series of *n*-alkanes (C8–C26). [c]SD = standard deviation. [d]Correct isomer not identified.

extraction with Et_2O from NaCl-saturated aqueous phase would help recover most of the organic compounds. Since eugenol accounts for a low percentage of the pure rose essential oil composition, and for a total mass of 1.25 g of essential oil, we considered that 20 mg/L of active HRP would be sufficient to oxidize eugenol and limit the oxidation of other alcohols. Such HRP concentration constitutes 20% of the concentration used for the milligram-scale experiments. After completion of the reaction, the aqueous reaction medium was saturated with NaCl and extracted with Et_2O. The formation of a brown insoluble material was observed, and it was easily removed from the reaction mixture by filtration. Gas chromatography analysis showed unambiguously that eugenol was missing in the HRP-modified oil (full chromatograms are given in the Supporting Information). It could be seen unambiguously that only the peak of eugenol is missing. Besides eugenol, 2-phenylethanol and citronellol also showed a drop in concentration, probably suffering from side reactions. The solvent was then evaporated, and a chromatogram was recorded again to show only quantitative differences, with a conservation of the qualitative composition. Detailed GC-FID and GC/MS analyses showed indeed a high conservation of the composition, with small quantitative differences in many instances within the relative error (Table 1). The reaction yield was 98% of modified essential oil, which could not be attained if substantial evaporation or degradation occurred.

The organoleptic properties of the HRP-modified essential oil obtained after evaporation were evaluated by triangular testing performed with a group of 12 nonexpert panelists. Two sets of samples were tested. The modified essential oil obtained in the presence of excess active HRP (milligram-scale experiments) could easily be identified (11/12 panelists). The modified essential oil obtained in optimized conditions (gram-scale experiment) was not significantly different from a sensory point of view, since only 6/12 panelists could make the difference. The optimized procedure clearly led to an improved olfactory quality of the HRP-modified essential oil.

In summary, we have shown that HRP could be used in a nonconventional fashion to remove eugenol from rose essential oil. The protocol involved a first phase of activation of the enzyme by H_2O_2, followed by quenching with catalase prior to essential oil addition to avoid the direct and nonselective oxidation of terpenoids by H_2O_2. Although small amounts of terpenyl alcohols could be degraded, the modified essential oil without eugenol was not significantly different from the pure nonmodified essential oil as observed by gas chromatography. Both samples could not either be discriminated by nontrained panelists, but only by professional perfumers. These results demonstrate the efficiency of an enzyme-based approach to selectively treat complex mixtures and could be extended to more challenging targets for the design of sustainable processes of detoxification of natural extracts.

■ ASSOCIATED CONTENT

Supporting Information

Strategy of the sequential use of HRP, H_2O_2, and catalase for the treatment of rose essential oil, gas chromatograms, comparison of the composition of the essential oils, and NMR spectra. This material is available free of charge via the Internet at http://pubs.acs.org.

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Funding

This work was supported by the University of Nice−Sophia Antipolis, the Chemical Institute of Nice (Innovative Projects Grant), and the CNRS.

ACKNOWLEDGMENTS

We are grateful to Dr. Elise Sarrazin for her assistance during sensory evaluation, the members of the APSM research group of the LCMBA for their participation in the panel, and Carole André, perfumer at Iris Consulting (Nice, France), and Pierre Benard, perfumer at Payan Bertrand S.A. (Grasse, France), for the olfactory evaluation. Professor Alain Burger is acknowledged for a critical reading of the manuscript. Professor Jonathan S. Dordick is acknowledged for his advice during the preparation of the manuscript and the suggestion of scale-up experiments.

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