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Identification of a Stale-Beer-like Odorant in Extracts of Naturally Aged Beer

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For a long time, beer staling has been a prime concern in brewery research. Yet, to improve flavor stability, better knowledge of all chemicals involved is still needed. From our aroma extract dilution analyses (AEDA) applied to naturally aged lager beers emerged an old-beer-like odorant at $RI_{CP-SIL\ 5\ CB} = 1532$ and $RI_{FFAP} = 2809$, with a FD value close to that of *trans*-2-nonenal (the well-known cardboard off-flavor found in aged beers). Specific phenol extraction, GC cold trapping, and mass spectrometry (electron impact and chemical ionization) enabled us to identify it as 4-vinylsyringol. Although already mentioned in some fresh beers, this compound had never been highlighted as involved in the aging process of lager beers.

KEYWORDS: Beer; storage; aging; stale; flavor; GC olfactometry; 4-vinylsyringol

INTRODUCTION

Beer staling has long been a prime concern for most brewers (*1*). Through storage, flavor appears to deteriorate greatly with time at a rate depending on beer composition (pH (*2*−*6*), oxygen (*7*, *8*), antioxidants (*9*, *10*), precursor concentrations (*4*, *6*, *11*−*14*), etc.) and storage conditions (packaging (*15*), temperature (*16*), light (*17*), etc.). Improvement of beer stability requires better knowledge of all chemicals involved.

In the past decade, many papers have stressed the importance of a few aged-beer off-flavors. Attention has especially been paid to *trans*-2-nonenal, which is released from protein adducts by acidic hydrolysis through aging (*13*, *18*). GC olfactometry (*4*) and sensorial analysis (*5*) have confirmed the key role of this cardboard flavoring in most beer brands. In some beers, however, other defects may be more pronounced, such as the typical onion odor of dimethyltrisulfide or the well-known "light-skunky" off-flavor of 3-methyl-2-butene-1-thiol. The former derives from 3-(methylthio)propionaldehyde and 3-(methylthio)propanol oxidation (*12*), while the latter comes mainly from hop isohumulone degradation (*19*). More rarely, β-damascenone (*4*, *11*, *20*), 3-(methylthio)propionaldehyde (*12*, *21*, *22*), and 2-furfuryl ethyl ether (*6*, *16*, *23*) can be responsible for consumer disappointment.

Controlling levels of all these compounds can sometimes be very hard, as some treatments that inhibit one off-flavor very effectively (e.g., sulfites mask *trans*-2-nonenal) may enhance other defects (e.g., dimethyltrisulfide increases significantly in the presence of sulfites (*9*)). Recently, Gijs et al. (2002) mentioned the presence of a strong "dentist-smoked-old beer" off-flavor at $RI_{CP-SIL\ 5\ CB} = 1532$ in a dichloromethane extract derived from a lager beer subjected to accelerated aging (5 days at 40 °C). It proved to be as strong as *trans*-2-nonenal. Lermusieau et al. (2001) have also mentioned its presence in a fresh beer hopped with Saaz pellets, but he did not detect it in unhopped beer (*24*).

The aim of the present work is to identify this old-beer-like odorant. GC olfactometry was applied to extracts of three lager beers stored for 3 and 6 months at 20 °C (more natural conditions than in the previous published experiments). Identification trials were further conducted on an overaged beer extract (10 days at 40 °C).

MATERIALS AND METHODS

Chemicals. Ethyl butanoate (ethyl butyrate, 99%) and 1-phenyl-2-ethanol (β-phenylethanol, 98%) were obtained from Janssen Chimica (Geel, Belgium). Dimethyltrisulfide (98%) was purchased from Acros Organics (Geel, Belgium). 8*E*-megastigma-3,5,8-trien-7-one (β-damascenone, 95%) was a kind gift from Haarman and Reimer GmbH (Nanterre, France). 3-Methyl-1-butyl acetate (isoamyl acetate, 99.7%), 2-methyl-3-furanthiol (85%), 3-(methylthio)propionaldehyde (methional, 99%), 4,5-dimethyl-3-hydroxy-2,5-dihydrofuran-2-one (sotolon, 97%), (*E*)-2-nonenal (*trans*-2-nonenal, 97%), *o*-aminoacetophenone (2′-aminoacetophenone, 98%), 2-methoxy-4-vinylphenol (4-vinylguaiacol, 98%), diethyl ether (99.9%), dodecane (external standard, 99.9%), eugenol (internal standard, 99.9%), and 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid, 99%) were purchased from Sigma-Aldrich (Bornem, Belgium). The 3-methyl-2-butene-1-thiol (isopentenylmercaptan) was obtained by chemical synthesis in our laboratory. Sodium sulfate (>99%), sodium chloride (99.5%), chloroform (>99%), and potassium hydroxide (85%) were purchased from Merck (Darmstadt, Germany). Hydrochloric acid 37% was obtained from Fisher Scientific (Leicestershire, UK). Methanol (99.9%) and dichloromethane (99.9%, redistilled twice prior use) were obtained from Romil (Cambridge, UK). Amberlite XAD-2 resin (Supelco, Bellefonte, PA) with a pore size of 9 nm and a specific area of 330 m^2/g were sequentially washed with methanol and diethyl ether (each for 4 h) in a Soxhlet and stored in methanol at 4 °C. Milli-Q water was used (Millipore, Bedford, MA).

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Beer Treatments, Extraction, and Isolation Procedures. Belgian commercial fresh lager beers (pH = 4.3−4.5) were aged in a dark room for 5 days at 40 °C (accelerated aging) or for 10 days at 40 °C (forced accelerated aging), and for 3 or 6 months at 20 °C (natural aging). Flavor XAD-2 and phenol liquid−liquid extractions (explained below) were carried out under red light. The extracts obtained before concentration to 0.5 mL (concentration factor = 100) were in all cases dried with anhydrous sodium sulfate, and 20 ppm dodecane was added (external standard). The final extracts were stored at −81 °C and analyzed by GC-FID, GC-MS, and GC-O.

Flavor XAD-2 Extraction to Obtain Fresh and Aged Beer Representative Extracts. Amberlite XAD-2 resin (2 g) was thoroughly rinsed with Milli-Q water (100 mL) and poured into a 100-mL Schott flask (Vel, Leuven, Belgium) containing 50 mL of beer. This mixture was shaken on a platform shaker at 200 rpm for 2 h at 20 °C. The content of the flask was then transferred to a liquid chromatography glass column (60 cm × 1 cm i.d.) ending with a coarse glass ball. The lower part was also filled with glass beads (3 g with a diameter of 3.5−4.5 mm and 1 g with a diameter of 0.8−1.2 mm) in order to retain the resin. The column was first rinsed with 4 × 25 mL of Milli-Q water in order to eliminate sugars and other water-soluble substances. Apolar aroma compounds were then eluted with 2 × 20 mL of diethyl ether at a flow rate of 0.75 mL/min.

Specific Liquid−Liquid Extraction for Phenols. Beer (50 mL), eugenol (internal standard, 5 ppm), 1 mL of 37% (v/v) hydrochloric acid, and 6.45 g of sodium chloride (to increase salting out) were mixed. After complete dissolution, 150 mL of chloroform/methanol (3:1, v/v) was added and the mixture stirred for 10 min at 1500 rpm. The lower organic solvent layer was retained, while the aqueous phase was extracted a second time in the same manner. The 300-mL organic phase was then shaken with 50 mL of 10% potassium hydroxide solution for 10 min at 1500 rpm. The upper aqueous phase (pH 13) was recovered, and the lower organic phase extracted a second time as described above. The pH of the aqueous phase was then adjusted to 9.0 with hydrochloric acid and extracted two times with 25 mL of dichloromethane after stirring for 10 min at 1500 rpm. The combined organic phases were further analyzed.

Cold Trapping. To isolate part of the GC effluent, the CP-SIL 5 CB GC column was connected to a T-junction, splitting the effluent between the cold trap and a throw gap (ratio 1:1). The trap consisted of an uncoated inactivated tube (51 cm × 0.53 mm i.d.) wrapped in a copper sleeve with two coiled ends immersed in Dewar vessels (Leuven, Belgium). The GC side was cooled with ice, while the other side was filled with liquid nitrogen so as to generate a temperature gradient inside the tube. After each 2-μL injection (30 injections required), an electronic switch allowed collection of the unknown compound between minutes 77 and 81.

Gas Chromatography Analyses Hyphenated with FID (GC-FID) or Olfactometric Detection (GC-O). Beer extract (1 μL) was analyzed on a Chrompack CP9001 gas chromatograph equipped with a splitless injector maintained at 250 °C; the split vent was opened 0.5 min postinjection. The carrier gas was nitrogen at a flow rate of 1 mL/min. Compounds were analyzed by using a wall-coated open tubular (WCOT) apolar CP-Sil 5 CB capillary column (50 m × 0.32 mm i.d., 1.2 μm film thickness) and/or a polar FFAP CB capillary column (WCOT, 25 m × 0.32 mm i.d., 0.3 μm film thickness). In each case, the oven temperature was programmed to rise from 36 to 85 °C at 20 °C/min, then to 145 °C at 1 °C/min, and finally to 250 °C at 3 °C/min. This temperature was further maintained for 30 min. To assess the olfactory potential of the extract, the column was connected to a GC-O port maintained at 250 °C. The effluent was diluted with a large volume of air (20 mL/min) prehumidified with an aqueous copper(II) sulfate solution. The original extracts were sniffed by three panelists in order to avoid overlooking odor-active areas. Complete AEDA was performed on each beer extract by two trained panelists. The extracts were diluted stepwise with diethyl ether (1 + 2 by volume). To control the system, the column was directly connected to an FID detector (250 °C) equipped with a Shimadzu CR6-A integrator.

Gas Chromatography Hyphenated with Mass Spectrometry (GC-MS). Electronic impact (EI) mass spectra were recorded at 70 eV (full scan with a mass range from 40 to 380 *m*/*z*) on a

Table 1. Compounds in Lager Beers I, II, and III with FD Values > 27 either in Fresh or in Aged (3 or 6 Months at 20 °C) Beer Extracts

		FD[b] of beer I			FD[b] of beer II			FD[b] of beer III			
			natural aging (months)			natural aging (months)			natural aging (months)		
RI[a]	individual odors	fresh	3	6	fresh	3	6	fresh	3	6	compound
774	fruity	9	27	27	3	9	9	1	27	81	ethyl butanoate (ethyl butyrate)[d]
807	hop	2187	2187	2187	2187	2187	2187	2187	729	2187	3-methyl-2-butene-1-thiol (isopentenylmercaptan)[e]
843	sweet, candy	27	27	27	27	27	27	27	27	27	3-methyl-1-butyl acetate (isoamyl acetate)[d]
846	nutty	729	2187	243	9	9	9	3	243	81	2-methyl-3-furanthiol[e]
863	potato	27	27	27	9	9	9	9	27	81	3-(methylthio)propionaldehyde (methional)[c]
957	rotting onion	81	243	729	27	81	243	27	2187	2187	dimethyltrisulfide[f]
1058	curry, maple syrup	27	27	243	9	1	3	-	243	81	4,5-dimethyl-3-hydroxy-2,5-dihydrofuran-2-one (sotolon)[f]
1079	floral	243	243	729	243	243	243	243	729	729	1-phenyl-2-ethanol (β-phenylethanol)[c]
1147	cardboard	27	81	243	27	81	81	81	243	729	(*E*)-2-nonenal (*trans*-2-nonenal)[c]
1262	grape	81	243	81	27	81	81	81	27	27	ortho-aminoacetophenone (2′-aminoacetophenone)[d]
1289	smoky	81	81	81	27	81	81	243	243	243	2-methoxy-4-vinylphenol (4-vinylguaiacol)[c]
1369	red fruits	27	81	81	27	81	81	81	2187	729	8*E*-megastigma-3,5,8-trien-7-one (β-damascenone)[c]
1532	dentist, smoky, tobacco, old beer	27	243	243	27	81	81	27	81	81	unknown
			243[g]			243[g]			2187[g]		

[a] RI, retention index on CP-SIL 5 CB. [b] FD = dilution factor (AEDA) = 3^{n-1} with *n*, the number of dilutions required for no odor to be perceived. [c] Identification by 4 methods: comparison of mass spectra, odors at the sniffing port, and the retention indexes on two columns (CP-SIL 5 CB and FFAP) with those of commercial products. [d] Identification by 3 methods: comparison of mass spectra, odors at the sniffing port, and the retention indexes on CP-SIL 5 CB column with those of commercial products. [e] Compounds tentativaly identified by 3 methods: comparison of the odors at the sniffing port and the retention indexes on two columns (CP-SIL 5 CB and FFAP) with those of pure compounds. [f] Compounds tentativaly identified by 2 methods: comparison of the odor at the sniffing port and the retention indexes on CP-SIL 5 CB column with those of the commercial products. [g] FD after an accelerated aging of 5 days at 40 °C.

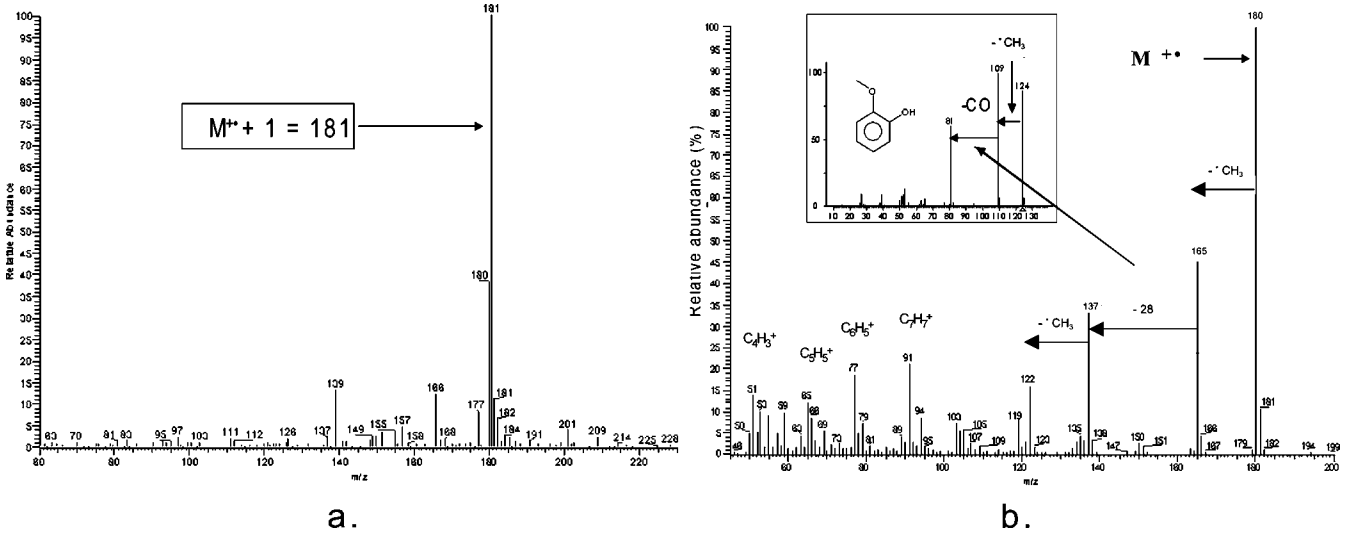
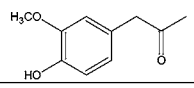
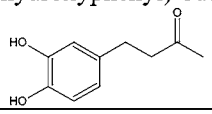
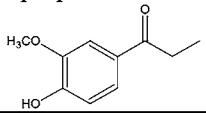


Figure 1. Mass spectrum of the unknown (XAD-2 beer I extract after cold trapping) obtained by GC-MS chemical ionization mode (**a**) and by GC-MS electronic impact mode (**b**).

Table 2. Characteristics of Three Suspected Compounds: Molecular Weight (M_W), RI on the Apolar Column, Fragmentation Pattern in MS Electronic Impact Mode (EI), and Odors at the Sniffing Port

Suspected compounds structure	*Mw*	*RI* [a]	*MS-EI (%)*	*Odors*
guaiacylacetone 	180	1488	137 [c](100), 180 [b](43), 122(10), 93 (6)	vegetable and clove
4-(3,4-dihydroxyphenyl)-butan-2-one 	180	1749	180 [b](100), 123(70), 137 [c](28), 91(17)	vegetable
propiovanillone 	180	1521	151(100), 180 [b](23), 123 [d](22), 52(13)	-

[a] RI, retention index on CP-SIL 5 CB column. [b] m/z = 180. [c] 137 = 180 − [CH_3–C=0]. [d] 123 = 180 − [CH_3–CH_2–C=0].

ThermoFinnigan Trace MS simple quadrupole mass spectrometer connected to a ThermoFinnigan Trace GC 2000 gas chromatograph equipped with a splitless injector. Chemical ionization (CI) mass spectra were recorded in the positive mode with a CH_4−N_2O (75:25, v/v) gas mixture on a TSQ 7000 Finnigan instrument. In both cases, the carrier gas was helium at a flow rate of 1 mL/min. Spectral recording was automatic throughout separation (Xcalibur software was used). Identification attempts were done by comparison with the NIST database or the odor at the sniffing port and the retention index on one or two columns (CP-SIL 5 CB and/or FFAP) of commercial (or synthesized) products.

RESULTS AND DISCUSSION

Key Flavors in Naturally Aged Beers. To determine potential key flavors responsible for beer staling, three Belgian commercial lager beers (I, II, and III; see **Table 1**) were subjected to natural storage for 3 or 6 months at 20 °C. Prior to GC-O analysis, the beers were extracted with Amberlite XAD-2 resin. The very representative samples (*24*) obtained in this manner were further analyzed by aroma extract dilution analysis (AEDA), a method initially used by Ullrich and Grosch (*25*). As previously proposed by Lermusieau et al. (2001), all FD values were compared to that obtained for 3-methyl-1-butyl acetate (FD = 27 in all our samples). This ester usually occurs in lager beers at a concentration close to its aroma threshold value (threshold = 1.60 ppm (*26*); concentration in beer I = 1.90 ppm). Therefore, FD values above 27 most probably indicate organoleptically active compounds in beer (**Table 1**).

Already before aging, 3-methyl-2-butene-1-thiol (FD = 2187) was perceived at dilutions much higher than 3-methyl-1-butyl acetate. At this level, 3-methyl-2-butene-1-thiol, issued from hops (*24*), seems to impart a pleasant hoppy flavor, while at higher concentrations, it is known to be responsible for the lightstruck off-flavor of lager beers (*27, 28*).

As expected (*4*), not only *trans*-2-nonenal (cardboard off-flavor, FD = 81−729) but also dimethyltrisulfide (onion flavor, FD = 81−2187), and β-damascenone (red fruit odor, FD = 81−2187) emerged as relevant to the sensory profile of the naturally aged beers (3 or 6 months at 20 °C). On the other hand, 3-(methylthio)propionaldehyde was found to be released only in beer III. The presence of sotolon in aged beers I and III, with an FD up to 243 after a few months at 20 °C, has also to be mentioned (already identified in a fresh beer **(***29***)**).

The unknown at $RI_{CP-SIL\ 5\ CB}$ = 1532 proved to be released through natural aging in our three beers (FD = 81−243 vs 27

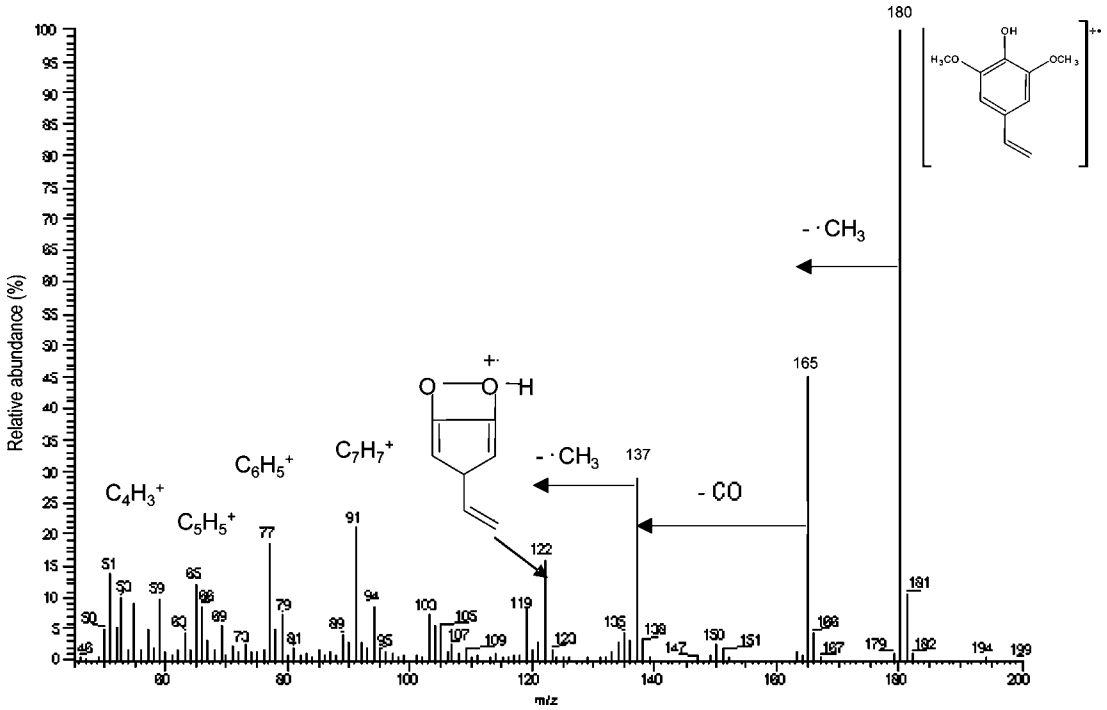


Figure 2. Proposed fragmentation pattern of 4-vinylsyringol synthesized by decarboxylation of the corresponding acid. Mass spectrum obtained by GC-MS electronic impact mode.

in fresh samples). Yet in samples subjected to accelerated aging, an FD between 243 and 2187 characterized this compound, suggesting a key role of temperature in its release. Its old beer-smoky-tobacco descriptors reinforced our desire to identify it.

Identification of the Unknown at $RI_{CP-SIL\ 5\ CB} = 1532$. To increase artificially the level of the unknown in an XAD-2 beer extract, we first applied accelerated aging for 10 days at 40 °C. The compound was further concentrated by means of the GC method proposed by Gallois (*30*). From RI = 1509 to RI = 1563, the eluent was diverted to a cold trap. Diethyl ether elution of this cold trap after thirty gas chromatographic injections enabled us to recover an extract with a very strong "phenolic-tobacco-old beer" odor. A retention index of 2809 was determined for this compound on the polar FFAP column. The unknown was recovered by applying a specific extraction procedure for phenolic compounds, indicating that its pK_a value is in the 9−13 range. The suspected phenolic structure was further confirmed by GC-MS analyses on the cold-trapped extract. By chemical ionization with methane−N_2O (75:25, v/v), a pseudomolecular ion of 181 ($M_w = 180$) was obtained (**Figure 1a**). As depicted in **Figure 1b**, electronic impact ionization led to the following fragmentation (unfortunately not listed in the NIST MS library): 180 (100), 165 (48), 137 (35), 91 (24), 77 (22). The stable molecular ion ($m/z = 180$) was consistent with an aromatic ring (**Figure 1b**). The highly favorable loss of 43 units ($m/z = 137$) indicated release of the CO and methyl moieties, as observed in many other phenols with an $m/z = 180$ (see **Table 2**). Yet our unknown was also characterized by the loss of a methyl radical ($m/z = 165$), as is guaiacol (**Figure 1b**), but not the phenolic methyl ketones depicted in **Table 2**. All of these data, plus the literature on tobacco, led us to suspect 3,5-dimethoxy-4-hydroxystyrene (*4*-vinylsyringol) (*31*). Decarboxylation of commercial 3,5-dimethoxy-4-hydroxycinnamic acid (sinapic acid) enabled us to confirm this identification (*32*). This was achieved by heating the powder in a glass tube with a burner until no bubble was released. The compound's mass fragmentation pattern is tentatively explained in **Figure 2**. Although already mentioned in some fresh beers (*33*−*35*), nobody has previously reported that 4-vinylsyringol may be released through the aging of lager beers.

In other food matrixes, two precursors of this compound releasing it by enzymatic decarboxylation or by hydrolysis were identified respectively as the sinapic acid (*36*) and the glycoside form (*37*). Therefore, in fresh beer, 4-vinylsyringol could arise from the decarboxylation of malt and hop sinapic acid. Its release through aging is probably due to acid hydrolyzation from the glycoside form, such as previously demonstrated for β-damascenone (*11*). Further investigations are now needed to sensorially characterize the impact of 4-vinylsyringol in aged beer, to isolate the suspected precursors in fresh beer, and to assess the influence of pH on them.

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