

Modulation of the Phytoestrogenicity of Beer by Monoterpene Alcohols Present in Various Hop Oil Fractions

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ABSTRACT

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The estrogenicity of beer, due to prenylated flavonoids, mainly 8-prenylnaringenin, can be modulated under suitable conditions, whereby monoterpene alcohols present in hop oils act as precursors for prenylation of flavonoids. Four hop oil fractions, 'citrusy', 'estery', 'floral', and 'spicy', respectively, were investigated as potential sources. The main constituents were separated and identified by gas chromatography – mass spectroscopy (GC-MS). They were related to 8-prenylnaringenin via their MS features, while the ability to deliver a prenyl residue was evaluated by selected ion monitoring of fragment ions m/z 69 and m/z 41. In particular, the citrusy hop oil fraction proved to be a rich source of precursors for prenyl residues and linalool, the main constituent in the citrusy and spicy hop oil fractions, was shown to be most prominent. The process was highlighted by an increase in the content of 8-prenylnaringenin on forced ageing of acidified beer spiked with naringenin and linalool.

Key words: Beer, GC-MS analysis, hop oil, monoterpene alcohols, phytoestrogenicity.

INTRODUCTION

Revealing various hop oil features and reactivities of important constituents presents an intriguing research topic related to beer quality. Analysis and profiling of hop oils give clues to varietal characterisation^{3,4,12} and the composition of hop oils has been targeted in a multitude of studies in order to better understand hop aroma and hop flavour in beer^{1,11}. During recent years, much attention has been paid to bioactivities of hop oils, mainly with respect to sedative and soporific properties^{6,21}, antibacterial actions^{2,13,15}, and inhibition of bone resorption¹⁸. As the main constituents of hop oils are monoterpenoids (composed of two prenyl or 3-methylbut-2-enyl units) and

sesquiterpenoids (three prenyl units), interferences must exist with the biosynthesis of hop compounds containing prenyl groups including humulones, Fig. 1-1, and lupulones²⁰ Fig. 1-2, as well as prenylated flavonoids, since they all are present in the lupulin glands of the female hop inflorescences. These compounds exhibit varying bioactivities including inhibition of the growth of breast cancer (MCF-7) cells, retardation of the cytochrome P450-mediated activation of procarcinogens, induction of the carcinogen-detoxifying enzyme, quinone reductase, and antioxidant actions^{10,17}.

Recently, we isolated and identified a very potent phytoestrogen from hops, 8-prenylnaringenin or 8-(3-methylbut-2-enyl)-5,7,4'-trihydroxyflavanone⁸ Fig. 1-4. The accompanying prenylated flavanones, isoxanthohumol Fig. 1-5, and 6-prenylnaringenin Fig. 1-6, are much less active⁹. The total content of prenylflavonoids in beer may add up to 1 mg/L. Additionally, instead of prenyl groups, geranyl (two prenyl units) chains may be introduced, but, as the estrogenicity of geranylated flavonoids is much weaker than that of prenylated flavonoids, these compounds are of minor importance⁹. Prenyl and geranyl groups are biosynthetically added in an activated form (e.g., as pyrophosphates) to aromatic rings in a Friedel-Crafts-type electrophilic substitution, which is directed and activated by the presence of phenolic groups. The building blocks, γ,γ -dimethylallyl pyrophosphate and the isomeric 2-isopentenyl pyrophosphate, may arise from either the mevalonate pathway or the deoxyxylulose pathway⁵, since they are both operative in plants. Prenyltransferases are involved, but prenylations at different positions of an aromatic structure catalysed by different enzymes have been described²². The fate of prenylation seems rather unclear and Zuurbier *et al.* proclaimed that "other prenylations of aromatic structures should occur as well"²². However, diverse mechanisms have not been detailed until now and alternative pathways remain unexplored. Thus, terpenoids could potentially deliver prenyl residues and non-enzymatic incorporation into flavonoids should be feasible, e.g., during beer storage. As a result, the phytoestrogenicity of beer could be modulated and we wish to describe investigations that confirm this premise. In this study, in addition to manipulated beers, various hop oil fractions were used to identify potential precursors or delivering agents of prenyl moieties based on monitoring of selected fragment ions in gas chromatography – mass spectroscopy (GC-MS).

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MATERIALS AND METHODS

Materials

Pure hop oil fractions were a gift of English Hop Products Ltd, Paddock Wood, Tonbridge, Kent TN12 6DQ, UK (kindly offered by Prof. Dr. Guido Aerts, Dr. Luc De Cooman, and ing. Koen Goiris of the Laboratory of Enzyme and Brewing Technology at the KaHo St.-Lieven, Ghent, Belgium). In common practice, hops are extracted using liquid or supercritical carbon dioxide, and the extract contains the essential oil in addition to humulones and lupulones. By adjusting conditions during extraction (liquid carbon dioxide at 14°C) an oil-rich extract is obtained, from which hop oil is molecularly distilled under high vacuum and at low temperature. Separation of the complex hop oil by column chromatography on alumina or silica affords four chemically and organoleptically distinct fractions, which are designated as 'citrusy', 'estery', 'floral', and 'spicy', respectively. The oil fractions are

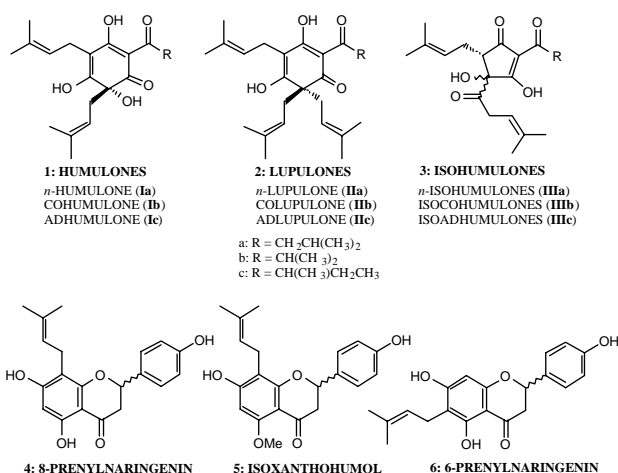


FIG. 1. Structures of humulones, lupulones, isohumulones, and prenylated flavanones.

TABLE I. Identification and retention time of main constituents in a citrusy hop fraction.

Peak number ¹	Compound	Retention time (min)
1	3-Methylbutan-1-ol	5.14
2	2-Methylbutan-3-ol	5.52
3	Hexan-1-ol	6.94
4	Heptan-1-ol	9.18
5	Octan-1-ol	11.93
6	Nonan-2-one	12.63
7	Linalool	12.86
8	Decan-2-one	15.87
9	Citronellol	16.92
10	Nerol	17.19
11	Geraniol	18.37
12	Undecan-6-one	18.47
13	Oct-1-en-3-yl acetate	19.22
14	Undecan-2-one	20.07
15	Dodecan-2-one	24.88
16	α -Humulene	27.66
17	Tridecan-2-one	30.13
18	Tetradec-2-yl acetate	30.69
19	Humulenol-2	37.21

¹ For chromatographic conditions, see Materials and Methods.

known as late hop essences, because the intent is to add these fractions at a late stage in the brewing process, i.e. during maturation or prior to packaging.

8-Prenylnaringenin was isolated from hops according to a procedure described in the literature¹⁴ and naringenin was supplied by Extrasynthèse, Genay, France. A commercial beer containing 8-prenylnaringenin (9.0 $\mu\text{g/L}$) was acidified to pH 2 and divided in two equal parts. One beer served as the control, while the other was spiked with linalool and naringenin (450 $\mu\text{g/L}$). The beers were kept at 60°C for three days and, subsequently, analyzed for the

TABLE II. Identification and retention time of main constituents in an estery hop fraction.

Peak number ¹	Compound	Retention time (min)
1	Methyl heptanoate	10.55
2	Methyl 7-methylheptanoate	12.31
3	Methyl octanoate	13.43
4	Methyl 7-methyloctanoate	15.60
5	Methyl 6-methyloctanoate	15.79
6	Methyl non-4-enoate	16.48
7	Methyl nonanoate	16.94
8	Methyl 3,7-dimethylocta-3,6-dienoate	18.65
9	Methyl 8-methylnonanoate	19.63
10	Methyl dec-4-enoate	20.73
11	Methyl deca-4,8-dienoate	21.01
12	Methyl geranate	21.31
13	Methyl decanoate	21.53

¹ For chromatographic conditions, see Materials and Methods.

TABLE III. Identification and retention time of main constituents in a floral hop fraction.

Peak number ¹	Compound	Retention time (min)
1	Methyl nonanoate	16.96
2	9-Methyldec-2-one	18.30
3	Dec-9-en-2-ol	19.15
4	Undecan-2-one	19.99
5	Methyl dec-4-enoate	20.65
6	Methyl deca-4,8-dienoate	20.90
7	Methyl geranate	21.32
8	Dodecan-2-one	24.72
9	α -Humulene	27.59

¹ For chromatographic conditions, see Materials and Methods.

TABLE IV. Identification and retention time of main constituents in a spicy hop fraction.

Peak number ¹	Compound	Retention time (min)
1	Linalool	12.82
2	Borneol	14.59
3	α -Terpineol	14.89
4	Decan-2-ol	15.78
5	Nerol	17.21
6	Geraniol	18.31
7	9-Methyldec-2-one	18.74
8	Undec-10-en-2-ol	19.42
9	Undecan-2-one	20.02
10	Undecan-2-ol	20.36
11	Caryolan-1-ol	33.72
12	Humulol	35.48
13	Humulenol-2	37.16
14	8-Methylundecan-2-ol	37.99
15	Caryophyllene oxide	38.28
16	Caryophyllenol	43.56

¹ For chromatographic conditions, see Materials and Methods.

content of 8-prenylnaringenin by high performance liquid chromatography (HPLC) as detailed previously¹⁴.

GC-MS

GC-MS analyses were carried out on a GCQ Finnigan quadrupole MS detector (Finnigan, San Jose, CA, USA), connected to a Finnigan GCQ gas chromatograph, which was equipped with an HP-5 MS (crosslinked 5% phenyl

methyl siloxane) fused silica capillary column (30 m x 0.25 mm i.d., film thickness 0.25 μm). Injections (1 μL) were carried out in the splitless mode at 220°C. The carrier gas was high-purity helium (Air Liquide, Liège, Belgium) at a flow rate of 1 mL/min. The oven temperature was programmed from 40°C to 100°C at 5°C/min, from 100°C to 200°C at 2°C/min, from 200°C to 260°C at 5°C/min, the final temperature being held for 5 min. The

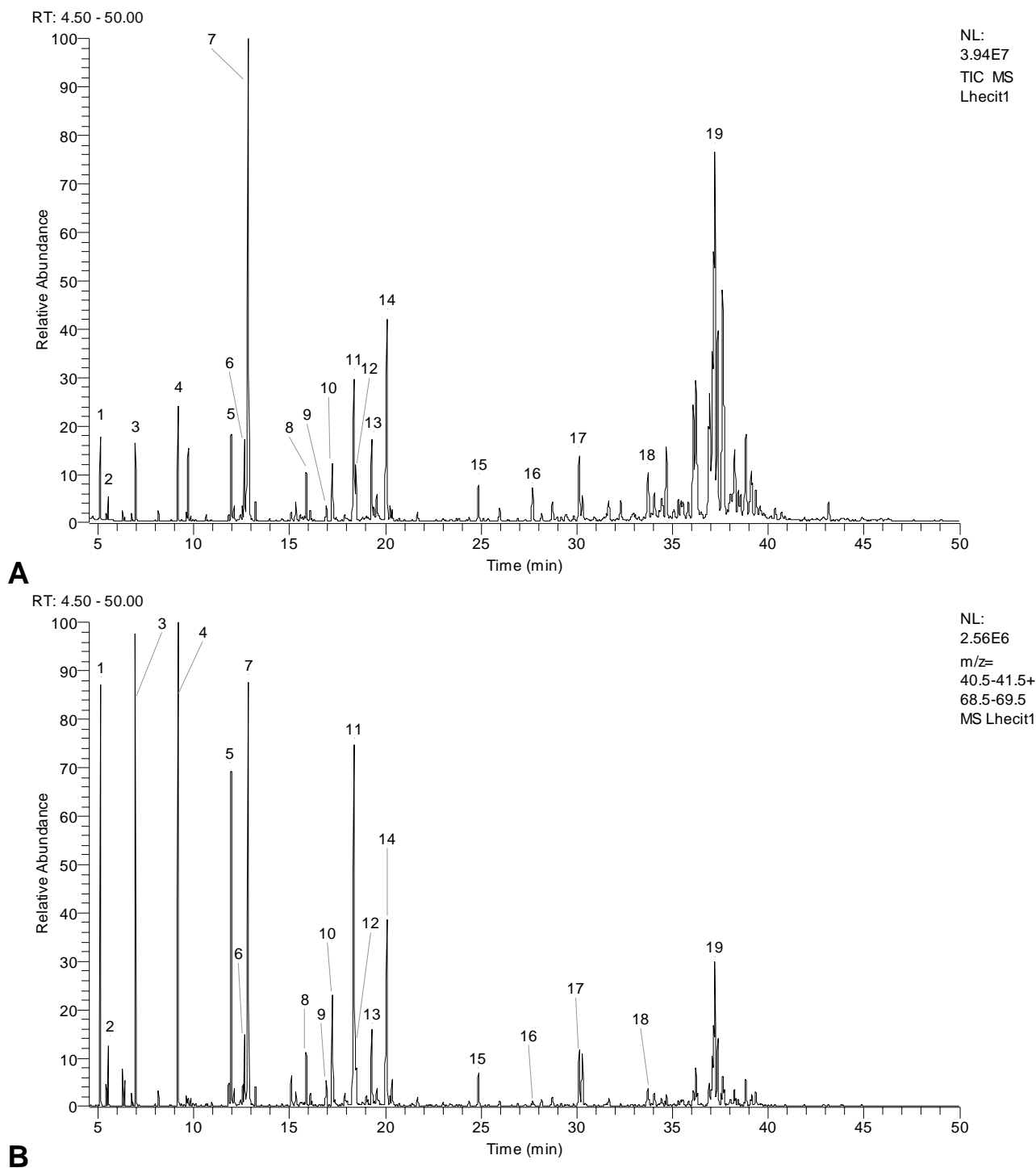


FIG. 2. Total ion chromatogram (A) and selected ion monitoring (SIM) (B) of the sum of ions m/z 41 and m/z 69 of a citrusy hop fraction. (For chromatographic conditions, see Materials and Methods.)

temperature of the transfer line was 260°C, the temperature of the filament (ion source) was 225°C and the electron voltage (electron impact mode) was 70 eV.

MS

The mass spectrum of 8-prenylnaringenin was obtained using a HP 59980A LC/MS interface optimized for a liquid inlet flow (methanol) of 0.4 mL/min and a helium nebulization gas flow of 1.5 L/min. The temperature of the solvation chamber was 40°C. A mass analyzer 5988A GC/LC MS system was used at an ionization potential of 70 eV.

RESULTS AND DISCUSSION

When the commercial beer was subjected to forced ageing, after adjusting the pH to 2, the content of 8-prenylnaringenin (9.0 µg/L) increased slightly, although not significantly. It was reasoned that, perhaps, the amounts of efficient prenyl-delivering agents (e.g., monoterpene alcohols) and of flavonoid substrates (e.g., naringenin) were insufficient to provide clear evidence for enhancement of the estrogenic activity of the beer. Attention was therefore turned to specific hop oil fractions ('citrusy', 'estery', 'floral', and 'spicy', respectively) in

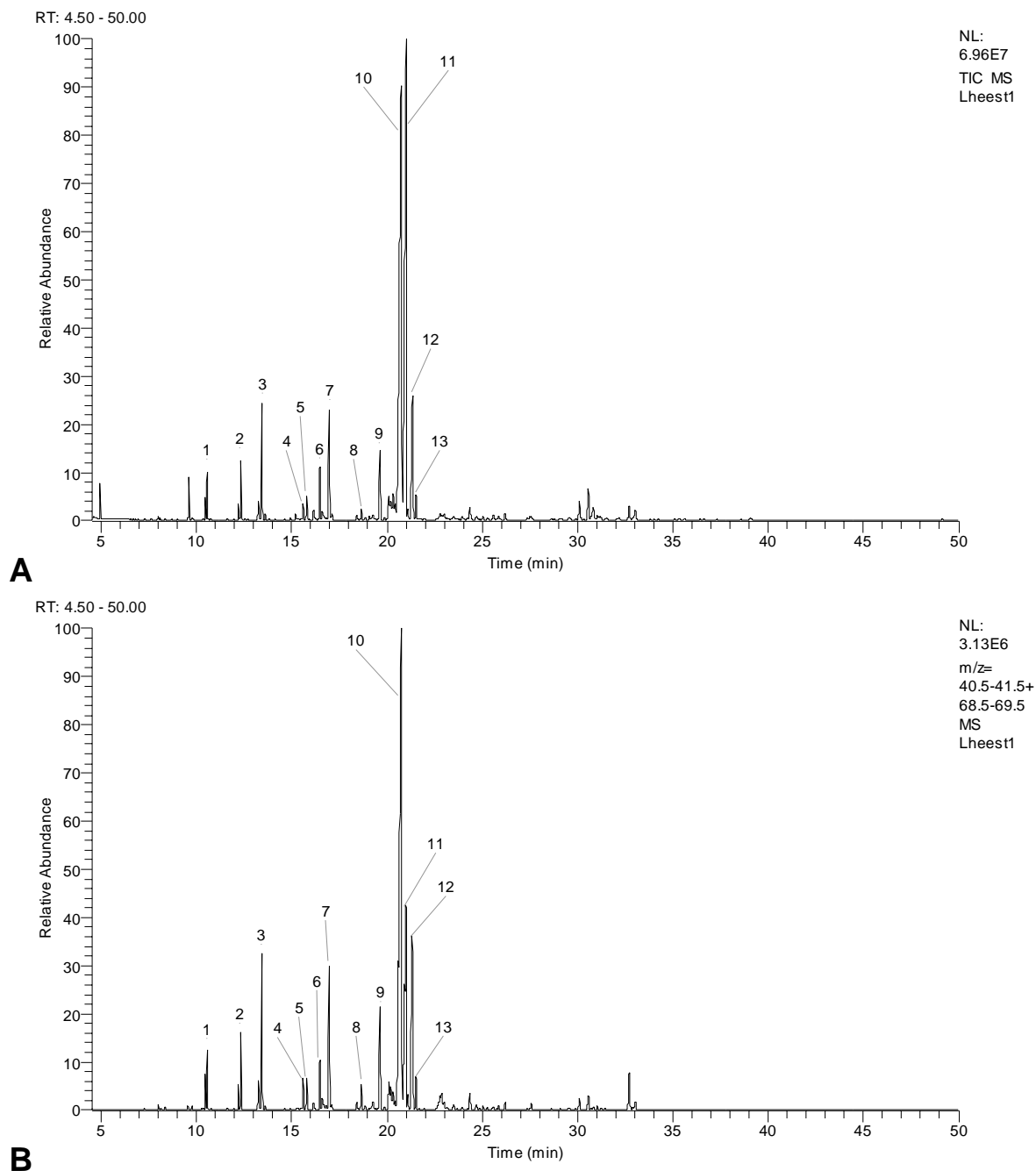


FIG. 3. Total ion chromatogram (A) and selected ion monitoring (SIM) (B) of the sum of ions m/z 41 and m/z 69 of an estery hop fraction. (For chromatographic conditions, see Materials and Methods.)

order to identify the capabilities of various hop oil constituents as precursors of prenyl fragments.

Analyses of these four hop oil fractions were done by GC-MS and the total ion chromatograms are shown in Figs. 2(A)-5(A). As full identification was not the purpose of the work, the identity of selected major components is given in Tables I-IV. Identification was achieved by comparison of the individual mass spectra with those stored in the NIST library with reference to retention indices and pure standards⁷. The citrusy fraction is composed mainly of alcohols, linalool being the major constituent, and ketones with undecan-2-one as a prevalent compound, while sesquiterpenes are also present in significant

amounts. With the exception of a few very minor peaks, the estery fraction comprises only methyl esters. Methyl dec-4-enoate and methyl deca-4,8-dienoate are predominant. The floral fraction is quite complex as esters, ketones, alcohols, ethers, as well as terpene hydrocarbons are present. Undecan-4-one is constitutively the most important compound. While linalool and undecan-2-ol are major constituents in the spicy fraction, the most prominent organoleptic features are derived from oxygenated sesquiterpenes¹⁹.

Attempts were made to correlate particular constituents of the hop oil fractions with the potent hop estrogen, 8-prenylnaringenin, by means of specific MS features. In

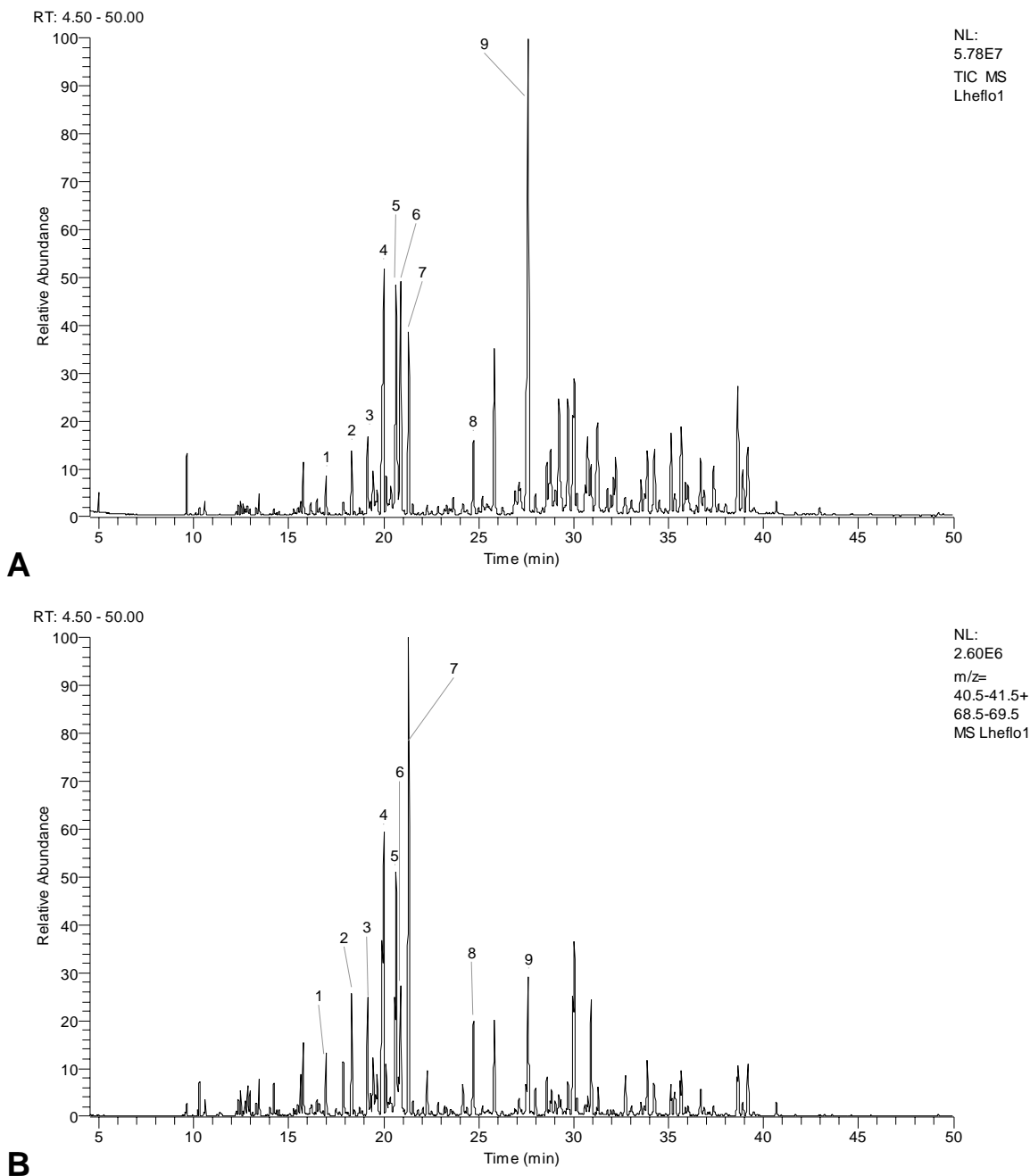


FIG. 4. Total ion chromatogram (A) and selected ion monitoring (SIM) (B) of the sum of ions m/z 41 and m/z 69 of a floral hop fraction. (For chromatographic conditions, see Materials and Methods.)

fact, fragment ions that are characteristic for the prenyl group in the EI spectrum of 8-prenylnaringenin, shown in Fig. 6, are a C_5H_9 -fragment (prenyl) at m/z 69 and a C_3H_5 -fragment at m/z 41. Appropriate correlation was made by selected ion monitoring (SIM) of the sum of these fragments in the mass spectra of compounds present in the hop oil fractions. Detection of these fragments would indicate that delivery of a prenyl group is in principle feasible as cleavage of the appropriate bond is evidenced.

Monitoring the sum of the fragment ions m/z 69 and m/z 41 in the citrusy fraction shows an ion chromatogram, Fig. 2(B), which significantly differs from the

total ion chromatogram displayed in Fig. 2(A). Mainly six constituents are apparent (relative abundance > 50%), the hemiterpene 3-methylbutan-1-ol (peak number 1), the linear alcohols hexan-1-ol (peak number 3), heptan-1-ol (peak number 4), and octan-1-ol (peak number 5), and the monoterpene alcohols linalool (peak number 7) and geraniol (peak number 11). Only linalool is a major compound in the citrusy fraction. The linear alcohols cannot supply a prenyl group as, indeed, masses 69 and 41 relate to $[(CH_2)_3CH=CH_2]^+$ and $[CH_2CH=CH_2]^+$ fragments, respectively, which are cleaved off after dehydration of the parent molecules. It is remarkable that sesquiterpenes

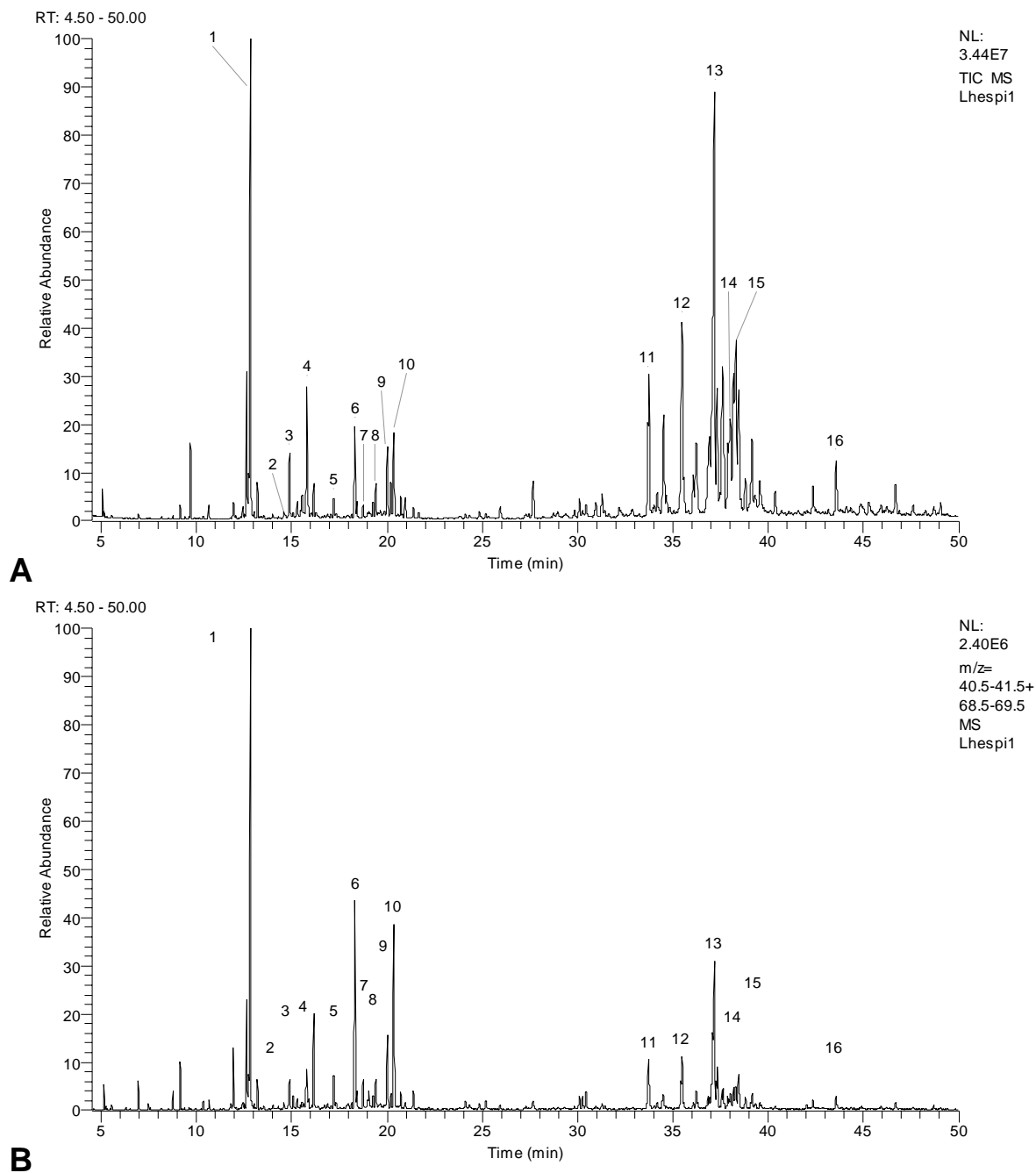


FIG. 5. Total ion chromatogram (A) and selected ion monitoring (SIM) (B) of the sum of ions m/z 41 and m/z 69 of a spicy hop fraction. (For chromatographic conditions, see Materials and Methods.)

such as humulenol-2 (peak number 19) are very weak contributors.

A predominant constituent in the estery fraction, methyl dec-4-enoate (peak number 10), also leads to intense fragment ions at m/z 69 and m/z 41, Fig. 3(B), while these features are much less evident in the doubly unsaturated methyl deca-4,8-dienoate (peak number 11). Apparently, cleavage of the ester group is sequenced by hydrogen transfer, while the presence of a double bond at C(8) does not lead to the appropriate ions. The weak prenyl-generating abilities of sesquiterpenes are confirmed on inspection of the SIM trace of the floral hop fraction, Fig. 4(B). α -Humulene (peak number 9) is the major constituent in the total ion chromatogram, Fig. 4(A), but only a minor compound in the SIM trace, reflecting the presence of masses 69 and 41. In this respect, methyl geranate (peak number 7), is, as expected, a proficient prenyl donor. Also in the spicy hop fraction Fig. 5(B), only linalool (peak number 1) is predominant thereby exceeding substantially the input of all other constituents.

Linalool, as well as all other monoterpene alcohols, may, in the presence of acidic material (e.g., humulones or isohumulones), or in acidic medium (e.g., beer), behave as an electrophile in a Friedel-Crafts-type substitution of a flavonoid (e.g., naringenin as a flavanone) by a 3-methyl-but-2-enyl (prenyl) residue and the presence of phenolic groups should activate the process leading to a prenylated flavanone such as 8-prenylnaringenin, see Fig. 7. The feasibility of the process was demonstrated by analysis of the forced-aged beer (pH 2) spiked with linalool and naringenin, as the concentration of 8-prenylnaringenin increased from 9.0 $\mu\text{g/L}$ to 12.4 $\mu\text{g/L}$. Thus, convincing

evidence is provided for the occurrence of in-situ prenylation of flavonoids, naringenin being a representative compound in connection to formation of 8-prenylnaringenin. In beer, next to 8-prenylnaringenin, other estrogenically active prenylated flavonoids (flavanones) may be formed thereby modulating the estrogenicity of beer.

CONCLUSIONS

Although the composition of hop oil fractions is complex, few constituents may deliver efficiently a prenyl fragment as evidenced by selected ion monitoring of fragment ions m/z 69 and m/z 41 in the respective mass spectra. In particular, linalool, the main constituent in the citrusy and the spicy fractions, should be considered as a prominent delivering agent - in appropriate conditions - of a prenyl group to flavonoids to form estrogenically active prenylated flavonoids (flavanones). Mainly the citrusy

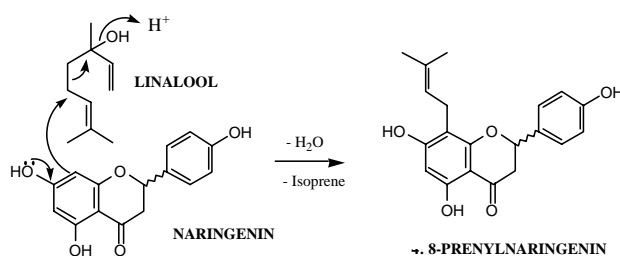


FIG. 7. Prenylation of naringenin by linalool in acidic conditions.

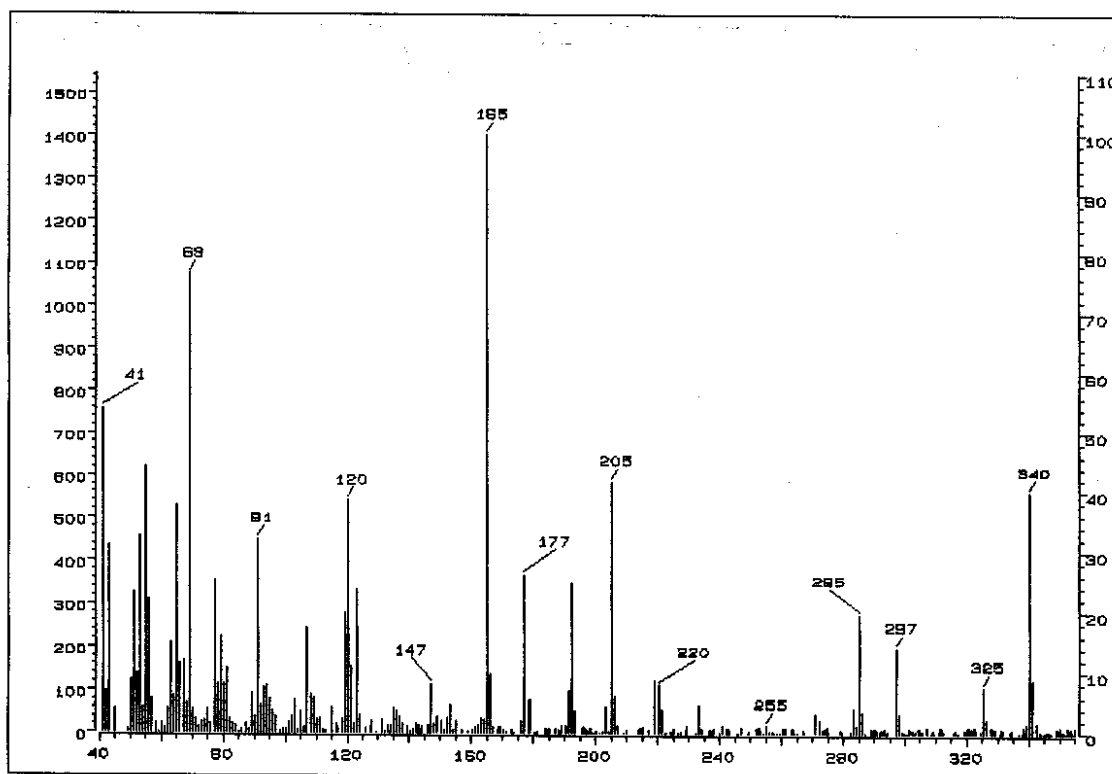


FIG. 6. Electron impact (EI) mass spectrum of 8-prenylnaringenin. (For conditions, see Materials and Methods.)

hop oil fraction could be a rich source of precursors for prenyl groups and the monoterpene alcohols citronellol, nerol, and geraniol, in addition to linalool, could modulate the estrogenicity of beer on storage.

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