Chapter 3

The Principal Flavor Components of Fenugreek (*Trigonella foenum-graecum* L.)

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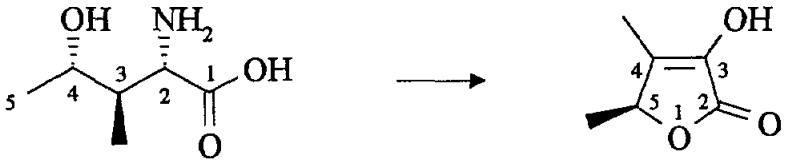
3-Hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone) was established as the character impact flavor compound of fenugreek on the basis of gas chromatography-olfactometry. Sotolone was found to occur predominantly in the (5*S*) enantiomeric form (95%) and to have a $\delta^{13}C_{PDB}$ value of -19.7‰. About 2-25 ppm sotolone were determined in fenugreek of different origins using the isotope dilution assay technique. Sotolone was generated in model systems by thermally induced oxidative deamination of 4-hydroxy-L-isoleucine (HIL) using different carbonyl compounds. Up to 24 mol% yields were obtained by boiling HIL and methylglyoxal as reactive α-dicarbonyl at pH 5 for 10 h. Strecker degradation of HIL was found to be a competitive reaction resulting in the formation of 3-hydroxy-2-methylbutanal. The lactone of HIL, 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone, was found to be a better precursor of sotolone. It generated about 36 mol% sotolone in the presence of methylglyoxal.

Fenugreek is the dried seed of *Trigonella foenum-graecum* L. (Fabaceae). The plant is an annual herb widely cultivated in Mediterranean countries and Asia (*1*). The pods contain about 10-20 yellowish seeds with an appetizing pleasing aroma. Toasted and ground fenugreek seed is an essential ingredient of curry powders and is often mixed with breadstuffs. It is used as a seasoning in food products such as pickles, chutneys, vanilla extracts, artificial maple syrup, and others.

Several volatile constituents have been reported in fenugreek (*2*), mainly terpenes and fatty acids. However, no systematic work has yet been published on compounds that contribute to the characteristic aroma of fenugreek. 3-Hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone) was suggested as an important volatile constituent of fenugreek due to its seasoning-like flavor note (*3*, *4*).

Sotolone is a powerful flavor compound found in several foods and spices (*5*). It contributes to the burnt/sweet note of aged sake (*6*), cane sugar (*7*), and coffee (*8*); to the spicy/curry note of lovage (*9*) and condiments (*10*); as well as to the nutty/sweet flavor of botrytized wines (*11*) and flor-sherry wines (*12*). The flavoring potential of sotolone is due to its low threshold values, that of 0.02 ng/l air (*8*), 0.3 μg/l water (detection/nasal, *10*), and 0.036 μg/l water (detection/retronasal, *13*).

The structural similarity between sotolone and 4-hydroxy-L-isoleucine (HIL), the most abundant free amino acid in fenugreek seeds, was pointed out (*14*, *15*). It was postulated that this unusual amino acid could be the precursor of sotolone in fenugreek (*15*) (Figure 1). This hypothesis has recently been supported by the fact that only the (5*S*) enantiomer of sotolone was found in fenugreek (*16*). This is in good agreement with the stereochemistry of HIL isolated from fenugreek, i.e. (2*S*,3*R*,4*S*) (*17*). However, these authors failed to discuss possible formation pathways.



(*2S,3R,4S*)-HIL (*5S*)-Sotolone

Figure 1. Stereochemistry of 4-hydroxy-L-isoleucine (HIL) and sotolone found in the seeds of fenugreek (*Trigonella foenum-graecum* L.).

The purpose of the present study was to identify those volatile compounds which significantly contribute to the seasoning-like note of fenugreek using the approach of sensory directed chemical analysis. Gas chromatography in combination with olfactometry and mass spectrometry have been used as key steps of this approach (*18*, *19*). The formation of flavor impact compound(s) was studied in model systems using the quantification technique Isotope Dilution Assay (*20*, *21*). The mechanistic study was based on a hypothetical pathway proposed for the formation of sotolone via thermally induced oxidative deamination of HIL (*10*).

Experimental

Materials. Commercially available fenugreek seeds of different geographical origins and fenugreek oleoresin were used. Sotolone was from Aldrich and diethyl 2-methyl-3-oxobutanedioate, L-isoleucine, methylglyoxal (40% in water), phenylglyoxal, 2,3-butanedione, and 2,3-pentanedione from Fluka. Solvents and other chemicals were of analytical grade from Merck.

Synthesis. 3-Amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone hydrochloride (ADF) and 4-hydroxy-L-isoleucine (HIL) were obtained as diastereomeric mixtures by photochemical chlorination of L-isoleucine (Figure 2a), i.e. (3*S*,4*R*,5*R*/3*S*,4*R*,5*S*) and (2*S*,3*R*,4*R*/2*S*,3*R*,4*S*), respectively (*17*, *22*, *23*). [5,6-^{13}C]-3-Hydroxy-4,5-dimethyl

2(5*H*)-furanone ([$^{13}C_2$]-sotolone) was prepared by condensation of diethyl 2-methyl-3-oxobutanedioate and [1,2-^{13}C]-acetaldehyde followed by lactonization and subsequent decarboxylation under strongly acidic conditions (Figure 2b) (*10*). The structures of the synthesized compounds were verified by elemental analysis, mass spectrometry (MS), and nuclear magnetic resonance spectroscopy measurements (^{1}H-NMR, ^{13}C-NMR) (*24*).

Gas Chromatography-Olfactometry. GC-O was performed on a Carlo Erba (Mega 2) equipped with a cold on-column injector, FID and a sniffing-port. Fused silica capillary columns of medium (DB-OV 1701) and high polarity (DB-FFAP) were used as previously described (*9*), both 30 m x 0.32 mm with a film thickness of 0.25 µm. The temperature program was: 50°C (2 min), 6°C/min to 180°C, 10°C/min to 240°C (10 min). Linear retention indices (RI) were calculated according to van den Dool and Kratz (*25*). The sensory significance of each odorant was evaluated and expressed as the flavour dilution (FD) factor (*18*).

Mass Spectrometry

Qualitative Analysis. Electron impact (EI) and positive chemical ionisation (PCI, ammonia) mass spectra were obtained on a Finnigan MAT 8430 mass spectrometer. MS-EI were generated at 70 eV and MS-CI at 150 eV with ammonia as the reagent gas. Non-volatile samples were directly introduced into the ion source held at 200 °C. Volatile components were introduced via a Hewlett-Packard HP-5890 gas chromatograph (GC-MS) using a cold on-column injection. DB-FFAP fused silica capillary columns were used (30 m x 0.25 mm, film thickness 0.25 µm). The carrier gas was helium (90 kPa). The temperature program was: 50°C (2 min), 4°C/min to 180°C, 10°C/min to 240°C (10 min).

Quantitative Analysis. Sotolone was quantified by isotope dilution assay using [$^{13}C_2$]-sotolone as internal standard (*10, 24*). Quantification experiments were performed with a HP-5971 GC-MS using the following conditions: DB-Wax capillary column (30 m x 0.25 mm, film thickness 0.25 µm); carrier gas: helium (100 kPa); splitless injection (250°C); temperature program: 20°C (0.5 min), 30°C/min to 100°C, 4°C/min to 145°C, 70°C/min to 220°C (10 min); EI ionisation at 70 eV; selected ion monitoring (SIM) of sotolone (m/z 128) and the labeled internal standard (m/z 130). The concentration of sotolone was calculated from the peak areas using a calibration factor of 1.1 (Figure 3). A good linearity was found in the concentration range 3-150 µg/ml. All samples were injected twice.

Fast Atom Bombardment (FAB-MS). This was applied to study the lactonization of HIL to ADF. FAB-MS was performed on a Finnigan MAT 8430 double focusing mass spectrometer. FAB ionisation was carried out with a saddle-field atom gun (Ion Tech, Teddington, UK) which was operated at 2 mA and 7-8 kV with xenon. Glycerol was used as matrix. The positive ions at m/z 130 (protonated molecular ion of ADF) and 148 (protonated molecular ion of HIL) were recorded.

Isotope Ratio Mass Spectrometry (GC-IRMS). This was performed with a Finnigan MAT delta S isotope MS coupled on-line with a Varian 3400 GC via a combustion interface. Isotope ratios were expressed as δ-values [‰] versus the PDP

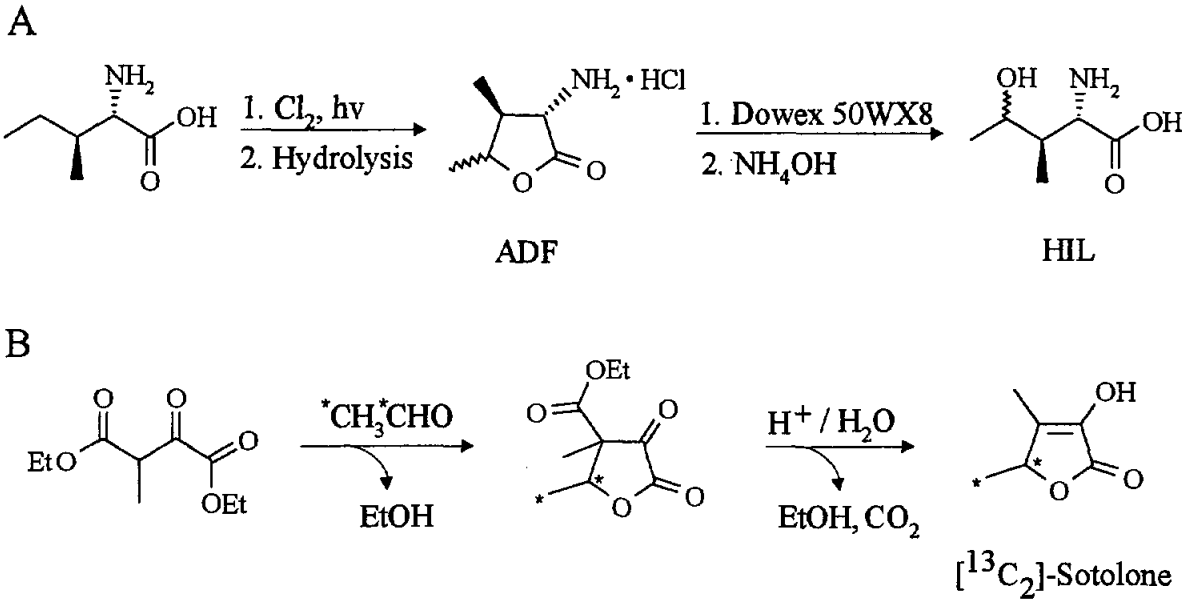


Figure 2. Synthesis of 4-hydroxy-L-isoleucine (HIL) and 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone hydrochloride (ADF) (**A**), and [5,6-^{13}C]-3-hydroxy-4,5-dimethyl-2(5*H*)-furanone ([$^{13}C_2$]-sotolone, used as internal standard) (**B**).

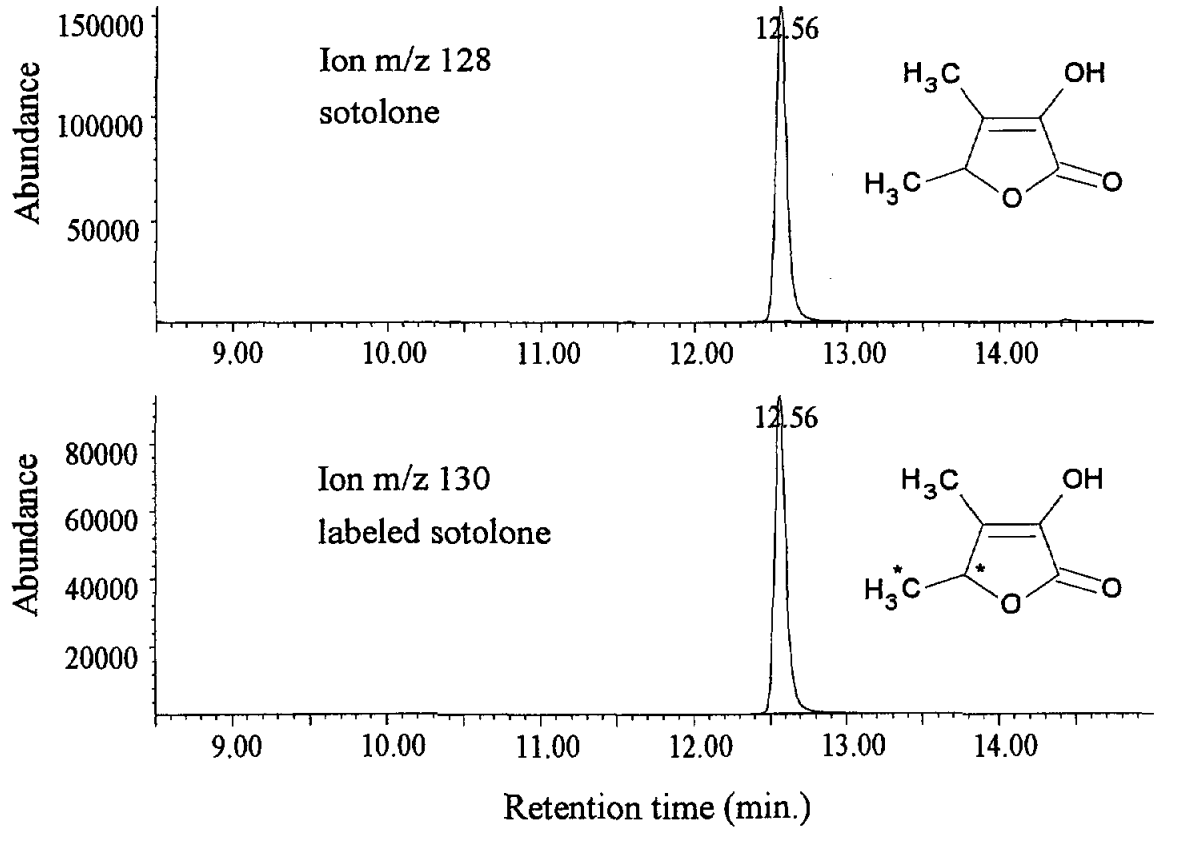


Figure 3. Quantification of sotolone by isotope dilution assay using $^{13}C_2$-sotolone as internal standard. The ions m/z 128 and m/z 130 were recorded by GC-MS operating in the selected ion monitoring mode. (Adapted from ref. 24.)

standard having a $[^{13}C]/[^{12}C]$ isotope ratio of 0.011237 for CO_2 yielded by combustion of fossil $CaCO_3$ (Peedee Belemnite). The GC was equipped with a DB-FFAP fused silica capillary column (30 m x 0.25 mm, film thickness 0.25 μm) using helium as carrier gas (100 kPa) and the split injection mode (220°C). The temperature program was the same as mentioned above.

Sample Preparation

Qualitative Analysis for GC-Olfactometry. The ground fenugreek seeds (100 g) were extracted with diethyl ether (Et_2O, 200 ml) by stirring the suspension for 5 h. The solvent was separated and the extraction was continued with additional solvent (200 ml) overnight. The extracts were combined, filtered and concentrated (80 ml) on a Vigreux column. Non-volatile by-products were separated by vacuum sublimation (VS), i.e. trapping volatiles under high vacuum conditions ($2 \cdot 10^{-4}$ mbar) into traps cooled with liquid N_2 (*21*). The sample was introduced drop-by-drop into the vacuum system to increase the yields. Then, Et_2O (30 ml) was added to the residue and the isolation procedure was repeated. The condensates of the traps were combined and concentrated to 1 ml on a Vigreux column.

Qualitative Analysis for GC-IRMS. Sotolone was isolated from fenugreek oleoresin. An aqueous solution of the oleoresin (35.5 g/100 ml) was extracted with Et_2O containing 5 % ethanol (5 x 100 ml). The organic phase was concentrated to 100 ml and extracted with Na_2CO_3 (0.5 mol/L, 3 x 100 ml). After acidification of the aqueous phase to pH 2 (HCl, 5 mol/L), sotolone was re-extracted with Et_2O (4 x 100 ml). The sample was dried over anhydrous Na_2SO_4 and concentrated to 20 ml. Sotolone was separated from nonvolatile by-products by sublimation in vacuum (10^{-2} mbar) (*21*). The condensates of the traps were collected and concentrated to 2 ml.

Qualitative Analysis for FAB-MS. HIL was dissolved in phosphate buffers (0.1 mol/L) with different pH values (pH 3.0, 4.0, 5.0, 6.0, and 7.0). The solutions were boiled for 1 h in sealed glass tubes. The samples were rapidly cooled down and directly analysed by FAB-MS.

Quantitative Analysis in Fenugreek Seeds (*10*). The material (5-10 g) was homogenized in water:ethanol (50 ml, 95:5). $[^{13}C_2]$-Sotolone (10-20 μg) was added as internal standard and the suspension was stirred for 30 min. After centrifugation (30 min, 10000 rpm), the supernatant was extracted with Et_2O. The acidic components were isolated with Na_2CO_3 (0.5 mol/L). The aqueous solution was acidified to pH 3 (5 mol/L HCl) and re-extracted with Et_2O. Finally, the organic layer was washed with saturated NaCl solution, dried over Na_2SO_4 and concentrated to about 0.2 ml using a Vigreux column and micro-distillation.

Quantitative Analysis in Model Experiments (*24*). HIL (2-10 mg) and ADF·HCl (2-10 mg) were each dissolved in a phosphate buffer (0.1 mol/L, pH 5.0). After adding the carbonyl reactant, the solution was boiled for 1 h in a sealed glass tube. The molar ratio of precursor to carbonyl was 1:10. Water and the internal standard ($[^{13}C_2]$-sotolone) were added to the cooled reaction mixture. The sample was saturated with NaCl and the pH adjusted to 4 (HCl, 1 mol/L). Sotolone was extracted from the reaction mixture with Et_2O for 8 h. The extract was dried (Na_2SO_4) and concentrated to 1 ml. All experiments were performed in duplicate.

Results and Discussion

Aroma Composition of Fenugreek. Liquid-liquid extraction using diethyl ether resulted in an aroma extract which represented the characteristic note of the original product, that i.e. is seasoning-like, spicy, herbaceous, and fenugreek-like. The representativeness of the samples before and after purification was checked by sensory evaluation.

GC-Olfactometry (GC-O) was used to detect the odor-active compounds present in the aroma extract of fenugreek. It is a simple but effective method to select those volatiles which contribute to the overall flavor (*18*, *19*). On the basis of GC-O, seventeen odorants were detected in the original aroma extract (Figure 4). An aroma extract dilution analysis (AEDA) was applied to classify the aroma composition into three groups having different sensory relevance, "high" (no. 17), "medium" (nos. 4, 6, 16), and "background" (nos. 1-3, 5, 7-15). Hence, identification experiments were focused on the odorants belonging to the first two groups. Compound no. 17 was of particular interest because of its high FD-factor and characteristic seasoning-like note.

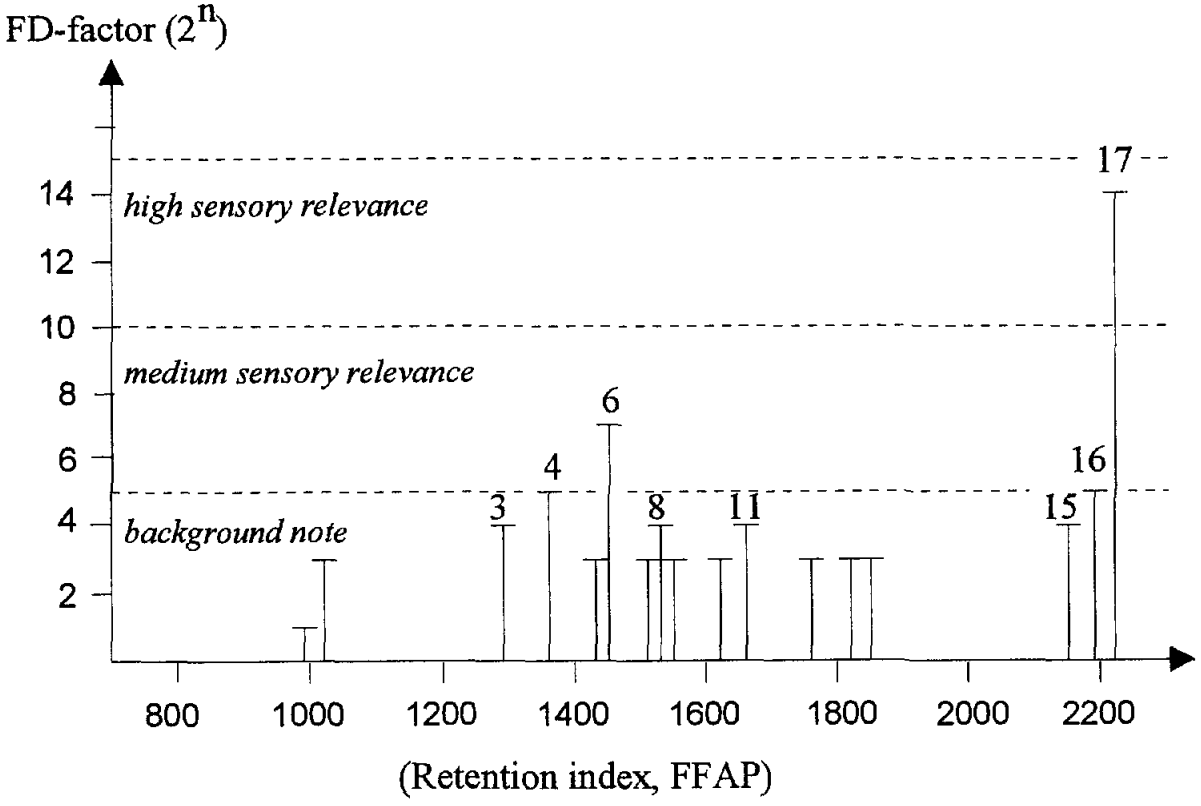


Figure 4. FD-chromatogram of an aroma extract obtained from fenugreek seeds.

The chemical structures (Figure 5) of the odorants were mainly elucidated by GC-MS (Table I). Compound no. 17 was identified as sotolone (Figure 6A). Sotolone is likely the character impact compound of fenugreek as indicated by the high FD-factor of 2^{14}, correspondingly low sensory threshold and characteristic aroma note.

Sotolone was detected by GC-O even after more than 10000-fold dilution of the original aroma extract. Its FD-factor was significantly higher than those of acetic acid (FD= 2^7), (*Z*)-1,5-octadiene-3-one (FD= 2^5), and 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone (FD= 2^5) which belong to the group with medium sensory relevance (Figure 4).

The FD-factors of the remaining compounds were lower. They most likely contribute to the background of the fenugreek flavor. These odorants are short chain fatty acids (nos. 10, 11, 14), lipid degradation products (nos. 3, 12), and alkylated methoxypyrazines (nos. 5, 7). All odorants listed in Table I, except nos. 15 and 17, were identified for the first time as constituents of fenugreek aroma.

Table I. Odor-active Compounds Detected in an Aroma Extract of Fenugreek Seeds on the Basis of GC-O (*n* is the number of dilution steps)

No	*Compound*	*Retention index*		*Aroma quality*	*FD-factor*
		FFAP	*OV-1701*	*(GC-O)*	*(2^n)*
1	Diacetyl[a]	990	680	Buttery	1
2	Unknown	1020	n.d.	Fruity, metallic	3
3	1-Octene-3-one[b]	1296	1065	Mushroom-like	4
4	(*Z*)-1,5-Octadiene-3-one[b]	1369	1090	Metallic, geranium-like	5
5	3-Isopropyl-2- methoxypyrazine[b]	1428	1145	Roasty, earthy	3
6	Acetic acid[a]	1445	785	Acidic, pungent	7
7	3-Isobuty-2- methoxypyrazine[b]	1518	1235	Roasty, paprika-like	3
8	Linalool[a]	1543	1193	Flowery	4
9	Unknown	1554	n.d.	Sulfury, roasty	3
10	Butanoic acid[a]	1624	968	Sweaty, rancid	3
11	Isovaleric acid[a]	1663	1025	Sweaty, rancid	4
12	Unknown	1760	n.d.	Fatty	3
13	Unknown	1823	n.d.	Flowery, citrus-like	3
14	Caproic acid[a]	1845	1163	Musty	3
15	Eugenol[a]	2163	1500	Spicy	4
16	3-Amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone[a]	2190	n.d.	Seasoning-like	5
17	Sotolone[a]	2210	1350	Seasoning-like	14

[a] Identification by comparison with the reference compound on the basis of retention indices, aroma quality and GC-MS.

[b] Identification by comparison with the reference compound on the basis of retention indices and aroma quality. The amounts were too small for verification by GC-MS.

n.d. not determined

Terpenes and terpenoid compounds do not play a major role. Only linalool was detected by GC-O (no. 8). Most of the terpenes identified by GC-MS were odorless at the concentration present in the aroma extract, i.e. α- and β-pinene, sabinene, 3-carene, menthol, β-terpineol, cineol, anethol, β-terpinyl acetate, 1-*p*-

menthen-8-yl acetate, carvone, and several sesquiterpenes. Further volatiles of low or no sensory relevance were 1-pentanol, 1-hexanol, 2-methyl-2-butene-1-ol, 2-methyl-2-butenal, 2-pentylfuran, formic acid, propanoic acid, and further longer chain fatty acids, γ-butyrolactone and several 5-alkylated γ-lactones, 3-amino-4,5-dimethyl-2(3*H*)-furanone, and others.

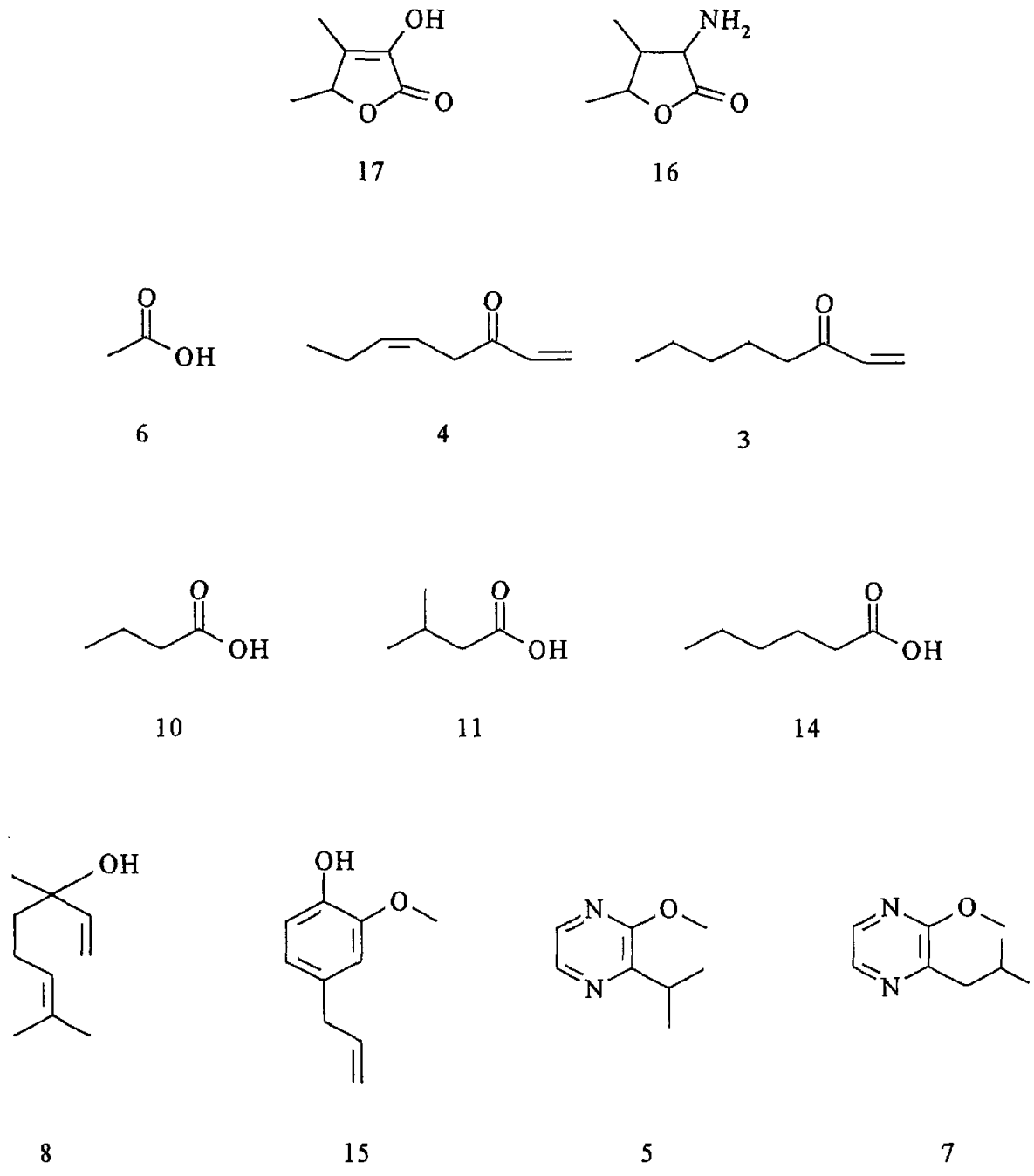


Figure 5. Sensory relevant compounds identified in the aroma extract of fenugreek. The numbers correspond to those in Table I.

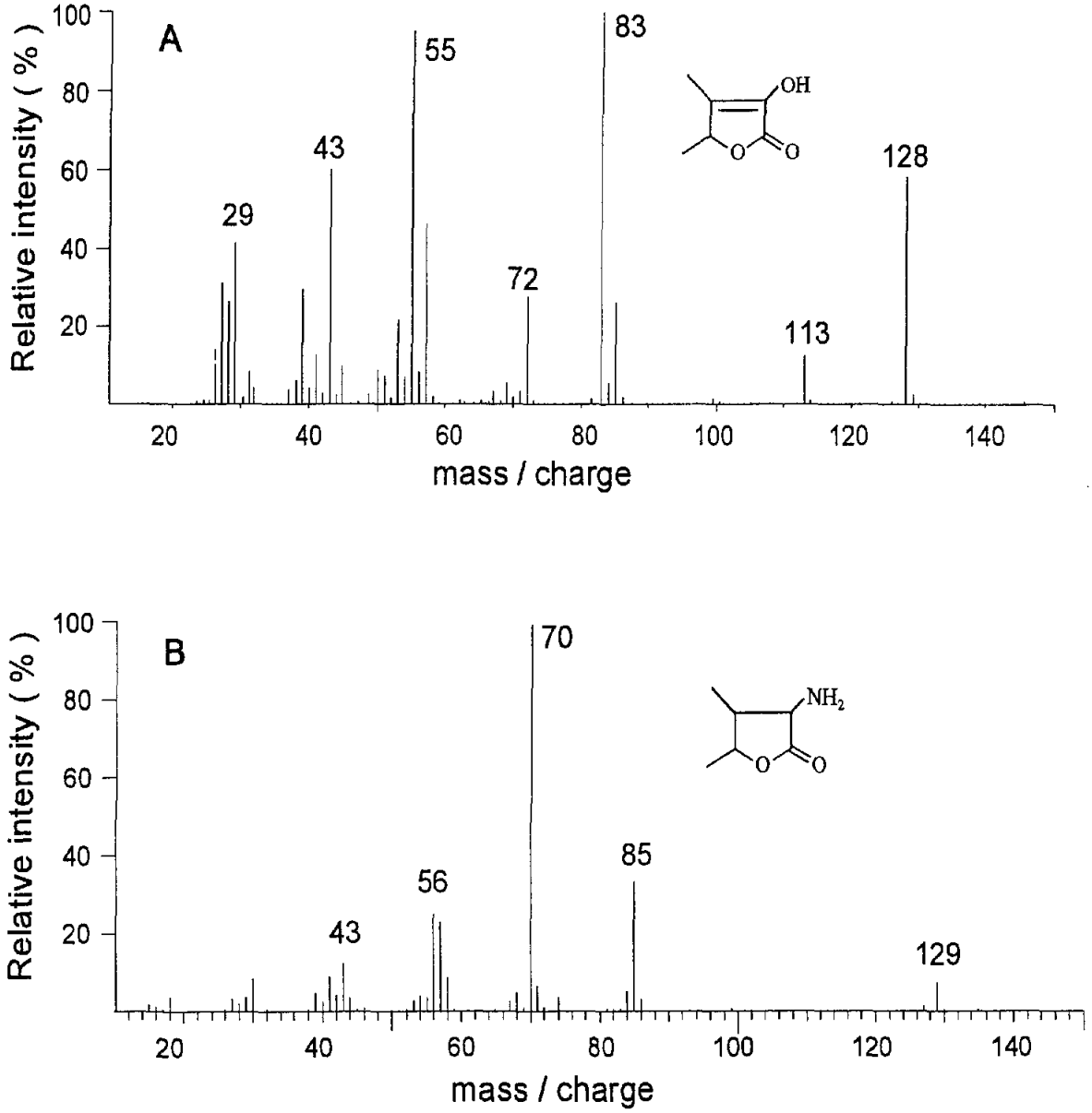


Figure 6. Mass spectra of 3-hydroxy-4,5-dimethyl-2(5*H*)-furanone (sotolone) (**A**) and of 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone (**B**).

Stereoisomeric Characterisation of Sotolone. In fenugreek seeds, sotolone occurs predominantly in the (5*S*) enantiomeric form (95%). This is in good agreement with the data reported by Sauvaire et al. (*16*).

The $\delta^{13}C$ values of natural and synthesized sotolone were determined by isotope ratio mass spectrometry (IRMS) using the GC combustion technique (*26*). As shown in Table II, natural sotolone was characterised by a $\delta^{13}C_{PDB}$ value of -19.7‰. On the contrary, a racemic mixture of synthesized sotolone showed a significantly higher value (-23.3‰).

Sotolone isolated from a commercial product revealed a $\delta^{13}C_{PDB}$ value of -22.3‰ indicating a mixture of natural and synthetic compounds. This was confirmed by chirospecific GC analysis resulting in a ratio of 65:35 for (5*S*):(5*R*). Hence, both

GC-IRMS and chirospecific GC suggest that about 2/3 of the sotolone found in the commercially available liquid seasoning was contaminated with synthetic sotolone.

Table II. $\delta^{13}C$ Values (‰ PDP) of Sotolone of Synthetic and Natural Origin

Sotolone	*$\delta^{13}C$ (‰)*
Synthetic	-23.30 ± 0.20
Natural (fenugreek oleoresin)	-19.69 ± 0.20
Natural (fenugreek seeds)	-19.75 ± 0.20
Liquid seasoning (commercial)	-22.28 ± 0.20

Quantitation of Sotolone in Fenugreek. The concentration of sotolone was determined by isotope dilution assay (*10*, *24*) using labelled sotolone as internal standard. As shown in Table III, the typical concentration range of sotolone was about 3-12 mg/kg fenugreek seeds. However, the amounts depend on the geographical origin. Fenugreek seeds from Egypt smelled more intensely and, in agreement with that, more sotolon was found in these samples. Some *Trigonella* species do not contain any sotolone as reported by Sauvaire et al. (*16*) and, consequently, they lack the characteristic seasoning-like note.

The sensory relevance of sotolone is due to its low threshold value of 0.3 μg/kg water (*10*). In fenugreek seeds, the concentration of sotolone is usually at least 3000 times higher than its threshold, thus indicating the sensory impact of sotolone to the overall flavor of fenugreek and products containing fenugreek. As shown in Table III, high amounts of sotolone were found in curry powder and some commercial liquid seasonings.

Table III. Concentration of Sotolone in Fenugreek and Products Containing Fenugreek

Sample	*Sotolone [mg/kg]*
Fenugreek, seed (Egypt, 1985)	25.1
Fenugreek, seed (Egypt, 1995)	12.2
Fenugreek, seed (Australia, 1991)	4.2
Fenugreek, seed (France, 1996)	3.3
Fenugreek, seed (India, 1996)	5.1
Fenugreek, seed (Turkey, 1995)	3.4
Curry powder (containing fenugreek)	39.7
Liquid seasoning A	1.4
Liquid seasoning B	88.9

Formation of Sotolone from Precursors

Precursors Present in the Aqueous Extracts of Fenugreek Seeds. The presence of glycosidically bound sotolone was examined by treating an aqueous extract of fenugreek with α- and β-glucosidases. As shown in Table IV, this enzymatic treatment did not significantly enhance the amounts of sotolone compared to the reference sample. On the other hand, boiling of the extract under acidic conditions (pH 2.4) for 1 h led to a more than 10 fold increase.

These trials indicate the presence of precursors in fenugreek that can be transformed to sotolone using specific conditions, particularly an acidic medium and heat treatment. 4-Hydroxy-L-isoleucine, known to be a characteristic amino acid of fenugreek (*14*, *15*), may be one of these precursors.

Table IV. Formation of Sotolone from Precursors Present in the Aqueous Extracts of Fenugreek Seeds.

Addition to the aqueous extract	*Reaction conditions*			*Sotolone*
	pH	*T [°C]*	*t [min]*	*[mg/kg]*
None	6.8	25	120	18.9
α-Glucosidase (200 U)	6.8	25	120	19.2
β-Glucosidase (200 U)	6.8	25	120	20.2
None	2.4	100	60	252.3

SOURCE: Adapted from ref. 10.

Formation of Sotolone from 4-Hydroxy-L-isoleucine (HIL). The formation of sotolone was studied in phosphate-buffered model systems (pH 5.0) by reacting HIL with different mono- and α-dicarbonyl compounds at 100°C for 1 h. Both 2,3-butanedione and 2,3-pentanedione formed only low amounts of sotolone (Table V). Higher yields were achieved with the α-ketoaldehydes methylglyoxal (7.4 mol%) and phenylglyoxal (2.5 mol%), producing about 70-200 times more sotolone than the corresponding reaction with the α-diketones. Monocarbonyl compounds, such as propionaldehyde and phenylacetaldehyde, generated less than 0.1 mol% sotolone (*24*, *27*).

Formation of Sotolone from 3-Amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone (ADF). The efficiency of ADF, the lactone of HIL, to generate sotolone was tested in the same model system as described above. As shown in Table V, significantly higher amounts of sotolone were generated from ADF as compared to HIL. Using methylglyoxal, the yields were increased from 64 μg (7.4 mol%) to 274 μg (35.9 mol%), thus indicating ADF to be a more efficient precursor than the free amino acid (HIL).

Table V. Formation of Sotolone from the Precursors 4-Hydroxy-L-isoleucine (HIL)[a] and 3-Amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone (ADF)[b] Present in Fenugreek Seeds.

α-Dicarbonyl	*Precursor[c]*		*Sotolone[d]*	*Yield*
	HIL	*ADF*	*[μg/mg HIL]*	*[mol%]*
2,3-Butanedione	+	-	0.34 ± 0.03	< 0.1
2,3-Pentanedione	+	-	0.30 ± 0.03	< 0.1
Methylglyoxal[e]	+	-	64.2 ± 0.3	7.4
Phenylglyoxal	+	-	22.2 ± 0.3	2.5
2,3-Pentanedione	-	+	5.4 ± 0.3	0.7
Methylglyoxal	-	+	274.4 ± 3.4	35.9

[a] Control experiment (without α-carbonyl) yielded less than 0.01 mol% sotolone.
[b] Control experiment (without α-carbonyl) yielded 0.03 mol% sotolone.
[c] The molar ratio of precursor to α-dicarbonyl was 1:10.
[d] Data are means of at least two experiments, each injected twice.
[e] Control experiment (without HIL) yielded 0.07 μg sotolone.

Mechanism of the Formation of Sotolone (Figure 7, pathway A). The data reported above confirm the hypothesis of the formation of sotolone by thermally induced oxidative deamination of HIL (*10*). Acid catalyzed cyclization of HIL leads to the corresponding lactone (ADF) which reacts with an α-dicarbonyl (e.g. methylglyoxal) to form a Schiff base (pathway A). Rearrangement and subsequent hydrolysis gives rise to sotolone. The data show that α-dicarbonyls are capable of generating sotolone from both HIL and the lactone ADF. However, the latter is more efficient in producing sotolone. Furthermore, the relatively low yields achieved with HIL indicate an alternative degradation pathway.

Strecker Degradation of HIL as a Competitive Reaction (Figure 7, pathway B). The lower yields obtained with HIL might be explained by a partial Strecker degradation of the amino acid HIL in the presence of an active α-dicarbonyl, e.g. methylglyoxal. As shown in Figure 7, the amino-carbonyl reaction of HIL and methylglyoxal results in a Schiff base which may either cyclize or decompose via decarboxylation. The Strecker aldehyde of HIL, 3-hydroxy-2-methylbutanal, is released by hydrolysis and this compound was tentatively identified by GC-MS (*24*) as a mixture of diastereomers.

In the sample based on HIL and methylglyoxal, the ratio of Strecker aldehyde to sotolone was about 1:2 at pH 5 (Table VI). Consequently, the Strecker degradation of HIL is a competitive reaction to the formation of sotolone. In contrast, only traces of Strecker aldehyde were detected in the sample containing the lactone ADF, i.e. about 50 times less than in the reaction with HIL. The formation of sotolone from ADF is the favoured reaction, most likely due to the blocked carboxyl group.

Table VI. Ratio of Sotolone to 3-Hydroxy-2-methylbutanal Formed in Model Reactions Based on Methylglyoxal and the Precursors 4-Hydroxy-L-isoleucine (HIL) and 3-Amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone (ADF)

pH	*HIL*	*ADF*
3	1 : 17	1 : 50
5	1 : 2	1 : 50
6	1 : 2	1 : 30
7	1 : 1.5	1 : 40

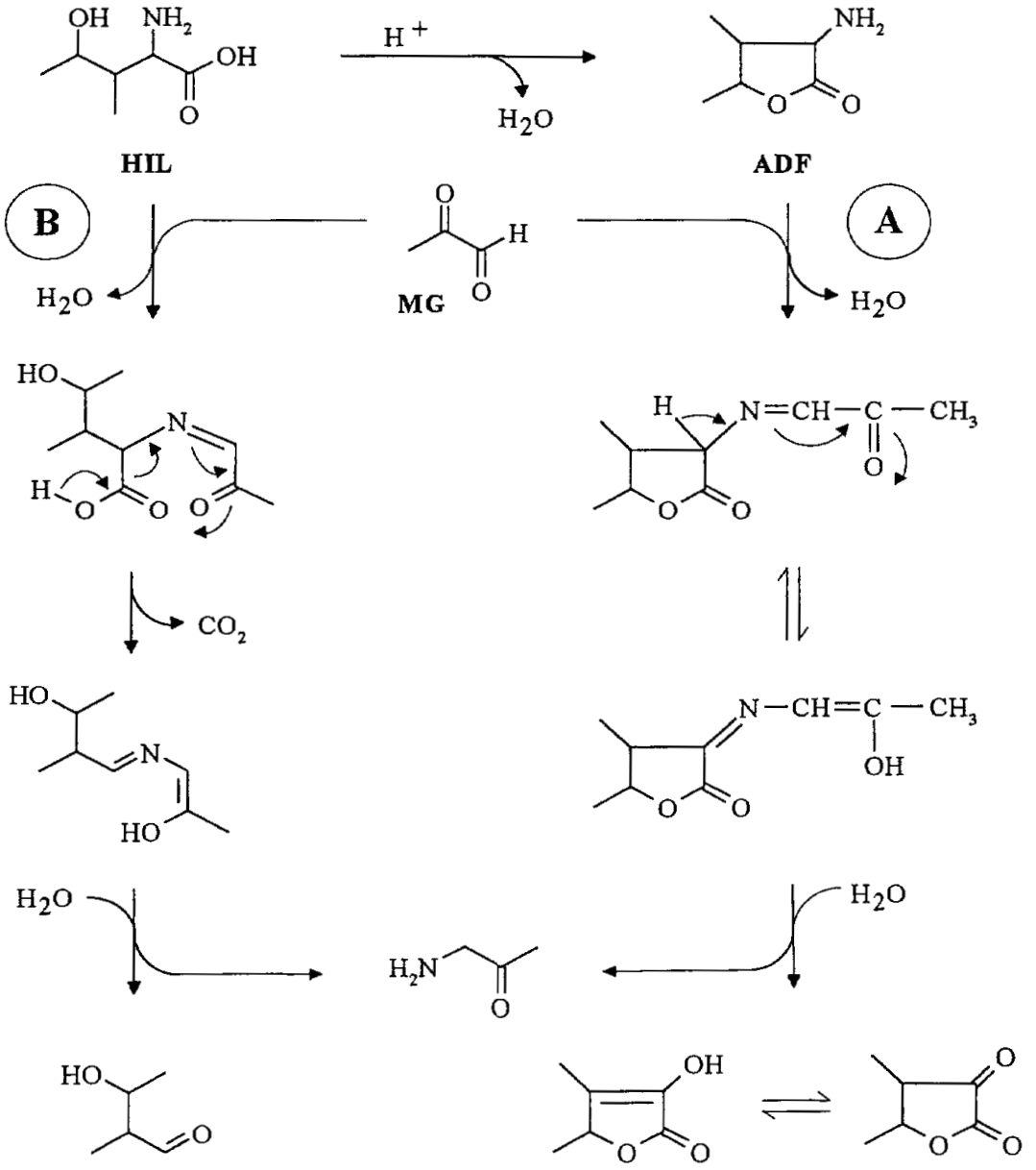


Figure 7. Formation of sotolone (pathway **A**) and 3-hydroxy-2-methylbutanal (pathway **B**) from 4-hydroxy-L-isoleucine (HIL) and 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone (ADF) using methylglyoxal (MG) as carbonyl reactant.

Influence of the pH. It is well known that both lactonization and the formation of the Schiff base strongly depend on the pH of the reaction medium. As shown in Figure 8, the lactonization step (HIL → ADF) was favored under acidic conditions and the yields were about 50 % at pH 3, but only 10 % at pH 5. On the other hand, Schiff bases are readily formed under neutral and slightly basic conditions.

The reactivity of the carbonyl compound is another crucial parameter for the formation of the Schiff base. α-Ketoaldehydes are much more efficient than α-diketones (Table IV) and α-keto acids, which generate only low amounts of sotolone from HIL (*27*).

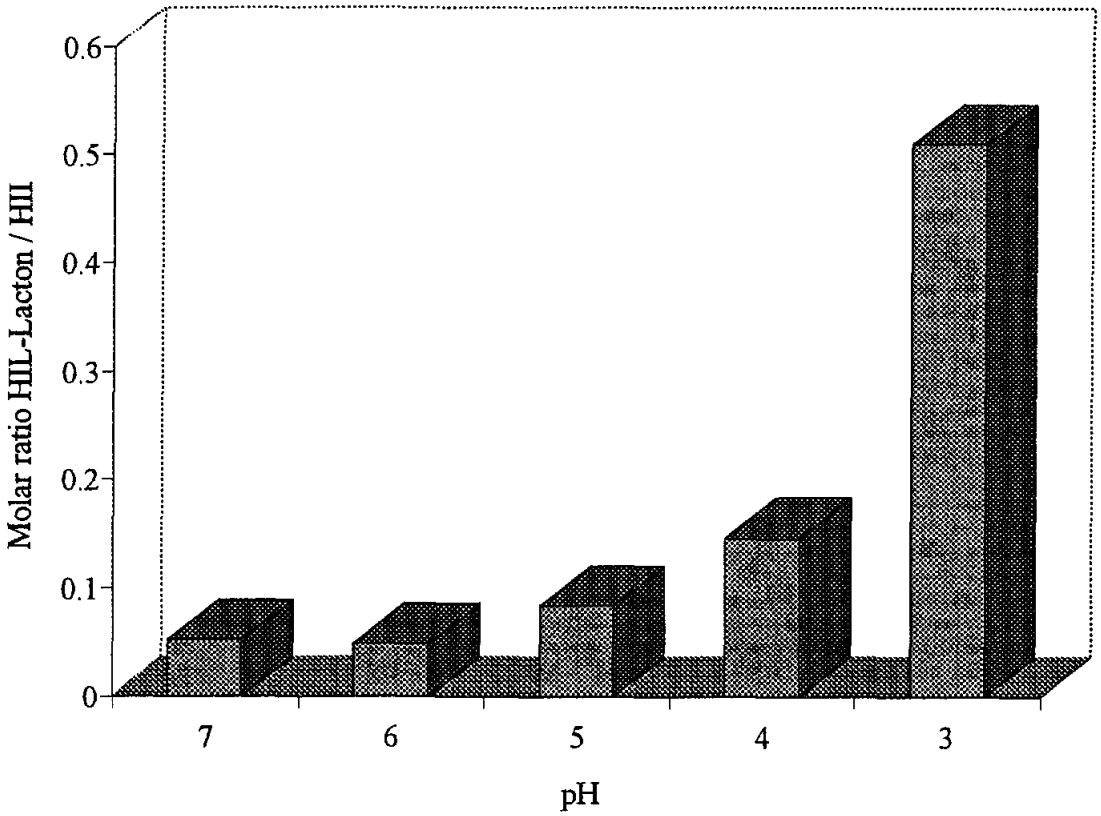


Figure 8. Lactonization of 4-hydroxy-L-isoleucine (HIL) as a function of pH.

To find the optimum pH, methylglyoxal was reacted with HIL and the lactone ADF, respectively. The reaction of methylglyoxal and HIL was favoured at pH 5 which apparently is the best compromise between the lactonization step and the reactivity of the amino group to form the Schiff base (Figure 7). Once the lactone (ADF) is formed, the amino-carbonyl reaction was favoured at pH 5-6. In general, the yields obtained with the lactone were significantly higher compared to the amino acid, particularly at pH 6 (40.2 mol%).

The reaction of HIL and methylglyoxal at pH 5 performed in water yielded 2.8 mol% sotolone compared to 7.4 mol% when using the phosphate buffered system. This suggests a catalytic effect of phosphate on the formation of sotolone from HIL.

Effect of Reaction Temperature and Time. The sotolone yields from HIL and methylglyoxal were strongly dependent on both reaction time and temperature. Significant amounts were generated above 70°C (Table VII). At a constant temperature of 100°C, the yield of sotolone continuously increased over a period of 10 h, with no significant increase thereafter (Table VIII). About 23 μg to 210 μg sotolone were generated from 30 min to 10 h which corresponds to 2.7 mol% and 23.8 mol%, respectively.

Methylglyoxal reacted with HIL at 50°C for 48 h resulted in 3.8 mol% sotolone, thus indicating that a long reaction time and mild reaction conditions are also suitable for generating significant amounts of sotolone. Therefore, hot climatic conditions might favour the formation of sotolone from HIL.

Table VII. Formation of Sotolone from HIL as a Function of the Reaction Temperature Using Methylglyoxal as Carbonyl Reactant [a]

Temperature [°C]	*Sotolone[b] [μg/mg HIL]*	*Yield [mol%]*
50	0.31 ± 0.01	0.03
60	1.15 ± 0.03	0.13
70	4.00 ± 0.05	0.46
80	12.5 ± 0.5	1.44
90	27.9 ± 1.8	3.2
100	64.2 ± 0.4	7.4

[a] Reaction conditions: phosphate buffer (0.1 mol/L, pH 5.0), 50-100°C, 1 h.
[b] Data are means of at least two experiments, each injected twice.

Table VIII. Formation of Sotolone from HIL as Affected by the Reaction Time Using Methylglyoxal as Carbonyl Reactant [a]

Time [h]	*Sotolone[b] [μg/mg HIL]*	*Yield [mol%]*
0.5	23.4 ± 0.8	2.7
1	64.2 ± 0.4	7.4
2	102.1 ± 4.5	11.7
5	170.2 ± 5.1	19.5
10	206.7 ± 10	23.8
15	208.3 ± 6.4	24.0
24	229.9 ± 5.3	26.4

[a] Reaction conditions: phosphate buffer (0.1 mol/L, pH 5.0), 100°C, 0.5-24 h.
[b] Data are means of at least two experiments, each injected twice.

Conclusion

The role of (*5S*)-sotolone as a character impact compound of fenugreek was corroborated and its formation from 4-hydroxy-L-isoleucine (HIL) via thermally induced oxidative deamination was substantiated. The lactone of HIL, 3-amino-4,5-dimethyl-3,4-dihydro-2(5*H*)-furanone (ADF), was found to be a better precursor than the amino acid. α-Ketoaldehydes were more effective in generating sotolone from both HIL and ADF than α-diketones. The reactivity of the dicarbonyl and the lactonization step are important parameters, particularly for the formation of the Schiff base. The transformation yields from HIL into sotolone greatly depend on the reaction conditions, such as temperature, time, pH and amount of dicarbonyl. High amounts of sotolone were obtained by boiling methylglyoxal with HIL for 10 h at pH 5 (24 mol%). Even better results (40 mol%) were achieved using ADF as precursor (1 h, pH 6), most likely due to inhibition of the Strecker degradation by the blocked carboxyl group.

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