Quantification of the Flavour Compounds 3-Hydroxy-4,5-dimethyl-2(5H)-furanone and 5-Ethyl-3-hydroxy-4-methyl-2(5H)-furanone by a Stable Isotope Dilution Assay

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A stable isotope dilution assay has been developed for the quantification of 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolone) and 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone (EHMF). The method was applied to fenugreek seeds, lovage, seasonings and soya sauce revealing that sotolone contributes more to the "hydrolyzed vegetable protein-like" flavour note than does EHMF. Boiling of the aqueous extracts of fenugreek or lovage under weakly acidic conditions significantly increased the amounts of sotolone.

The flavour compounds 3-hydroxy-4,5-dimethyl-2(5H)-furanone (sotolone) and 5-ethyl-3-hydroxy-4-methyl-2(5H)-furanone (EHMF), both exhibit intense odours which are described as "hydrolyzed vegetable protein-like" or "curry-like".

EHMF was first reported by Sulser et al [1] as a degradation product of the α-amino acid threonine and was later proposed by the same group [2] as the flavour principle of seasonings prepared from plant protein hydrolysates. In a study on the sensory properties of several alkyl substituted 3-hydroxy-2(5H)-furanones, Sulser et al. [2] reported sotolone as a powerful synthetic odorant for seasonings. Later, the flavour compound was identified in different foods, e.g. raw cane sugar [3], fenugreek seeds [4] and flor sherry wines [5, 6]. By application of an aroma extract dilution analysis, Blank et al. [7] reported sotolone and EHMF, both of which show the low odour threshold of 0.3 µg/L water, as important contributors to the flavour of roasted coffee. Sotolone was recently found to be responsible for the seasoning-like flavour of lovage extract [8].

On the basis of quantitative data, sotolone was recently established as important odorant in sherry wines [9]. It was determined by means of a two-dimensional-capillary GC method using undecane as internal standard. The quantification of cyclic enolones like sotolone and EHMF may lead to incorrect results; e.g. losses due to their inefficient extraction from more complex matrices or due to their instability during HRGC [6, 10] are not compensated if an internal standard of different structure is used in the quantification method. As recently shown for 4-

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hydroxy-2,5-dimethyl-3(2H)-furanone [11], a stable isotope dilution assay is an accurate method for the quantification of such polar compounds.

The aim of the following investigation was to develop a stable isotope dilution assay for sotolone and EHMF, and to apply it for the analysis of some food seasonings as well as fenugreek seeds and lovage.

Experimental

Materials. Fenugreek seeds (*Trigonella foenum graecum L.*) from Egypt and Australia were kindly supplied by Prof. Dr. J.K.P. Weder (Institute of Food Chemistry, Technical University of Munich). Dry leaves and roots of lovage (*Levisticum officinale*) were purchased from a local pharmacy; fresh lovage was harvested in a Munich garden. The seasonings were purchased from local shops.

Synthesis. Sotolone and EHMF were synthesized according to the literature [2]. [^{13}C]-3-Hydroxy-4,5-dimethyl-2(5H)-furanone ([^{13}C]-sotolone) and [^{2}H]-5-Ethyl-3-hydroxy-4-methyl-2(5H)-furanone ([^{2}H]-EHMF) were synthesized following the routes for the respective unlabelled compounds [12] as shown in Figure 1.

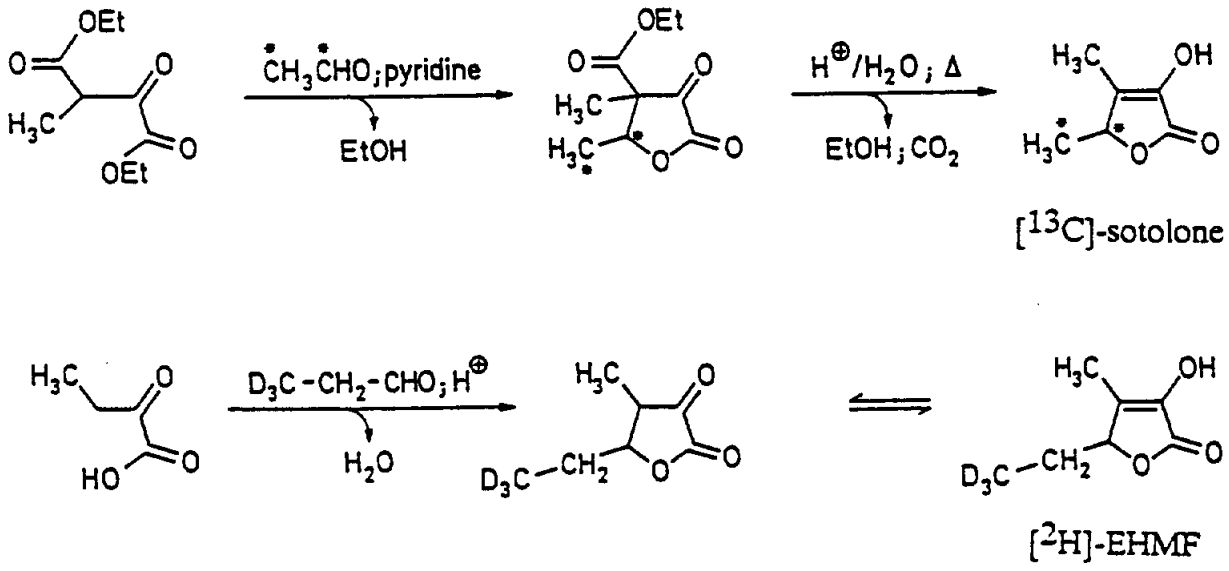

Figure 1 : Syntheses of labelled sotolone and EHMF

The unlabelled compounds and the corresponding labelled internal standards showed the following Ei-mass spectra (m/z; % relative abundance). Sotolone : 83(100), 55(72), 128(57, M^+), 43(55), 57(40), 72(16), 85(14), 39(12); [^{13}C]-sotolone : 85(100), 57(94), 130(56, M^+), 45(45), 86(15), 74(15), 58(15); EHMF : 97(100), 57(78), 69(57), 41(48), 142(40, M^+), 113(37), 85(34), 86(28), 39(18), 55(17); [^{2}H]-EHMF : 100(100), 72(59), 57(55), 145(39, M^+), 113(38), 85(38), 60(38), 43(38), 89(26), 42(22), 41(21).

Quantitative analysis. Method I (samples high (>0.5 mg/kg) in sotolone). The material (5 g) was homogenized (Ultra-turrax, Jahnke & Kunkel, Oberstaufen, Germany) in water/ethanol (50 ml; 95 + 5 v/v) which contained 10µg of [^{13}C]-sotolone . After stirring for 30 min at room temperature, the suspension was centrifuged (30 min; 10000 rpm) and the supernatant was then extracted with diethyl ether (3 x 50 ml). To isolate the acidic compounds, the ether solution was extracted with sodium carbonate (0.5 mol/L; 2 x 75 ml), the aqueous solution was then acidified to pH 3.0 (5 mol/L HCl) and extracted with diethyl ether (2 x 75 ml). The organic layer was washed with saturated aqueous sodium chloride (75 ml), dried over Na_2SO_4 and finally concentrated to about 0.15 ml.

Method II (samples low (<0.5 mg/kg) in sotolone and EHMF). The material (100 g) was homogenized in water/ethanol (400 ml; 95 + 5 v/v) containing [^{13}C]-sotolone (30 µg) and [^{2}H]-EHMF (30 µg). After stirring at room temperature, the suspension was centrifuged (30 min; 10000 rpm) and the supernatant was extracted with diethyl ether (3 x 400 ml). The combined organic layers were dried over Na_2SO_4 and then concentrated to about 150 ml by distilling off the solvent on a Vigreux-column (50 cm x 1 cm). The volatiles and the solvent were separated from the non-volatile material by sublimation in vacuo [13]. From the ether solution condensed in the traps, the acidic compounds were isolated as described above.

Quantification by mass chromatography. The samples were applied by the "on-column" technique on a fused silica capillary (30m x 0.32mm, FFAP, 0.25 µm; Fisons Instrument, Mainz, Germany) and analyzed in the chemical ionization mode (MS-CI) with isobutane as reactant gas using the mass spectrometer 8230 (Finnigan, Bremen, Germany). From the integrated abundances of the ions m/z 129 (sotolone) and m/z 131 ([^{13}C]-sotolone) and of the ions m/z 143 (EHMF) and m/z 146 ([^{2}H]-EHMF) found in five trials with different ratios of labelled and unlabelled compounds, calibration factors of R = 0.96 $\pm$ 0.06 (sotolone) and R = 1.06 $\pm$ 0.07 (EHMF) were calculated.

Results and Discussion

In preliminary experiments mixtures of sotolone (30 µg), [^{13}C]-sotolone (30 µg), EHMF (30 µg) and [^{2}H]-EHMF (30 µg), dissolved in water/ethanol (100 ml, 95 + 5, v/v) and containing seasoning C (No. 3, cf. Table II) were analyzed using method II. The recoveries of sotolone and EHMF found in three trials were better than 92 %.

Fenugreek seeds and lovage. At first the method was applied to quantify sotolone and EHMF in fenugreek seeds and lovage (Table I). The data indicated high concentrations of sotolone especially in the fenugreek seed variety from Egypt. In fenugreek seeds from Australia the concentration of the odorant was lower by a factor of six (cf. Nos. 1 and 2, Table I).

Table I: Concentration (mg/kg) of sotolone and EHMF in fenugreek seeds and lovage

No	Sample	Sotolone	EHMF
1	fenugreek seed (Egypt, 1985)	25.12	< 0.05 [a]
2	fenugreek seed (Australia; 1991)	4.15	< 0.05 [a]
3	fresh lovage (leaf)	0.05	< 0.01 [b]
4	fresh lovage (stem)	0.03	< 0.01 [b]
5	dried lovage (leaf)	0.84	< 0.05 [a]
6	dried lovage (root)	0.34	< 0.05 [a]

[a] detection limit of method I
[b] detection limit of method II

In both seeds, EHMF was not detectable (detection limit of method I: 50 µg/kg). As the odour thresholds of sotolone and EHMF are nearly identical [7], it can be assumed that the latter odorant does not contribute to the characteristic fenugreek flavour. Furthermore, it is interesting to note that sotolone seems to be relatively stable in the seed, since the sample from Egypt was already seven years old.

Compared to fenugreek seeds, the concentration of sotolone in lovage leaves or stems was significantly lower (cf. Nos. 3 and 4 to nos. 1 and 2; Table I). Even in the dried lovage material the sotolone concentration was lower by a factor of nearly thirty than in the Egyptian fenugreek seeds (cf. Nos. 5 and 1; Table I). As in fenugreek, EHMF was not detectable in the lovage samples.

Seasonings. Seasonings produced on the basis of hydrolyzed plant proteins are widely used to intensify the flavours especially of soups and sauces. To estimate their contribution to these flavours, sotolone and EHMF were quantified in four different commercial products.

As summarized in Table II, the concentration of sotolone varied significantly in the four samples. Seasoning D (No. 4; Table II) contained very high amounts of the odorant, while lower concentrations were found in seasonings A and B (No. 1 and 2; Table II). Seasoning C, which was labelled "for vegetarians" did not contain sotolone. EHMF was only present in seasoning A, but the amount was a factor of ten lower than that of sotolone (No. 1; Table II). The data suggest that sotolone, but not EHMF, is mainly responsible for the typical odour of such seasonings. The high level of sotolone in seasoning D suggests that it was prepared using fenugreek seeds. Also in soy sauce sotolone was present in significant amounts, at a level of nearly six higher than those of EHMF (No. 5, Table II).

The flavour of sotolone is sometimes described as "curry-like". As shown in Table II, a commercial curry powder contained very high concentrations of sotolone (No. 6). Fenugreek flour, which was indicated on the label, is most likely the source of the sotolone present in the curry powder.

Table II: Concentrations (mg/kg) of sotolone and EHMF in commercial seasonings, soy sauce and curry powder

No	Product	Sotolone	EHMF
1	seasoning A	1.41	0.14
2	seasoning B (containing lovage)	0.26	< 0.05
3	seasoning C (manufactured from yeast and vegetables)	< 0.05	< 0.05
4	seasoning D	88.92	< 0.05
5	soy sauce	0.47	0.08
6	curry powder (containing fenugreek powder)	39.73	n.a
7	commercial lovage extract	7.58	< 0.05

n.a. not analyzed

A commercial lovage extract was relatively high in sotolone (No. 7; Table II). Its level was a factor of nine higher than that of a lovage powder (cf. No. 5; Table I) obviously indicating that during processing further amounts of sotolone are liberated from the plant material.

Liberation of sotolone from precursors. In plant materials, a proportion of the odorants bearing a hydroxy group is sometimes present in a bound form, e.g. as glucoside. To test whether fenugreek seeds and lovage contain, besides free sotolone, a bound form, aqueous extracts were treated either with α- and β-glucosidases or under weakly acidic conditions.

As shown in Table III treatment of the extracts (either fenugreek or lovage) with α- or β-glucosidase did not significantly enhance the amounts of sotolone compared to the untreated sample (cf. Nos. 2 and 3 to No. 1). On the other hand, boiling of the extract under acidic conditions for 60 min led to an increase by a factor of more than twelve (fenugreek seeds) or four (lovage), respectively.

Table III: Liberation of sotolone from precursors in aqueous extracts of lovage and fenugreek seeds

No	Additions to the aqueous extract	Reaction conditions pH	T°C	time (min)	Sotolone (mg/kg) fenugreek	lovage
1	none	6.8	25	120	18.89	0.047
2	α-Glucosidase (200 U)	6.8	25	120	19.21	0.041
3	β-Glucosidase (200 U)	6.8	25	120	20.24	0.055
4	none	2.4	100	60	252.28	0.248

Two reaction routes can be discussed to explain these results. As α- and β-glucosidases were not active, sotolone was not bound to glucose. However, these

experiments did not rule out that it is bound to other sugars, and that such O-glycosides were cleaved by acid hydrolysis. An alternative pathway starts with 4-hydroxy isoleucine, which has been detected in fenugreek by Sauvaire et al. [14]. As suggested in Figure 2, lactone formation followed by a Strecker reaction would directly yield sotolone from this amino acid.

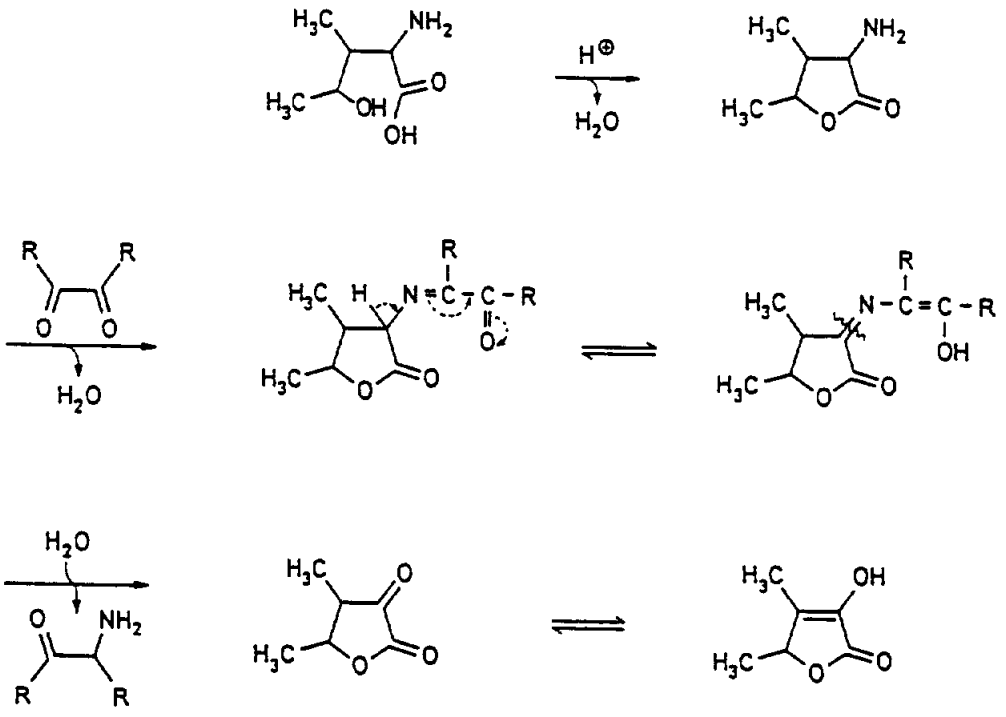

Figure 2 : Possible pathway leading from 4-hydroxy isoleucine to sotolone

Conclusion

A stable isotope dilution has been developed for sotolone and EHMF. Its major advantage in comparison to other methods is that losses of the analytes during isolation and handling are corrected by internal standards having the same physical and chemical properties as the analytes. The data reveal that sotolone contributes much more to the "hydrolyzed vegetable protein-like" odour note of fenugreek seeds, lovage and seasonings than does EHMF.

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