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Potential of Gas Chromatography−Orthogonal Acceleration Time-of-Flight Mass Spectrometry (GC-oaTOFMS) in Flavor Research

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Gas chromatography−orthogonal acceleration time-of-flight mass spectrometry (GC-oaTOFMS) is an emerging technique offering a straightforward access to a resolving power up to 7000. This paper deals with the use of GC-oaTOFMS to identify the flavor components of a complex seafood flavor extract and to quantify furanones formed in model Maillard reactions. A seafood extract was selected as a representative example for complex food flavors and was previously analyzed using GC−quadrupole MS, leaving several molecules unidentified. GC-oaTOFMS analysis was focused on these unknowns to evaluate its potential in flavor research, particularly for determining exact masses. *N*-Methyldithiodimethylamine, 6-methyl-5-hepten-2-one, and tetrahydro-2,4-dimethyl-4*H*-pyrrolo-[2,1-*d*]-1,3,5-dithiazine were successfully identified on the basis of the precise mass determination of their molecular ions and their major fragments. A second set of experiments was performed to test the capabilities of the GC-oaTOFMS for quantification. Calibration curves were found to be linear over a dynamic range of 10^3 for the quantification of furanones. The quantitative data obtained using GC-oaTOFMS confirmed earlier results that the formation of 4-hydroxy-2,5-dimethyl-3(2*H*)-furanone was favored in the xylose/glycine model reaction and 2(or 5)-ethyl-4-hydroxy-5(or 2)-methyl-3(2*H*)-furanone in the xylose/alanine model reaction. It was concluded that GC-oaTOFMS may become a powerful analytical tool for the flavor chemist for both identification and quantification purposes, the latter in particular when combined with stable isotope dilution assay.

KEYWORDS: GC-oaTOFMS; flavor; furanones

INTRODUCTION

In 1955, Gohlke and McLafferty (cited in ref *1*) realized the first coupling between a gas chromatograph and a mass spectrometer, offering to the analytical community one of their most powerful spectroscopic techniques. Nowadays, gas chromatography−mass spectrometry (GC-MS) is the method of choice for the analysis of complex mixtures. In flavor research, GC-MS is the workhorse of any flavor chemist, and each year several hundred publications discuss results obtained by GC-MS. However, as flavor research deals with key odorants that usually occur in trace amounts, often embedded in extracts containing volatile compounds at much higher concentrations, GC-MS suffers sometimes from a lack of selectivity and/or sensitivity, making identification of unknown compounds difficult in such complex mixtures. Despite the availability of mass spectral libraries containing about 400000 mass spectra, identification of unknown flavor molecules remains a challenge. Researchers have tried to overcome the complexity of flavor mixtures by increasing the resolving power of their analytical techniques using, for example, high-resolution capillary gas chromatography (*2*) or GC−tandem mass spectrometry (*3*, *4*). However, capillary gas chromatography coupled with high-resolution mass spectrometry (HR-MS) has rarely been used. Capillary GC-HR-MS has been performed up to now either with double-focusing magnetic sector instruments (*5*) or with FTICR machines (*6*), which are very expensive and need well-trained operators. Recently, gas chromatography−orthogonal acceleration time-of-flight mass spectrometry (GC-oaTOFMS) has been shown as an emerging technique offering a straightforward access to a resolving power up to 7000 (*7*). For GC-MS analysis orthogonal acceleration time-of-flight provides many advantages over conventional scanning mass spectrometers. In oaTOFMS ions formed in a continuous ion source are accelerated focused into a parallel ion beam. As the ions traverse an orthogonal sampling region a sudden voltage pulse is applied, ejecting a

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portion of the beam orthogonally. This packet of ions is then accelerated into the time-of-flight drift region. Ions of different *m*/*z* values have different velocities and hence arrive at a detector at different times relative to the orthogonal acceleration pulse. By precisely recording these arrival times a time-of-flight mass spectrum is produced. The axial ion beam is typically sampled at between 10000 and 100000 times per second; individual time-of-flight spectra are generally summed before storage to disk. As the initial energy spread of the ions is generally very low in the orthogonal direction compared with the axial direction, the spread of ion arrivals for a particular *m*/*z* value is minimized and high mass resolution may be obtained. The addition of a reflectron device, to increase the flight time of the ions, may further improve the mass resolution of the oaTOF analyzer. This elevated resolution reduces mass interferences, increasing the selectivity of the instrument. The oaTOF analyzer simultaneously samples ions of all *m*/*z* values, unlike scanning instruments where ions are detected or ejected sequentially. This provides a particular advantage for GC-MS analysis, in which analyte concentration and composition change rapidly as components elute from the GC column. Spectra produced are representative of the composition of the analyte regardless of how quickly the concentration of the analyte changes. Spectral "skew", which may be apparent with rapidly eluting analytes using scanning instruments, is avoided using oaTOFMS. The high duty cycle of the oa-TOFMS (typically 30%) results in significantly improved sensitivity for full mass range data, compared to conventional scanning instruments. In addition, the precise and stable relationship between an ion's arrival time and the square root of its mass allows good mass accuracy with only a single internal reference mass. Finally, benchtop GC-oaTOFMS systems like the one used in this study are easy to operate and do not need highly experienced users.

This paper will present identification and quantification experiments that have been performed with complex seafood flavor extracts and model Maillard reactions.

MATERIALS AND METHODS

Chemicals. D-Xylose, glycine, L-alanine, methylmercaptan, dimethylamine, phenyl isocyanate, and thionyl chloride of highest purity (>99%) were obtained from Fluka (Buchs, Switzerland). 4-Hydroxy-2,5-dimethyl-3(2*H*)-furanone (HDMF) and 2(or 5)-ethyl-4-hydroxy-5(or 2)-methyl-3(2*H*)-furanone (EHMF) were from Fluka and Givaudan (Dübendorf, Switzerland), respectively. 4-Hydroxy-2(or 5)-[^{13}C]methyl-5(or 2)-methyl-3(2*H*)-[2(or 5)-^{13}C]furanone ($^{13}C_2$-HDMF) and 2(or 5)-([2,2,2-$^2H_3$]eth-1-yl)-4-hydroxy-5(or 2)-methyl-3(2*H*)-furanone ($^2H_3$-EHMF) were synthesized as recently described (*8*). The isotopic content of the labeled compounds was 99%. Anhydrous sodium sulfate, dipotassium hydrogen phosphate dihydrate, and diethyl ether were from Merck (Darmstadt, Germany). The organic solvents were purified by slow distillation on a Vigreux column (1 m × 1 cm).

Seafood Flavor Extract. This was prepared from a commercially available liquid seafood sample using the SAFE technique (*9*), which allows the careful isolation of volatiles from complex mixtures. The aqueous seafood flavor concentrate (50 mL) was adjusted to pH 9 with NaOH (20 mL, 2 N). Methylene chloride was added (50 mL), and the nonvolatile compounds were separated from this mixture by evaporation of the volatiles, water, and organic solvent under high vacuum. The organic phase was separated from the aqueous layer, dried with Na_2SO_4, and concentrated to 0.6 mL by microdistillation (*10*) to obtain the SAFE extract. Chemical synthesis of *N*-methyldithiodimethylamine was performed by adapting procedures described by Mukaiyama et al. (*11*) and Kulikovskaya et al. (*12*).

Maillard Reaction Samples. Sample preparation was performed as recently described (*13*) with some modifications. In a 15 mL Pyrex tube, xylose (750 mg) and glycine (375 mg) or alanine (446 mg) were dissolved in a phosphate buffer (5 mL, 0.2 mol/L K_2HPO_4, pH 6.0). The tube was sealed with a screw cap and heated at 90 °C for 1 h in an oil bath under stirring with a magnetic stirrer. The reaction mixture was rapidly cooled with tap water. Then, water (100 mL) and the labeled internal standards (73.0 μg of $^{13}C_2$-HDMF and 18.9 μg of $^2H_3$-EHMF) were added to the dark brown reaction mixture, which was then saturated with NaCl (35 g). The pH was adjusted to 4 (aqueous HCl, 2 mol/L), and the neutral compounds were continuously extracted with diethyl ether (50 mL) overnight using a rotation perforator (Normag, Weinheim, Germany). The organic phase was separated, dried over sodium sulfate at 4 °C, and concentrated to 1 mL using a Vigreux column (50 cm × 1 cm) and a microdistillation device. All experiments were performed in duplicate.

Low-Resolution GC-MS. Conventional GC-MS analysis was performed using a gas chromatograph (HP-5890, Agilent, Geneva, Switzerland) equipped with a splitless injector heated at 260 °C and coupled with a quadrupolar mass spectrometer (HP-5970, Agilent) operated in electron impact ionization mode at 70 eV. Acquisitions were carried out over a mass range of 10−300 Da. Separation was performed on a 100% dimethyl polysiloxane stationary phase (Agilent Ultra-1 PONA, 50 m × 0.20 mm i.d., 0.5 μm film thickness). Helium was used as the carrier gas with a constant flow rate of 0.6 mL/min. The oven was programmed as follows: 20 °C (0.5 min), 70 °C/min to 60 °C, 4 °C/min to 240 °C. The temperature of the transfer line was held at 280 °C during the chromatographic run. The same chromatographic equipment and conditions were used to detect odorous compounds by GC−olfactometry (*14*). Flame ionization detection was performed in parallel to GC-O.

High-Resolution GC-oaTOFMS. All experiments were performed using a Micromass GCT mass spectrometer (Manchester, U.K.) operated in electron impact ionization mode at 70 eV. The GCT is a benchtop, orthogonal acceleration, reflectron, time-of-flight mass spectrometer capable of elevated resolution (7000 full-width at half-maximum height definition). Acquisitions were carried out over a mass range of 50−450 Da with an acquisition rate of one spectrum per second at a resolution of 7000 (fwhm). The source temperature was held at 180 °C. Exact masses were determined using a lock mass at *m*/*z* 201.9609 obtained after continuous infusion of chloropentafluorobenzene during the GC program.

GC analyses were performed using a gas chromatograph (HP-6890, Agilent) equipped with a splitless injector heated at 280 °C and a DB-5MS (J&W Scientific, Folsom, CA) capillary column (5% phenyl, 95% dimethyl polysiloxane stationary phase, 20 m × 0.18 mm i.d., 0.18 μm film thickness). Helium was used as the carrier gas with a constant flow rate of 1.0 mL/min. The oven program was 40 °C (2 min) and then 4 °C/min to 250 °C. The temperature of the transfer line was held at 250 °C during the chromatographic run.

Quantification Experiments. The calibration curve for $^{13}C_2$-HDMF was established with standard mixtures containing defined amounts of unlabeled and labeled compounds in different ratios following the procedure described by Guth and Grosch (*15*). Samples for establishing the calibration curve were injected four times and for quantifying HDMF in the Maillard model reactions, twice.

RESULTS AND DISCUSSION

Identification of Unknown Flavor Molecules. Seafood flavors are known to be very complex, composed of many volatile compounds that occur in a wide concentration range (*16*−*18*). In general, a number of volatile compounds can be identified by conventional GC−quadrupole MS on the basis of retention index and fragmentation pattern. However, in the case of unknowns or compounds for which no reference mass spectrum is available, positive identification remains a challenge to the flavor chemist. Therefore, a seafood sample was selected as a representative example for complex food flavors, which had previously been analyzed by conventional GC−quadrupole MS. Using this technique, several molecules remained unknown. GC-oaTOFMS analysis was focused on these unknowns to evaluate its potential in flavor research, particularly for determining exact masses.

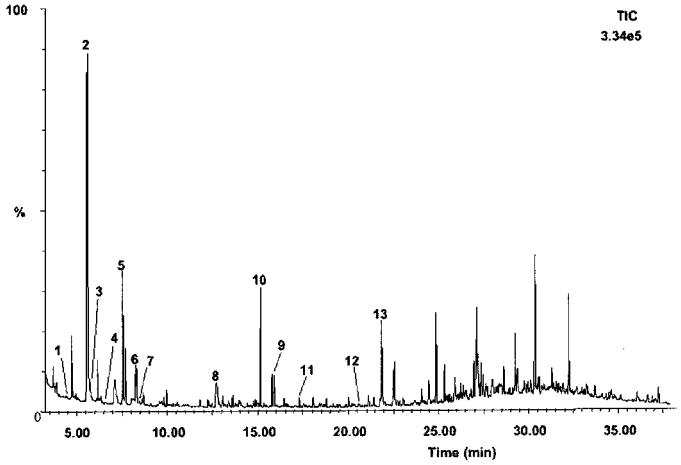

Figure 1. Total ion chromatogram obtained after GC-oaTOFMS analysis of a seafood extract. Numbers correspond to compounds identified by exact mass measurement.

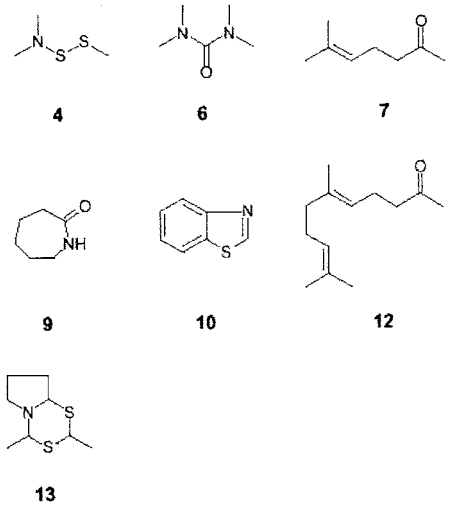

Figure 2. Chemical structures of volatile compounds identified in the seafood aroma extract.

Figure 1 shows the GC-oaTOFMS chromatogram obtained. Some unknown compounds of interest were examined either after selection by GC-O on the basis of their sensory characteristics or because of their weak abundance to test the GC-oaTOFMS sensitivity. Their exact mass spectra and the elemental composition of the major mass ions were determined. For each measured mass ion, a theoretical composition was calculated within a window of ±2 mDa. The results are summarized in **Table 1**, and the structures of all compounds studied are presented in **Figure 2**.

Because of its sensitivity, GC-oaTOFMS allows exact mass measurements of low intensity ions, for example, ions at *m*/*z* 61, 83, and 67 for compounds **4**, **9**, and **10**, respectively. From our past experience, this has never been achieved so easily using a double-focusing sector field instrument. The latter, however, works at a higher resolution power (from 10000 to 35000) and is more accurate as compared to the TOF instrument.

Straighforward measurement of the exact mass of low-intensity ions enables the analyst to propose an elemental composition of each ion obtained in the mass spectrum and, therefore, to pursue structure identification on the basis of not only the molecular ion but also all fragments. This is a valuable piece of information as fragmentation is directly linked to the chemical structure, thus allowing a higher degree of confidence in the identification of unknowns.

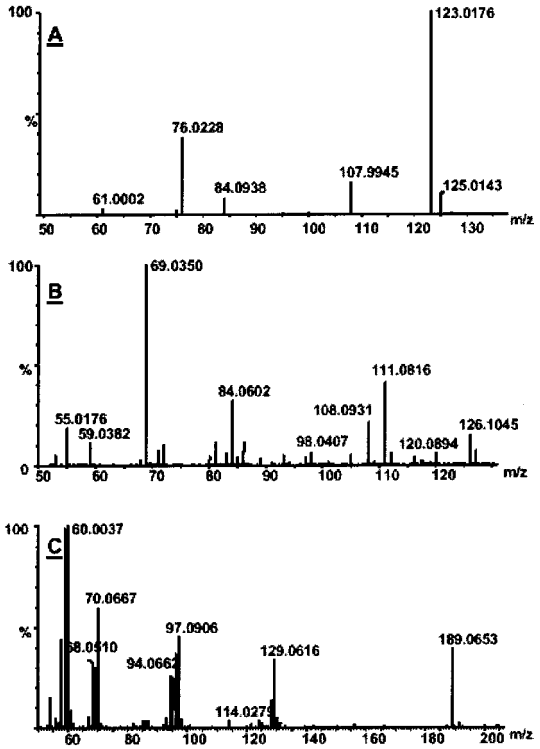

Figure 3. Mass spectra of compound **4** identified as *N*-dimethyldithiodimethylamine (A), compound **7** identified as 6-methyl-5-hepten-2-one (B), and compound **13** identified as tetrahydro-2,4-dimethyl-4*H*-pyrrolo[2,1-*d*]-1,3,5-dithiazine (C).

The deviations observed between the measured mass and the calculated mass of the proposed structure are on average below 1 mDa. These results are in agreement with the data recently published with hybrid instruments (quadrupole−oaTOFMS) working with an electrospray source and showing that good mass accuracies were obtained in MS and MS/MS operation of the TOF instrument (*19*, *20*). The mass accuracy obtained in our experiments is sufficient to obtain the "true" elemental composition identified in the first hit in >90% of the cases. This result is valid because low molecular weight molecules were investigated and, moreover, because the identity of these molecules can be confirmed by other techniques such as GC-O and GC coupled with specific detectors indicating the presence of specific elements. However, this shows that the GC-oaTOFMS technology could be a valuable tool for flavor identification as the compounds of interest have molecular weights around 200 Da with fragment ions in the low mass range.

The mass spectrum of compound **4** (**Figure 3A**) is a typical example of the information that can be obtained by GC-oaTOFMS. The mass spectrum displayed an intense molecular ion measured at *m*/*z* 123.0176 and small fragment ions at *m*/*z* 107.9945 and 76.0228. The molecular ion measured at 123.0176 Da is in agreement with the theoretical formula $C_3H_9NS_2$. The two fragment ions measured at *m*/*z* 107.9945 and 76.0228 allow the theoretical formulas $C_2H_6NS_2$ (theoretical mass 107.9942, difference of 0.3 mDa) and C_2H_6NS (theoretical mass 76.0221, difference of 0.7 mDa), respectively, to be proposed. From these data, the new structure *N*-methyldithiodimethylamine was proposed.

To assess this proposal, we synthesized the *N*-methyldithiodimethylamine following the route depicted in **Figure 4**. Bis-(dimethylamine) sulfoxide **I** was obtained by reacting dimethylamine with thionyl chloride in chloroform. In the presence of phenyl isocyanate, **I** resulted in the betaine **II**, which reacted

Table 1. Investigated Peaks Detected in the Seafood Flavor Extract and Measured at a Resolution of 7000 Using the GC-oaTOFMS[a]

peak	measured mass	relative ion intensity (%)	proposed composition	theoretical mass	deviation (mDa)	identification	odor descriptor
4	123.0176	100.0	$C_3H_9NS_2$	123.0176	0.0	methyldithiodimethylamine	sulfury and alliaceous
	107.9945	18.4	$C_2H_6NS_2$	107.9942	0.3		
	76.0228	31.0	C_2H_6NS	76.0221	0.7		
	61.0002	1.5	CH_3NS	60.9986	1.6		
6	116.0943	39.7	$C_5H_{12}N_2O$	116.0950	–0.7	tetramethylurea	odorless
	72.0446	100.0	C_3H_6NO	72.0449	–0.3		
7	126.1045	12.7	$C_8H_{14}O$	126.1045	0.0	6-methyl-5-hepten-2-one	fruity and blue cheese
	111.0816	40.2	$C_7H_{11}O$	111.0810	0.6		
	108.0931	19.4	C_8H_{12}	108.0939	–0.8		
	84.0602	37.3	C_5H_8O	84.0575	2.6		
	69.0350	100.0	C_4H_5O	69.0340	1.0		
9	113.0834	100.0	$C_6H_{11}NO$	113.0841	–0.7	ϵ-caprolactam	odorless
	84.0572	41.9	C_5H_8O	84.0575	–0.3		
	83.0506	5.9	C_5H_7O	83.0497	0.9		
	67.0552	7.4	C_5H_7	67.0548	0.4		
	56.0261	15.9	C_3H_4O	56.0262	–0.1		
	55.0187	35.7	C_3H_3O	55.0184	0.3		
10	135.0138	100.0	C_7H_5NS	135.0143	–0.5	benzothiazole	slightly rubbery
	108.0037	21.7	C_6H_4S	108.0034	0.3		
	83.0506	5.9	C_5H_7O	83.0497	0.9		
	67.0552	7.4	C_5H_7	67.0548	0.4		
12	194.1683	12.2	$C_{13}H_{22}O$	194.1671	1.2	geranyl acetone	floral and fruity
	176.1576	10.7	$C_{13}H_{20}$	176.1565	1.1		
	161.1331	10.4	$C_{12}H_{17}$	161.1330	0.1		
	151.1122	91.5	$C_{10}H_{15}O$	151.1123	–0.1		
	136.1254	10.6	$C_{10}H_{16}$	136.1252	0.2		
	133.1017	8.3	$C_{10}H_{13}$	133.1017	0.0		
	126.1036	6.2	$C_8H_{14}O$	126.1045	–0.9		
	125.0969	48.4	$C_8H_{13}O$	125.0966	0.3		
	121.1022	17.2	C_9H_{13}	121.1017	0.5		
	108.0922	11.6	C_8H_{12}	108.0939	–1.7		
	107.0864	52.7	C_8H_{11}	107.0861	0.3		
	93.0706	23.1	C_7H_9	93.0704	0.2		
	69.0706	100.0	C_5H_9	69.0704	0.2		
13	189.0653	40.0	$C_8H_{15}NS_2$	189.0646	0.7	tetrahydro-2,4-dimethyl-4*H*-pyrrolo[2,1-*d*]-1,3,5-dithiazine	musty and roasty, crustacean-like
	129.0616	28.1	$C_{10}H_9N_2O_2$	189.0664	–1.1		
	128.0541	10.0	$C_6H_{11}NS$	129.0612	0.4		
	97.0906	60.5	$C_6H_{10}NS$	128.0534	0.7		
	96.0822	26.2	$C_6H_{11}N$	97.0891	1.5		
	95.0737	29.8	$C_6H_{10}N$	96.0813	0.9		
	94.0662	34.7	C_6H_9N	95.0735	0.2		
	70.0667	41.6	C_6H_8N	94.0657	0.5		
	69.0591	33.7	C_4H_8N	70.0657	1.0		
	68.0510	31.3	C_4H_7N	69.0578	1.3		
	60.0037	100.0	C_4H_6N	68.0500	1.0		
	58.9960	93.7	C_2H_4S	60.0034	0.3		
	57.9884	34.5	C_2H_3S	58.9955	0.5		
	56.9808	19.5	C_2H_2S	57.9877	0.7		
	54.0358	11.44	C_2HS	56.9799	0.9		
			C_3H_4N	54.0344	1.4		

[a] For each peak, a tentative identification was proposed considering all ions measured.

with methylmercaptan to form *N*,*N*-dimethyl *N*-phenyl urea **III**, *N*-methylthiosulfoxy dimethylamine **IV**, and *N*-methyldithiodimethylamine **V**. Although the target compound **V** was not the main product obtained, its spectrum was identical to that of compound **4** (data not shown).

These results confirmed the chemical structure of *N*-methyldithiodimethylamine **4** as proposed after interpretation of the fragmentation pattern and considering the elemental composition obtained by GC-oaTOFMS analysis. Moreover, and because of its sensitivity, GC-oaTOFMS allowed the detection of a weak fragment at *m*/*z* 61.0002. Sulfur-containing compounds often exhibit such a typical mass peak corresponding to the fragment C_2H_5S (theoretical mass of 61.011196, difference of 10.99 mDa). The exact mass measurement indicated that this odd-electron fragment is correlated with a formula CH_3NS (theoretical mass of 60.9986, difference of 1.6 mDa) and could be formed by a methyl group rearrangement leading to the odd-electron ion $[CH_3{-}N{=}S]^{\bullet+}$.

Compound **7** was described as fruity by the GC-O analysis, and its mass spectrum is presented in **Figure 3B**. The measurements of the ions at *m*/*z* 126.1045, 111.0816, 108.0931, and 84.0602 were the basis for suggesting the structures $C_8H_{14}O$, $C_7H_{11}O$, C_8H_{12}, and C_5H_8O, respectively. They support the identification of compound **7** as 6-methyl-5-hepten-2-one. For the ion measured at *m*/*z* 69.0350, the closest theoretical formula is C_4H_5O (69.0340; mass difference of 1.0 mDa), whereas the

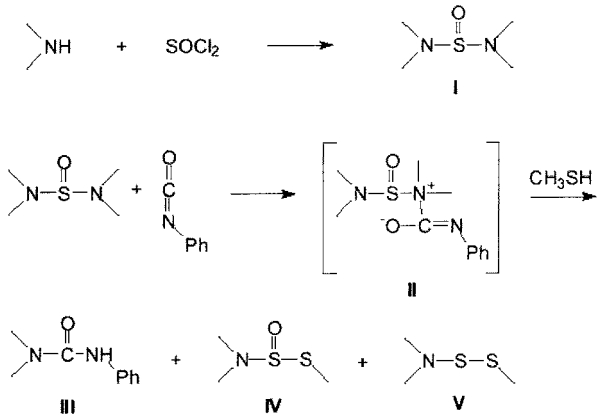

Figure 4. Synthesis pathway of *N*-methyldithiodimethylamine.

alternative structure C_5H_9 can be ruled out (69.0704; mass difference of 35 mDa). The presence of oxygen in this fragment suggests the migration of the double bond to the conjugated position followed by allylic cleavage. Compound **7** is probably isomerized prior to the formation of the even-electron ion at *m*/*z* 69 (M-57).

Compound **13** (**Figure 3C**) is a very potent and well-known compound often present in seafood. It was easily identified as tetrahydro-2,4-dimethyl-4*H*-pyrrolo[2,1-*d*]-1,3,5-dithiazine because of the typical fragmentation of dithiazines. The various mass peaks generated by the fragmentation of these byciclic dithiazines have been described by Werkhoff (*21*). The ions at *m*/*z* 129.0616, 97.0906, and 70.0667 correspond to the formulas $C_6H_{11}NS$, $C_6H_{11}N$, and C_4H_8N. Unlike the spectrum obtained from quadrupole mass spectrometers (from Werkhoff's article or from our own acquisition), where no clear mass peak indicates the alkyl group in 2-position, a specific peak (*m*/*z* 60.0037) of the spectrum from the GC-TOF shows the side chain associated with the fragment CH−S of the ring. Surprisingly, this ion is even the base peak of the mass spectrum obtained with the GC-TOFMS and confirms the presence of a methyl group in the 2-position.

Quantitative Experiments. The potential of the orthogonal acceleration TOFMS technology for quantitative measurements has recently been investigated using small molecules separated by reversed phase liquid chromatography and ionized into an atmospheric pressure ionization interface (*22*). With this instrumental setup, it has been shown that excellent sensitivity and a linear dynamic range of nearly four decades were obtained. As capillary GC requires faster acquisition rates than HPLC (because of their respective peak widths), we evaluated our GC-oaTOFMS for quantitation. This work has been carried out with furanones because of their low sensory threshold and therefore their important contribution to many flavors, even if present at low concentrations. We applied the isotope dilution assay (IDA) as quantitation method (*8*, *15*, 23), which is based on the use of labeled internal standards to minimize losses of labile aroma compounds, such as furanones, during sample preparation. Calibration curves were found to be linear over a dynamic range of 10^3 with correlation coefficients >0.9997 and all values comparable to those determined with quadrupole instruments (*8*). The method was applied to Maillard model reaction mixtures. The obtained data confirmed earlier results (*8*); that is, higher amounts of HDMF were found in the system xylose/glycine, and the formation of HEMF was favored in the xylose/alanine model reaction (**Table 2**). The variation coefficients are from about 5 to 30%, depending on the concentration, which is mainly due to the limited repeatability of Maillard reaction samples. A particular advantage of monitoring exact masses for quantification purposes is the possibility to discriminate interfering compounds having the same nominal masses. Furthermore, not only can the molecular mass be used but also smaller fragments having a characteristic elemental composition for the given compound.

Table 2. Quantification of HDMF and HEMF in Model Maillard Reactions[a]

Maillard system	HDMF	HEMF
xylose/glycine	16.6 ± 4.0	0.3 ± 0.1
xylose/L-alanine	4.7 ± 0.5	8.5 ± 0.6

[a] Results are expressed in μg/mmol sugar and are the mean of six measurements.

In conclusion, GC-oaTOFMS may become a powerful analytical tool for the flavor chemist for both identification and quantification purposes, in particular when combined with the IDA method.

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