Automated Solid-Phase Microextraction–Gas Chromatography–Mass Selective Detection Investigations of Carbonyl–Amine Reaction Pathways

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Abstract

Employing automated solid-phase microextraction–gas chromatography–selected ion monitoring-mass selective detection, mechanistic information on the formation of volatile components associated with carbonyl–amine chemistries in microwave heat-treated natural products is gathered. Employing ^{13}C uniformly labeled amino acids and sugars, pathways for the formation of Strecker aldehydes, pyrazines, and furan derivatives are delineated in a heat treated licorice suspension. The results are among the first pieces of information providing unambiguous reaction pathways for the production of these compounds in heated natural products.

Introduction

Embedded within all natural products, both plant and animal alike, are two abundant classes of compounds: nitrogen, containing molecules such as amino acids and amines, and monosaccharides, such as fructose and glucose. Although the major roles of these reagents in living tissue are well established, their roles in the heat treatment of natural products is also very important. That is, under the appropriate conditions, these carbonyl- and amine-containing reagents react to produce a complex series of volatile, semivolatile, and nonvolatile compounds, some of which have very powerful sensory impact at very low concentrations (1). In particular, the reaction between amino acids and sugars was first investigated in detail by Maillard around the turn of the century, and the reaction resulting from his pioneering work (that is, the reaction between amino acids and sugars) is generally referred to as the Maillard reaction (2–6). Reaction variables such as pH, moisture content, temperature, concentration, and time can be used to produce (in model and natural systems) a wide array of volatile, semivolatile, and nonvolatile compounds, including aldehydes, ketones, pyrazines, pyridines, furans, and diketones. (7–27).

Extensive sample work-up via solvent extraction, liquid–liquid extraction, headspace, or cold trapping of the reaction products followed by analysis using either gas chromatography–mass spectrometry (GC–MS) or high-performance liquid chromatography (HPLC) have been the traditional approaches used to characterize volatile and nonvolatile carbonyl–amine and Maillard reaction products (28). An alternative approach to the use of the extensive sample work-up is solid-phase microextraction (SPME) (29). Since its introduction, SPME has been applied across a diverse array of analytical determinations, including organic solvents in water (30), explosives (31), flavors (32–34), pesticides (35), and Maillard reaction products (37,38). These applications have shown SPME to be a convenient and efficient extraction method. Theory and applications have shown the fibers provide quantitative analysis in equilibrated and nonequilibrium situations (36). The introduction of an automated SPME (AutoSPME) injection system has further advanced the usefulness of the SPME approach.

Recent findings based on results from AutoSPME–GC–selected ion monitoring (SIM)-MSD analyses provided the first evidence of the unambiguous assignment of a mechanism for the formation of pyrazines in microwave heat-treated natural product suspensions (39). The results from this investigation delineated the roles of selected nitrogen sources in the production of volatile and semivolatile pyrazines in heat-treated cocoa and licorice suspensions.

The results reported here further expand the understanding of the mechanisms involved in the synthesis of Strecker aldehydes, pyrazines, and furan derivatives in heat-treated natural products. Through the use of ^{13}C labeled compounds in conjunction with analysis by AutoSPME–GC–SIM-MSD, the role (or roles) of selected amino acids and sugars in the synthesis of volatile compounds in heat-treated natural products is delineated.

Experimental

Reagents

The pyrazines were obtained from Aldrich Chemical Company (Milwaukee, WI). The ^{13}C labeled compounds were obtained from Cambridge Isotope Laboratories (Andover, MA). All reagents were used as received.

Licorice powder was obtained from Mafco Worldwide Corporation (Camden, NJ) and used as received.

Sample Preparation

Suspensions

Heat-treated licorice powders were prepared as 15% aqueous suspensions by adding 4.5 g of the powder to 25.5 g of deionized water within a vessel especially designed for microwave heat treatment vide infra. After gently swirling, the suspension was placed in the microwave for heat treatment.

Microwave system

The microwave reactions were performed in a CEM Corporation (Matthews, NC) model MES-1000 microwave extraction oven. The microwave power was set at the maximum, 950 ± 50 watts, at a frequency of 2450 MHz. The reactions were performed in sealed vessels at 175°C for a period of 30 min. The reaction temperature was attained slowly over a period of 10 min, employing the software capabilities of the CEM instrument. The pressure (100–150 psi) of the sealed vessel was monitored but not controlled. Specially designed microwave transparent-lined vessels (CEM Corporation) were employed and assembled strictly following the manufacturer's instructions. The sealed vessels were placed on a turntable within the oven. The turntable was cycled back and forth during the course of the run to ensure even distribution of the microwaves. After heat treatment, the samples were allowed to return to room temperature prior to opening. Once removed from the microwave, the samples were either analyzed immediately or stored in a refrigerator prior to analysis.

Analysis instrumentation and methodology

AutoSPME–GC–MSD

A Varian (Santa Clara, CA) 8200 CX AutoSampler with SPME III Sample Agitation was mounted atop a Hewlett-Packard (Palo Alto, CA) HP 5890 series II Plus GC equipped with an HP 5972 MSD operating either in the electron impact mode at 70 eV or in the SIM mode. The GC was fitted with a DB-Wax (J&W Scientific, Folsom, CA) fused-silica column (30 m × 0.25-mm i.d., 0.5-µm film thickness). The MSD interface and GC injection port temperatures were 250°C. The GC oven was temperature-programmed from 40 to 140°C at 5°C/min, then to 220°C at 10°C/min and held there for 4 min. The injection port was fitted with a narrow-bore liner. Splitless injections were made, and the split was opened after 1 min. The fiber was automatically submerged in the solution, vibrated for 0.75 min, removed, injected, and held in the injection port for 30 min, employing the parameters set via the operating software. This 30-min holding time was selected to simplify the timing commands of the various components of the instrument configuration. Fresh samples were used for every injection. Under these operating conditions, no fiber performance degradation was noted for at least 100 injections. After approximately 100 injections, the injection port was replaced. For 6 replicate injections of the same solution contained in 6 separate vials, the SIM area count for methylpyrazine had a percent relative standard deviation (%RSD) value of 6.5.

SPME fibers for these automated injections were obtained from Supelco (Bellefonte, PA) and employed strictly following the manufacturer's instructions. Polydimethylsiloxane (PDMS), carbowaxdivinylbenxene, carboxen/PDMS, and PDMS/divinylbenzene SPME fibers were evaluated in this study much in the same manner as previously described (48). The PDMS SPME fiber, having a film thickness of 100 µm, was selected based on the consistency of response, lack of measurable carryover, and extraction capability.

Prior to analysis by AutoSPME–GC–MSD, the aqueous heat-treated suspensions were manually filtered through a Whatman (Milford, MA) Autovial equipped with a 0.45-µm PVDF (polyvinylidenefluoride) filter designed for use with aqueous solutions. Then, 1.7 mL of the filtered solution was added via a Rainin (Woburn, MA) EDP Plus Motorized Microliter Pipet to 1.8-mL vials equipped with Teflon-lined septa. Strict attention to consistant addition of 1.7 mL was necessary to obtain reproducible results. The 1.7-mL volume was selected to minimize the headspace inside the 1.8-mL vial yet allow for enough room for extensive movement of the solution during the vibration of the fiber. No further optimization experiments of the vial volume were attempted. The charged vials were loaded on the sample carousel and automatically sampled employing the instrumentation software provided by Varian and HP. In some cases, it was necessary to dilute the 15% heat-treated suspensions with water to obtain reproducible fiber performance.

SIM was used for the analysis of selected pyrazines and furans in the heat-treated suspensions. The C2, C3, and C4 notations preceding the pyrazines are used to denote a class of pyrazines. For example, C2 pyrazines would include substituted pyrazines, such as all of the dimethylpyrazines as well as ethylpyrazine. In all of these cases, the pyrazines have two carbons (C2) attached in some fashion to the fundamental pyrazine molecule. Identical arguments are used for the C3 and C4 terms.

Results and Discussion

Recent information on the treatment of natural products in microwave ovens has yielded some insights into the impact of reaction parameters on the production of volatile and semi-volatile carbonyl–amine and Maillard reaction products (40–46). However, no fundamental mechanistic information has appeared from these studies. This report represents some of the first fundamental mechanistic information available on microwave heat-treated natural products directed specifically at understanding the production of Strecker aldehydes, pyrazines, and sugar (carbonyl) thermal degradation processes.

Mechanistic studies: ^{13}C Strecker aldehyde formation

One of the volatile byproducts of the reaction of certain amino acids with sugars is the Strecker aldehyde. When valine, leucine,

isoleucine, phenylglycine, and phenylalanine react with fructose, for example, at elevated temperatures, the following aldehydes are produced, respectively: 2-methylpropanal, 3-methylbutanal, 2-methylbutanal, benzaldehyde, and benzeneacetaldehyde (7). Figure 1 provides a pictorial representation of the relationship between the structure of two selected amino acids and their resulting Strecker aldehyde. To ascertain if the reaction mechanism was viable in licorice heat-treated suspensions, selected uniformly labeled ^{13}C amino acids were added to the licorice suspension and, following microwave heat treatment, the resulting total ion chromatograms from the AutoSPME–GC–MSD experiment were examined for the presence of the corresponding uniformly labeled ^{13}C aldehyde. The SPME fiber was preferably immersed in the sample rather than being suspended in the headspace above the sample, mainly because shorter exposure times were required to extract a sufficient amount of material. The presence of a relatively large quantity of 2-methylpropanal (retention time, approximately 3.5 min) in the heat-treated aqueous licorice suspension containing 1% (w) uniformly labeled ^{13}C valine was observed (Figure 2). If the 2-methylpropanal originated from the uniformly labeled ^{13}C valine, then the mass spectrum of the parent ion should have a mass-to-charge ratio of 76 versus 72 for the naturally abundant 2-methylpropanal. The mass spectrum of the 2-methylpropanal in the uniformly labeled ^{13}C valine/licorice reaction mixture revealed the parent ion of 2-methylpropanal to be *m/z* 76, thus confirming that the 2-methylpropanal appearing in the ^{13}C valine/licorice reaction mixture was derived from the uniformly labeled ^{13}C valine (Figure 3). Similar findings were discovered for the other amino acids capable of yielding Strecker aldehydes. These results confirm for the first time an unambiguous link between the presence of Strecker aldehydes and amino acids in a microwave-assisted heat-treated natural product. These results with the labeled amino acid are very similar to those obtained by Arnoldi, et al. (47) using naturally abundant amino acids in lipid formulations.

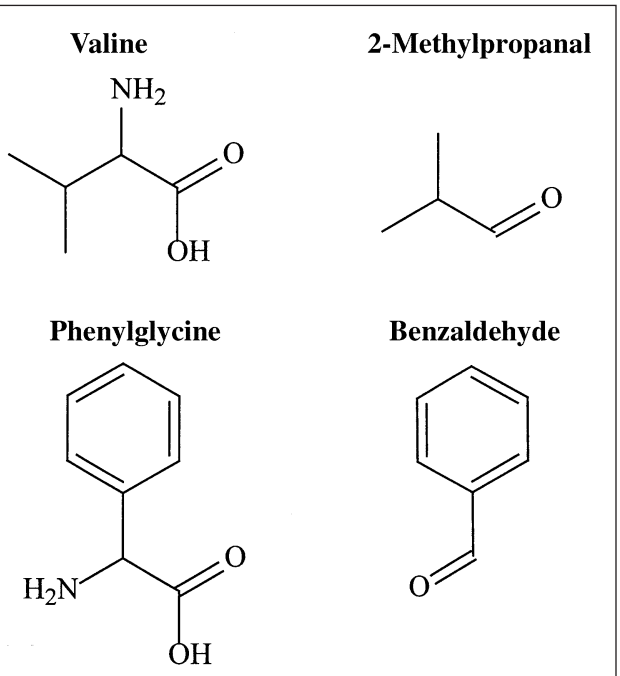

Figure 1. Relationship between selected amino acids and corresponding aldehydes.

Mechanistic studies: ^{13}C pyrazine formation

Sugars such as glucose and fructose have long been confirmed in model studies as the sources of the carbons that appear in the pyrazine structures (16). To date, no evidence has appeared establishing this link in a microwave heat-treated natural product. Thus, 1% uniformly ^{13}C labeled glucose and fructose were added to two licorice suspensions, respectively, followed by microwave heat treatment. An accepted mechanism for the synthesis of pyrazines involves the coupling of two intermediate molecules to yield the resultant pyrazine (Figure 4). Thus, methylpyrazine can be envisioned as arising from one molecule (A) containing 2 carbon atoms and another molecule (B) containing 3 carbon atoms. Should the source of these carbons now found in the completed methylpyrazine molecule arise from a precursor compound containing uniformly labeled ^{13}C , then the resulting methylpyrazine would possess increases in mass of either 2 or 3 amu, depending on the extent of incorporation of the 2- or 3-carbon-containing units. For example, naturally abundant methylpyrazine displays a molecular ion at *m/z* 94. Should the methylpyrazine be produced from a carbon source uniformly ^{13}C labeled, then increases in mass abundances would display themselves in the form of the presence of ions at *m/z* 96, 97, or 99, corresponding to the incorporation of a 2-, 3-, or both a 2- and 3-carbon-containing unit. Similar arguments and

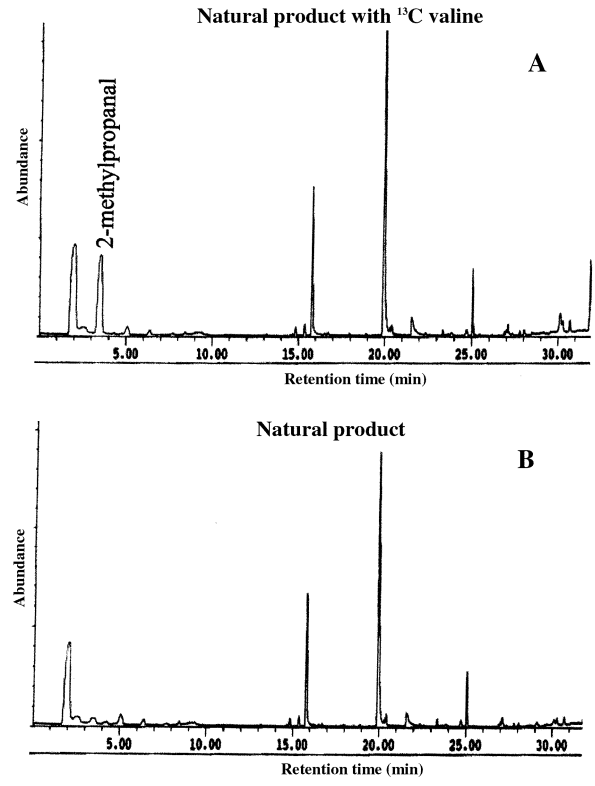

Figure 2. Total ion chromatograms from AutoSPME–GC–MSD analysis of aqueous natural products with (A) and without (B) the addition of ^{13}C valine.

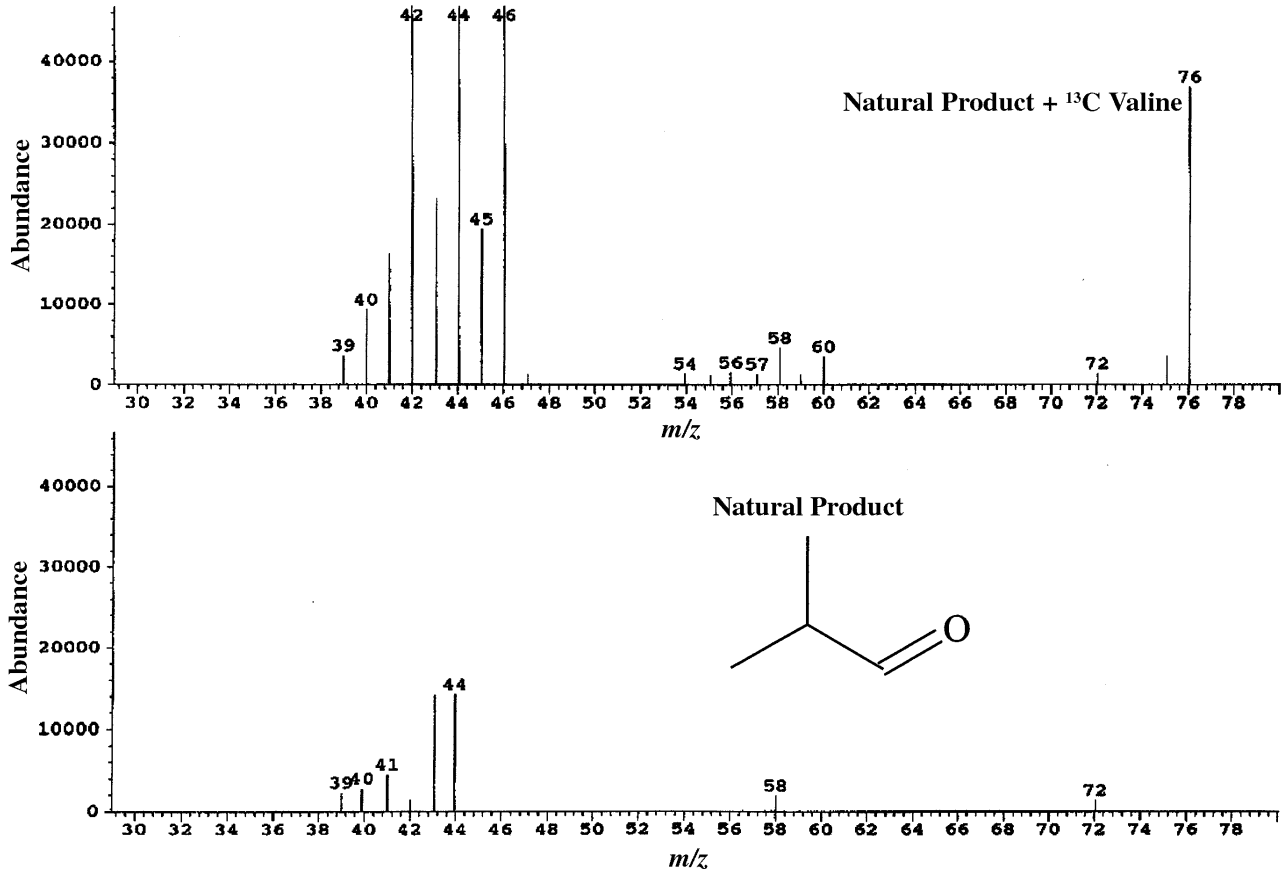

Figure 3. Mass spectra of 2-methylpropanal present in the aqueous natural products with and without the addition of ^{13}C valine.

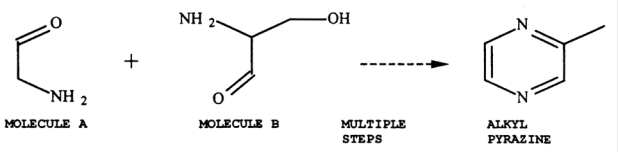

Figure 4. Mechanism for the synthesis of pyrazines.

deductions can be made for all of the pyrazines found in the heat-treated suspensions based on their individual structures.

Thus, an array of microwave assisted heat-treated licorice suspensions containing 1% (w) uniformly labeled ^{13}C glucose and fructose were prepared (Table I). The percent ion abundances for selected pyrazines produced during the microwave assisted heat-treatment of a licorice suspension clearly confirmed the incorporation of the ^{13}C label into the pyrazine molecules. The possible presence of naturally abundant sugars at the percent level in licorice prevents the absolute assignment of the degree of ^{13}C label incorporation; however, this does not preclude the confirmation of the ^{13}C label incorporation. For example, in a few cases, the data did reasonably indicate that fructose was more effective than glucose in yielding its carbon atoms for incorporation into the pyrazine molecules. For example, in the case of 2-ethyl-6-methylpyrazine, the abundance of ions attributable to ^{13}C label incorporation were consistently much greater from fructose than glucose. The same might be said for 2,6-dimethylpyrazine as well. For the remaining pyrazines, no clear indications regarding preferential incorporation of fructose or glucose residues were evident.

The data also provides for further insight into the building of the pyrazine molecules from the sugar sources. The ion abundances from methylpyrazine strongly indicated that at least one 2-carbon unit from both uniformly ^{13}C labeled glucose and fructose was incorporated into the molecule, evidenced by the observed increase in abundance of the ions at *m/z* 96 relative to the abundance of methyl pyrazine with no added label. Adequate changes in the abundance of *m/z* 97 confirm the incorporation of one 3-carbon unit as well. The evidence for simultaneous incorporation of both a 2- and 3-carbon into methylpyrazine was not as clear. Thus, the mechanistic pathway depicted by Figure 4 seems very clearly viable in light of these findings.

For 2,6-dimethylpyrazine, a symmetrical molecule (the evidence for incorporation of the carbon label from both sugar sources) was rather convincing. A dramatic increase in the abundance percentage due to *m/z* 111 relative to the natural sample was found for both the fructose and glucose samples (Table I). This observation was also consistent with Figure 4 in that, should one side of the 2,6-dimethylpyrazine be built from a 3-carbon unit with ^{13}C atoms, then an increase in molecular weight of 3 should be observed (i.e., *m/z* 108 to *m/z* 111). For 2-ethyl-6-methypyrazine (an asymmetrical molecule), the labeled carbons from both the glucose and fructose found their way into the molecule, as evidenced by increased ion abundances at *m/z* 125 and 126. The increase at *m/z* 125 corresponds to the

Table I. Percent Ion Abundances from Selected Pyrazines in Heat-Treated Licorice Suspensions Containing Uniformly ^{13}C-Labeled Sugars

Ions monitored (*m/z*)	Natural	U ^{13}C Glucose*	U ^{13}C Fructose
Methylpyrazine			
94	98.72	92.32	92.09
96	0.55	3.91	3.02
97	0.36	3.23	4.42
99	0.37	0.54	0.46
2,6-Dimethylpyrazine			
108	82.55	78.61	76.51
109	8.87	8.58	8.71
110	3.24	3.57	3.74
111	2.76	6.74	8.48
114	2.58	2.51	2.57
Ethylpyrazine			
107	4.78	4.44	4.45
108	82.21	78.69	76.77
109	7.07	7.04	6.93
110	1.4	2.36	2.26
111	1.17	4.33	6.13
112	1.15	1.15	1.25
113	1.12	0.97	1.03
114	1.09	1.02	1.19
2,3-Dimethylpyrazine			
108	77.96	68.67	70.05
109	8.45	12.69	11.47
110	3.83	6.47	5.83
111	3.4	5.37	5.64
112	3.25	3.88	4.06
114	3.11	2.91	2.95
2-Ethyl-6-methylpyrazine			
121	55.44	51.41	41.94
122	30.93	29.86	26.47
123	4.07	5.37	6.17
124	1.97	4.07	5.79
125	1.84	3.12	6.17
126	1.84	2.21	4.56
128	1.93	1.95	4.27
129	1.97	2.03	4.65
2-Ethyl-5-methylpyrazine			
121	45.89	42.29	20.96
122	27.57	26.3	47.9
123	5.89	6.26	8.04
124	4.11	5.86	4.96
125	4.02	5.8	6.16
126	4.02	4.53	3.93
128	4.21	4.43	3.93
129	4.3	4.62	4.11
2-Ethyl-3,6-dimethylpyrazine			
135	25.82	23	22.49
136	19.28	18.29	17.82
137	9.29	9.23	9.34
138	7.75	8.36	8.48
139	7.75	8.54	8.48
140	7.23	7.49	7.61
141	8.26	8.36	8.3
143	7.23	7.49	7.44
144	7.4	9.23	10.03

* U ^{13}C Glucose, uniformly labeled carbon-13 glucose.
† U ^{13}C Fructose, uniformly labeled carbon-13 fructose.

incorporation of one uniformly labeled 3-carbon unit, and the increase at *m/z* 126 corresponds to the incorporation of one uniformly labeled 4-carbon unit. There appeared to be strong indications (at least in the case of the fructose experiment) that there was an increase in the number of molecules built from simultaneous incorporation of both one uniformly labeled 3-carbon unit and one uniformly labeled 4-carbon unit. Notice for 2-ethyl-6-methypyrazine, an ion abundance percentage of 4.65 for *m/z* 129 in the uniformly labeled fructose experiment, compared with an abundance of 1.97 for the natural system. The corroboration of incorporation of the ^{13}C atoms into a C4 pyrazine such as 2-ethyl-2,6-dimethylpyrazine was not as firm as with the other lower-molecular-weight pyrazines. There was only a very slight indication that one 3-carbon unit may have been incorporated into the molecule (i.e., a slight increase in the abundance of *m/z* 139 relative to the natural sample).

Mechanistic studies: ^{13}C furfurals formation

Accompanying the formation of Strecker aldehydes and pyrazines during the heat treatment of natural products is the formation of selected volatile and semivolatile furan derivatives. These furans can form from a variety of pathways, but one of the dominant ways involves the thermal degradation of sugars. When heated, fructose and glucose have been shown to form furan derivatives such as furfural, 5-methylfurfural, 5-hydroxymethylfurfural, and furan methanol (1) in model and natural systems. Furfural is a typical caramelization product of sugars and is one of the most widely distributed food flavor constituents. It has a characteristic "toasted" penetrating odor and is used as a flavor additive (52,53). Thus, based on its sensory impact, knowledge of the source and subsequent distribution of furfurals would be of benefit to the design of new aroma/flavor formulations.

The addition of uniformly ^{13}C labeled fructose and glucose at the 1%-level (w) to the licorice suspension was also used to examine the possible production of furfurals through the conversion of these reagents to uniformly labeled furfural, 5-methylfurfural, and furanmethanol during the microwave-assisted heat treatment. The SIM approach was essentially the same as employed with the labeled N studies, involving the construction of SIM tables from the ion abundance pattern of the compounds in the control samples and samples with the ^{13}C labeled fructose and glucose. For example, the ion pattern associated with furfural in the natural state is dominated by *m/z* 94, 95, and 96, with some minimal contribution from ions at *m/z* 99, 100, and 101. Should the furfural be derived from the ^{13}C labeled fructose and glucose, then increases in the ion abundances at *m/z* 99, 100, and 101 would be expected in the SIM chromatograms. Examination of the AutoSPME–GC–SIM-MSD data for the three compounds in the licorice heat-treated samples containing the uniformly ^{13}C labeled fructose and glucose indicated a conversion of the labeled compounds to the three compounds examined (Table II). For example, 1.58% of the furfural in the uniformly ^{13}C labeled fructose–licorice heat-treated experiment was found to be uniformly labeled. Likewise, 1.30% of the 5-methylfurfural was found to be uniformly labeled in the ^{13}C labeled glucose–licorice heat-treated experiment. Thus, direct evidence exists for the conversion of fructose and glucose to fur-

Table II. Percent of Uniformly ^{13}C Labeled Furan Derivatives in Heat-Treated Licorice Suspensions with Uniformly Labeled ^{13}C Fructose and Glucose

Furan derivative	Sugar added	
	Fructose	Glucose
Furfural	15.80	0.87
5-Methylfurfural	2.53	1.30
Furanmethanol	2.96	5.83

furals in these heat-treated licorice experiments. The determination of the absolute degree of conversion of sugars to furfurals in natural products is difficult because of the presence of thermally labile, naturally occurring mono- and disaccharides in the natural products.

Conclusion

Mechanistic information on the formation of volatile components associated with the Maillard and carbonyl (sugar)–amine chemistries in microwave-assisted heat-treated natural products has been gathered by employing SPME–GC–SIM-MSD. The effectiveness of selected ^{13}C sources as sources for the formation of selected volatile pyrazines in microwave-assisted heat-treated natural products was convincingly demonstrated using AutoSPME–GC–SIM-MSD. Employing uniformly ^{13}C labeled fructose and glucose, a portion of the carbons found in the pyrazines were shown to arise from the added sugars. The conversion of portions of the added sugars to the comparable furan derivatives was observed. These results have shed new light on the possibilities of designing and synthesizing new aroma/flavor formulations from natural products via microwave-assisted heat treatment, and they confirm that AutoSPME–GC–MSD is a viable approach for the study of selected important reaction mechanisms involving the production of key sensory components in heated natural products.

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