An Automated Solid-Phase Microextraction–Gas Chromatography–Mass Selective Detection Approach for the Determination of Sugar-Amino Acid Reaction Mechanisms

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Abstract

Confirmation of the proposed mechanisms of Strecker aldehyde involvement in the production of branched-chain alkyl-substituted pyrazines is easily accomplished using an automated solid-phase microextraction–gas chromatography–mass selective detection (AutoSPME–GC–MSD) approach. The method is relatively elementary with minimal sample preparation. Microwave heated aqueous formulations require only the addition of NaCl to "salt-out" the analytes prior to analysis by AutoSPME–GC–MSD. A 100-µm polydimethylsiloxane fiber with accompanying fiber vibration can extract ample quantities of the compounds of interest in as little as 1 min. Such sensitivity negates the requirements for time and reagent-consuming sample extraction via organic solvents or concentration using heat. This approach, therefore, should find additional expanded applications in establishing reaction pathways.

Introduction

Manual and automated solid-phase microextractions (SPME), in combination with separation and detection by gas chromatography (GC) and mass spectrometry, have been reported to provide excellent qualitative and quantitative analyses of a wide array of analytes dispersed in a number of different matrices (1–12). SPME is a relatively new, solvent-free method of sample preparation that involves analyte extraction followed by the thermal desorption of analytes usually within the heated injection port of a GC. The performance characteristics of a number of SPME fibers for the extraction and determination of water-soluble Maillard reaction products have been described in some of these earlier reports (4,5). The volatile products of Maillard and other sugar–nitrogen reactions, as well as sugar thermal degradation products, have been closely linked to the sensory characteristics of cooked materials such as peanuts, bread, and coffee for some time (13,14). Some of the more recent work (15–17) on unraveling the mechanisms associated with the formation of the volatile materials has involved headspace approaches and the use of organic solvent extraction of the reaction mixture followed by concentration and analysis by GC–mass selective detection (MSD). These works have shown that the nature of the nitrogen source influences the quantity and type of pyrazines formed. The amount and type of pyrazines have been linked to sensory attributes of sugar–amino acid reaction formulations. SPME has been employed in the detection of low-molecular-weight pyrazines in a series of sugar–amino acid (glucose–glycine) model microwave reactions (18). To date, however, no report employing automated SPME (AutoSPME) as a sample preparation technique for the determination of reaction mechanisms in sugar–amino acid reactions has appeared. This report will describe how AutoSPME in combination with GC and MSD (AutoSPME–GC–MSD) can be effectively employed in the understanding of reaction mechanisms between amino acids and sugar sources in the production of relatively high-molecular-weight pyrazines.

Experimental

Instrumental

The AutoSPME–GC–MSD analyses were performed using a Varian Instruments (Walnut Creek, CA) 8200 vibrating SPME III autosampler fitted with a selected SPME fiber from Supelco (Bellefonte, PA) mounted atop a Hewlett-Packard (Palo Alto, CA) 5890 GC. The GC was fitted with either a J&W Scientific (Folsom, CA) DBWAXETR capillary column (30 m × 0.25-mm i.d., 0.25-µm film thickness) or a J&W Scientific DB1701 capillary column (30 m × 0.32-mm i.d., 1-µm film thickness). A linear velocity of approximately 35 mL/min was employed. The AutoSPME injections were performed in the splitless mode. The splitter was opened 1 min after injection.

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Table I. AutoSPME Results for Specific Pyrazines in Selected Microwave-Heated Formulations

Pyrazine type detected via AutoSPME–GC–MSD	HFCS–DAP	HFCS–DAP–LEU	HFCS–DAP–ILE	HFCS–DAP–VAL
Pyrazine	3	3		
Methyl	3	3	3	3
2,5-Dimethyl	3	3	3	3
2,6-Dimethyl	3	3	3	3
Ethyl		3	3	3
2,3-Dimethyl	3	3	3	3
2-Ethyl-6-methyl	3	3	3	3
2-Ethyl-5-methyl	3	3	3	3
Trimethyl	3	3	3	3
Vinyl	3			
Propyl				
2-Methyl-5-(1-methylethyl)		3		
2-Methylpropyl				
2,6-Diethyl		3	3	3
3-Ethyl-2,5-dimethyl		3	3	3
2-Ethyl-3,5-dimethyl			3	
2,5-Diethyl		3	3	3
2,3-Diethyl				
2,3-Dimethyl-5-ethyl		3		
2-Ethyl-3,5-dimethyl				
2-Vinyl-6-methyl	3			
2-Vinyl-5-methyl		3	3	
2,3,5-Trimethyl-6-ethyl		3		
2,6-Dimethyl-3-vinyl(tent)		3		
2-Methyl-6-propyl				
2-Methyl-3-propyl				
2-Methyl-3-(2-methylpropyl)isomers				3
Tetramethyl		3		3
2-Ethenyl-6-methyl+3,5-diethyl-2-methyl		3	3	3
2,3-Diethyl-5-methyl+2,3-diethyl-6-methyl				3
2,5-Dimethyl-3-(2-methylpropyl)isomers			3	3
3,5,6-Trimethyl-2-(2-methylpropyl)isomers				3
2,3-Dimethyl-3-propyl			3	
2,3-Dimethyl-5-propyl				
2,6-Dimethyl-6-propyl				
2-Methyl-3-(1-propenyl)tent				
2-(2-Methylbutyl)			3	
2-(3-Methylbutyl)		3		
2-Methyl-3-(2-methylbutyl)isomers			3	
2-Methyl-3-(3-methylbutyl)isomers		3		
2-Methyl-6-(1-propenyl)tent				
2,5-Dimethyl-3-(2-methylbutyl)isomers			3	
2,5-Dimethyl-3-(3-methylbutyl)isomers		3		
2,5-Dimethyl-6,7-dihydro-5H-cyclopenta		3	3	
Methylcyclopenta			3	3
Dimethylcyclopenta				
2,3,5-Trimethyl-6-(3-methylbutyl)		3		
2,5,6-Trimethyl-3-(2-methylpropyl)isomers			3	
Cyclopenta				
2,5,6-Trimethyl-3-(2-methylbutyl)isomers				
2,5,6-Trimethyl-3-(3-methylbutyl)isomers				
2,5-Dimethyl-3-propenyl				
Methylcyclohexa		3		3
Dimethylcyclohexa		3	3	

To a 1.8-mL vial equipped with a poly(tetrafluoroethylene) (PTFE)/silicon-lined septum was added 1.7 mL of the sample. The fibers were immersed at room temperature (~24°C) in 1.7 mL of sample for 1 min with vibration prior to injection. The GC oven temperature was held at an initial value of 40°C for 1 min, then programmed to 220°C at 2°C/min. The GC injection port and MSD interface were held at 250°C. The mass spectrometer was operated in the electron impact mode at 70 eV.

The 100-µm polydimethylsiloxane (PDMS) SPME fibers were activated, stored, and handled strictly following the manufacturer's instructions.

Reagents

High fructose corn syrup (HFCS) was obtained from National Starch and Chemical Company (Bridgewater, NJ). Diammonium phosphate (DAP), sodium chloride, isoleucine (ILE), valine (VAL), and leucine (LEU) were obtained from Aldrich Chemical Company (Milwaukee, WI). ^{13}C labeled leucine (^{13}C LEU) was obtained from Cambridge Isotope Labs (Andover, MA). All reagents were used as received.

Sample preparation

To generate the reaction products between the amino acids and sugars, a microwave-based heat treatment protocol was employed (19). To 30 mL of water was added 0.81 g of HFCS, 0.25 g of DAP, and 0.25 g of LEU. The solution was microwave heated at 175°C in a sealed microwave-permeable vessel for 30 min. The special vessel was obtained from CEM Corporation (Matthews, NC) and assembled closely following the manufacturer's instructions. A ramp time of 10 min was used to bring the solution from room temperature to 175°C. The microwave reactions were performed in a CEM Corporation model MES-1000 system. The microwave power was set at maximum (950 ± 50 watts at a frequency of 2450 MHz). A turntable located inside the oven cycled back and forth during the run to insure even adsorption of the microwave energy.

Microwave heat-treated samples were either analyzed immediately after returning to room temperature or stored in a freezer (–18°C) prior to analysis.

Just prior to analysis by AutoSPME, 2.5 mL of the sample was placed in a 20-mL vial, followed by the addition of approximately 1 g of NaCl. The vial was then placed on a vortex mixer, followed by filtration through a Whatman 0.45-µm Autovial Filter. Then, 1.7 mL of the clear solution was transferred to a 2.0-mL screw top clear vial with a hole cap and PTFE/silicone septum (Supelco) using a Rainin Autopipet.

The vial was sealed and placed in the Varian autosampler "puck".

Data collection and analysis

The operation of the AutoSPME autoinjector/tower was controlled via software provided by Varian. The Varian software was also employed to activate the automated collection of mass spectral data via the Hewlett-Packard mass spectral data collection software.

Compound identification was facilitated through the use of GC retention time databases and mass spectral search results from both the Wiley and NBS libraries of mass spectral data available with the GC software package.

Results and Discussion

Recently, some specific, relatively high-molecular-weight pyrazines were found in reaction systems containing sugar sources and the amino acids phenylalanine and ILE (15). In particular, the report described a modified headspace procedure followed by GC–MSD analysis for the determination of pyrazines with branched alkyl chains attached. Pyrazines containing branches such as 2-methylbutyl- were detected in the reactions containing the ILE. It was postulated that the Strecker aldehyde from ILE, 2-methylbutanal, was incorporated into the pyrazine molecules. Based on additional work (17) with similar systems involving organic solvent extraction followed by analysis using GC–MSD, a mechanism for the incorporation of the Strecker aldehyde moiety into the pyrazine molecule was proposed. Based on our previous experiences with manual and automated SPME, it seemed that the AutoSPME approach would possibly provide a simpler, less labor- and reagent-intensive method for investigations of this type. Thus, AutoSPME–GC–MSD trials were initiated to examine their ability to afford meaningful data and information on the nature of the reaction products of sugar–amino acid reactions.

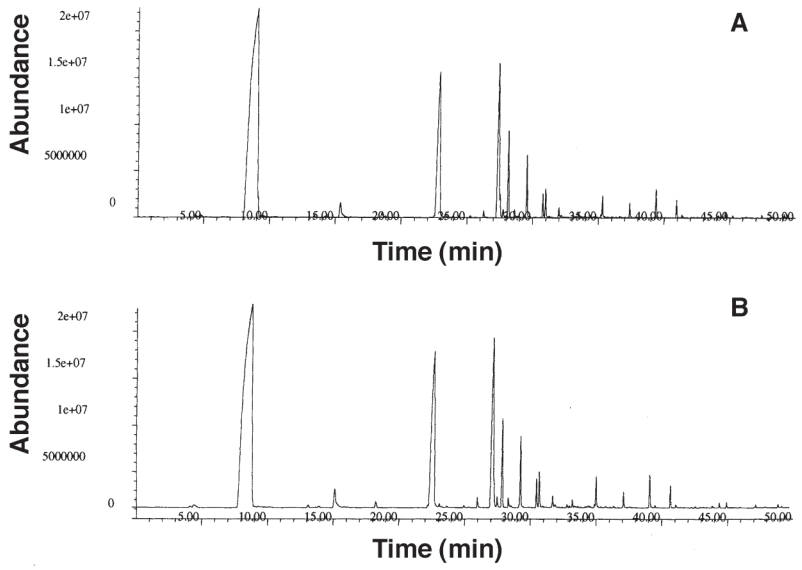

Figure 1. Total ion chromatogram of selected heated formulations: ^{13}C leucine (A) and leucine (B).

Table I contains a list of those pyrazines detected via AutoSPME–GC–MSD for selected microwave heat-treated HFCS–DAP and HFCS–DAP–amino acid formulations. The compound identifications were established through the use of retention time databases and mass spectral search results from both the Wiley and NBS mass spectral databases. The qualitative nature of the HFCS–DAP formulation was much less complex that the qualitative nature of the formulations containing amino acids. The ammonium ion contained within the DAP compound must serve as a nitrogen source in the formation of the pyrazines. This observation is consistent with earlier findings with selected ammonium salts in pyrazine formation (20). The level of DAP was held constant in all formulations for consistency.

The qualitative results obtained from this AutoSPME–GC–MSD approach for the HFCS–DAP–ILE formulation agree very well with those previously obtained using a headspace approach (15). In particular, the presence of pyrazines containing the 2-methylbutyl substituent was evident in both cases, strongly indicating the incorporation of the Strecker aldehyde 2-methylbutanal in the pyrazine structure. In a very similar fashion, the formulation containing valine was found to contain pyrazines having the 2-methylpropyl substituent attached, most probably resulting from incorporation of the Strecker aldehyde of valine, 2-methylpropanal, into the pyrazine structure.

Having established clearly the observation that Strecker aldehydes are incorporated into the pyrazine structures, a reasonable hypothesis for LEU-containing reactions would be the discovery of pyrazines containing the 3-methylbutyl substituent. This substituent would be derived from the Strecker aldehyde of LEU, 3-methylbutanal. Examination of the data in Table I would strongly indicate that the 3-methylbutyl group was incorporated into some of the pyrazines. The trends observed in branched alkyl chain pyrazine substituents for LEU, ILE, and VAL, coupled with the absence of these groups in the formulation without the amino acids, provides overwhelming evidence for reaction pathways involving the Strecker aldehydes.

To establish unambiguous confirmation of these reaction pathways, a series of microwave reactions employing ^{13}C labeled LEU and unlabeled LEU were performed. AutoSPME–GC–MSD was subsequently used to examine the resulting formulations for the presence of branched-chain pyrazines containing the ^{13}C label. The chromatograms shown in Figure 1 definitely indicate that the products of the formulations containing the labeled and unlabeled LEU were essentially identical. Thus, a direct correlation between the characteristics of the compounds derived from the ^{13}C label LEU experiment with the unlabeled LEU was possible.

The large peak located at approximately 8 min (Figure 1) was 3-methylbutanal. The mass spectra taken for the 3-methylbutanal in the labeled and unlabeled LEU formulations can be found in Figure 2. The pattern of the ions attributable to 3-methylbutanal containing uniformly labeled ^{13}C was readily distinguishable from those resulting from the unlabeled 3-methybutanal. In addition, the fragmentation pattern displayed by the

uniformly labeled ^{13}C 3-methylbutanal was incrementally higher in mass-to-charge ratio. For example, the *m/z* of 61 strongly suggests a C3 chain from 3-methylbutanal containing uniformly labeled ^{13}C atoms. This ion would result from the loss of a carbonyl group from uniformly ^{13}C labeled 3-methylbutanal. Similar arguments can be made for the *m/z* of 75 appearing in the ^{13}C leucine experiment compared with the *m/z* of 71 appearing in the unlabelled leucine experiment. Thus, the presence of 3-methylbutanal in the LEU-containing formulations can unambiguously be assigned to the Strecker degradation of the LEU. This result establishes one reaction pathway for the amino acid. The observation also points to the availability of a ^{13}C labeled Strecker aldehyde that could be subsequently incorporated into a pyrazine molecule. Should this occur, then systematic increases in molecular weight should be observed in pyrazines containing 3-methylbutanal components.

The branched-sidechain pyrazines of interest were found between retention times 35 and 45 min (Figure 1). No additional pyrazines having retention times greater than 50 min could be positively identified. The component eluting at 37.4 min served to unambiguously confirm, for the first time, the incorporation of an amino acid alkyl sidechain into the pyrazine molecule. Specifically, the mass spectrum obtained from the pyrazine at 37.4 min in the formulation with unlabeled LEU is illustrated in Figure 3A and is correctly identified as 3-methylbutylpyrazine. The mass spectrum from the component at 37.4 min in the formulation containing ^{13}C leucine is also contained in Figure 3B but is easily recognized as being different from its counterpart.

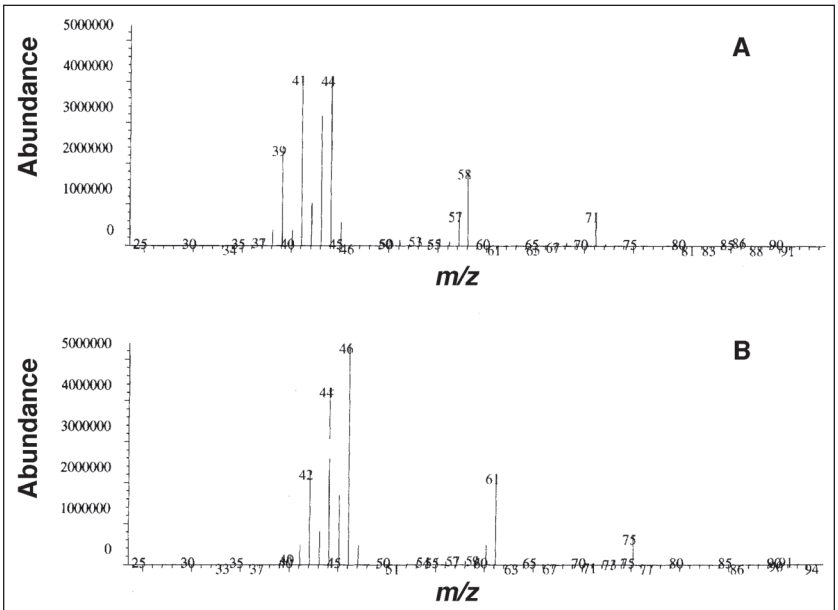

Figure 2. Mass spectra of 3-methylbutanal from selected heated formulations: leucine (A) and ^{13}C leucine (B).

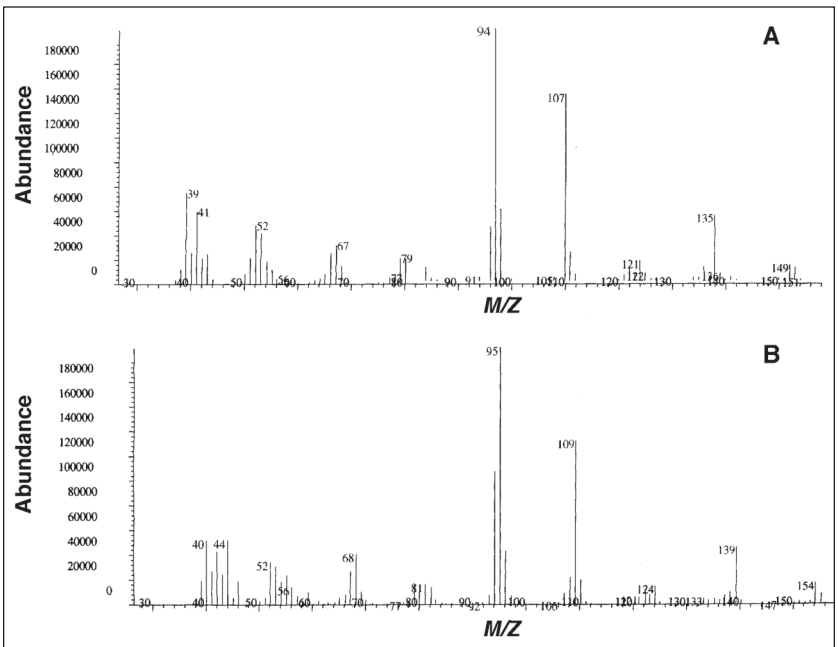

Figure 3. Mass spectra of 3-methylbutylpyrazine from selected heated formulations: leucine (A) and ^{13}C leucine (B).

The mass spectrum of the 3-methylbutylpyrazine found in the ^{13}C labeled LEU formulation contains ions (*m/z*) that have higher values than the mass spectrum of the 3-methylbutylpyrazine from the unlabeled LEU formulation. Interpretation of the fragmentation pattern associated with the unlabeled component is consistent with the systematic loss of *m/z* 14 or the loss of a methylene group from the parent molecule. Thus, the molecular ion of a 3-methylbutylpyrazine containing five ^{13}C atoms should be 154, whereas the molecular ion of a 3-methylbutylpyrazine containing five ^{12}C atoms should be 149. This was observed. Subsequent sequential loss of methylene groups should produce ions differing in *m/z* of 4, 3, 2, and 1 units. This is exactly what was observed in the comparison of the two mass spectra (Figure 3). Another way of confirming the incorporation of the label was to calculate the difference in the relative abundance of the ions. For example, for *m/z* 94 and 95, the abundances for 3-methylbutylpyrazine in the unlabeled formulations were 85.5 and 7.6%, respectively. Conversely, ions at *m/z* 94 and 95 for the 3-methylbutylpyrazine from the labeled experiment have abundances of 13.5 and 80.2%, respectively. Therefore, unambiguous proof has been provided, for the first time, directly implicating the incorporation of amino acid alkyl side chains into pyrazines under the operating conditions employed herein. This is a second reaction pathway for the amino acid.

In addition to the presence of 3-methylbutylpyrazine, methyl and dimethyl-3-methylbutylpyrazine analogues were detected in both formulations using AutoSPME–GC–MSD. The mass spectra of these higher-molecular-weight derivatives, wherein ^{13}C labeled LEU was employed, followed the same trends described above for the 3-methylbutylpyrazine.

Conclusion

AutoSPME–GC–MSD has been shown to be an uncomplicated approach with minimal sample preparation for the effective confirmation of proposed mechanisms of Strecker aldehyde involvement in the production of branched-chain alkyl-substituted pyrazines. Microwave-heated aqueous formulations require only the addition of NaCl prior to analysis by AutoSPME–GC–MSD. Ample quantities of the compounds of interest are extracted by a 100-µm PDMS fiber with vibration in a little as 1 min. Such sensitivity negates the time-consuming requirements for sample extraction using organic solvents or concentration using heat. This analytical approach should find additional applications in the area of reaction mechanism understanding.

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