Table I. Recovery of Bromide Ion from Tissue

| tissue | fortification, μmol/g | detected,[a] μmol/g | % recovery |
|---|---|---|---|
| mouse plasma | 0 | ND[b] | |
| | 0.5 | 0.430 ± 0.017 | 86.0 |
| | 1.0 | 0.740 ± 0.075 | 74.0 |
| | 1.5 | 1.562 ± 0.089 | 104.1 |
| mouse whole blood | 0 | ND[b] | |
| | 0.5 | 0.317 ± 0.033 | 63.3 |
| | 1.0 | 0.759 ± 0.037 | 75.9 |
| | 1.5 | 1.431 ± 0.155 | 95.4 |
| mouse liver | 0 | ND[c] | |
| | 0.5 | 0.342 ± 0.102 | 68.4 |
| | 1.0 | 0.573 ± 0.102 | 57.3 |
| | 3.5 | 2.835 ± 0.116 | 81.0 |
| | 6.2 | 5.679 ± 1.090 | 91.6 |
| | 8.2 | 6.749 ± 0.918 | 82.3 |

[a] Mean ± standard error. [b] ND, no bromide ion detected. [c] ND, no bromide ion detected by indicator-TLC or X-ray fluorescence.

pending on the type of sample used, the background level, and the quantity of sample available for analysis. This system is very useful for tissue samples from small laboratory samples, particularly mice.

Aside from evaluating blood samples, this TLC system allows measurement of liver bromide ion concentrations. Measuring ion concentrations in the liver is generally complicated by the large number of interfering ions, lipids, and proteins. The centrifugation procedure removes the larger molecules and the TLC procedure separates the interfering ions. Bromide levels in human serum can be analyzed if the samples are handled in a manner similar to that for the liver samples. High lipid tissues are more difficult to spot and analyze, particularly when concentrated to very small volumes. This problem is minimized with ultracentrifugation of the sample. Some presently unidentified ionic compounds may also increase the background levels of the sample.

As seen in Figure 1, there is a slight background interference when water only is spotted. This background may also be present without spotting a sample and may be due to the variable thickness of the TLC plate. This background was determined and subtracted from our samples for each experiment. Since there is a slight variation for each experiment, the peak areas should be calibrated for each experiment with a new standard curve.

This TLC procedure has been tested with chloride and bromide ions, and it is our feeling that it may be useful for other halides. The type of procedure may be applicable to other systems where different compounds are separated and where an indicator is used for detection. Modification of an existing TLC procedure may increase the sensitivity of the system and allow a more precise means of quantification.

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# A Fully Automated High-Performance Liquid Chromatographic Procedure for Isolation and Purification of Amadori Compounds

A semipreparative reversed-phase high-performance liquid chromatographic (HPLC) method was developed for the separation and purification of Amadori compounds from crude extracts of Maillard reactions. The Amadori compounds alanine–fructose (Ala–Fru), leucine–fructose (Leu–Fru), hydroxyproline–fructose (Hyp–Fru), and tryptophan–fructose (Trp–Fru) were isocratically separated on a $NH_2$-bonded silica gel column by using methanol–water (80:20) as the mobile phase. The collected fractions containing the Amadori compounds were concentrated and chromatographed a second time under the same conditions for further purification. Pure products were obtained by crystallization from anhydrous methanol of the fractions obtained after evaporation. The purities of the Amadori compounds were tested by thin-layer chromatography (TLC) and HPLC; their structures were confirmed by infrared spectrometry, $^{13}C$ NMR, and mass spectrometry.

Amadori compounds (1-amino-1-deoxy-2-ketoses) occurring in the early stages of the nonenzymatic browning Maillard reaction, have been isolated from several biological materials (Abrams et al., 1955; Anet and Reynolds, 1957; Heyns and Paulsen, 1959; van den Ouweland et al., 1979).

Some of their properties explain the growing interest in these compounds: they are considered as potential "natural antioxidants" (Hodge, 1955; Eichner, 1975) and precursors of the aroma and flavor of processed foods. An important development has been made in the chemistry of aromas in Maillard model systems (Tressel, 1979; Po-

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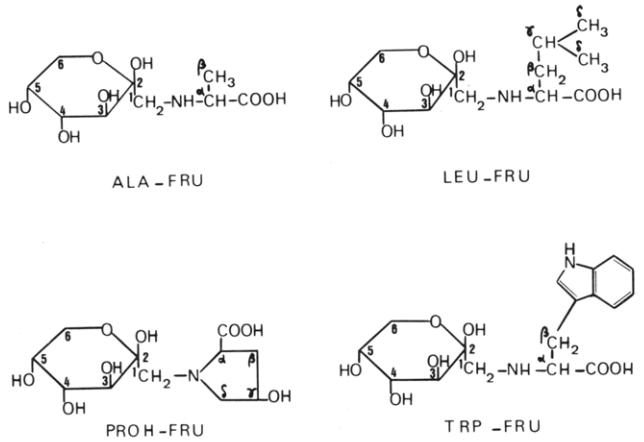


**Figure 1.** Structure of the Amadori compounds: Ala–Fru, alanine–fructose; Leu–Fru, leucine–fructose; PrOH–Fru, hydroxyproline–fructose; Trp–Fru, tryptophan–fructose.

korny et al., 1979), and thermolysis of Amadori compounds themselves has been investigated by some authors (Mills and Hodge, 1976; Mills, 1979; Shigematsu et al., 1977). In recent years, problems of toxicity in food processing have begun to receive much attention, and mutagenicities of browning products from model systems (Shibamoto et al., 1981a–c; Spingarn and Garvie, 1979; Pintauro et al., 1980) and of some substances derived from Amadori compounds (Heyns et al., 1979) have been tested.

Study of the behavior of isolated Amadori compounds should permit, in the future, a better approach to the understanding of the early stages of the Maillard reactions.

For this purpose and also to make available authentic samples for analysis of Amadori compounds in processed foods or submit them to further biological investigations, we have developed a new method (Moll and Gross, 1981) for their preparation from the reaction mixture. Instead of the classical ion-exchange chromatographic separations (Abrams et al., 1955; Hashiba, 1976) that are tedious and time consuming, the HPLC procedure we have previously described, using a $C_{18}$-bonded silica gel column and water as the eluent, presents the following advantages: Amadori compounds are directly obtained as free products, in good recoveries, and with low solvent cost.

A more general procedure, tested with success in all the browning systems that we have investigated, uses a $NH_2$-bonded silica gel column and a methanol–water (80:20) mixture. We used this technique of separation of Amadori compounds in a completely automated chromatographic system. As an example, we present the results obtained with the four compounds: Ala–Fru, Leu–Fru, Hyp–Fru, and Trp–Fru (Figure 1).

## EXPERIMENTAL SECTION

**Reagents.** Amino acids and glucose were from Fluka (Buchs, Switzerland). Water and methanol as eluents for the HPLC analyses were very high purity grade. Samples and solvents for the HPLC analyses were passed through a 0.45-$\mu$m Millipore filter (Millipore Corp., Bedford, MA). Thin-layer plates were coated with Polygram Sil G silica gel (Macherey, Nagel & Co., Düren, Federal Republic of Germany).

**Preparation of Model Maillard Browning System.** The general procedure for the preparation of the model reactions was the following: 0.010 mol of the L-amino acid (alanine, leucine, hydroxyproline, or tryptophan) was dissolved in 200 mL of methanol with an excess of D-(+)-glucose monohydrate (0.015–0.030 mol) and refluxed for 1 h. After being cooled the solution was evaporated to dryness under reduced pressure at 15 °C. The residue

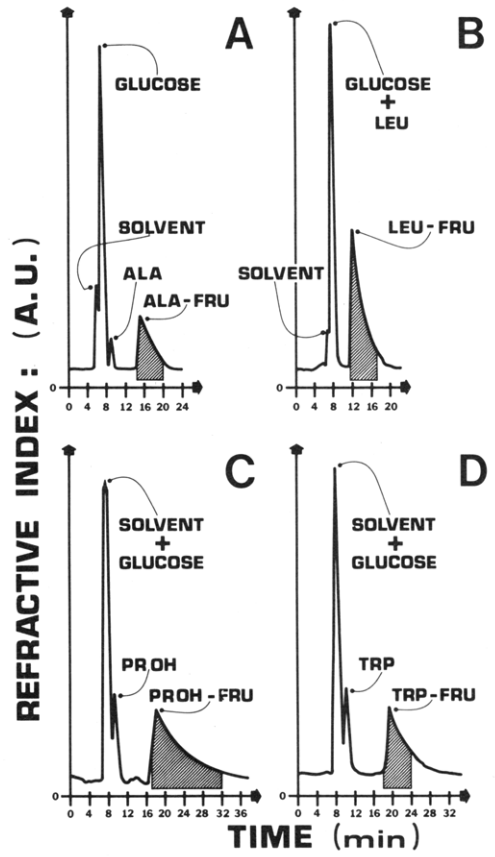


**Figure 2.** HPLC profiles of the crude Maillard reactions between glucose and alanine (A), leucine (B), hydroxyproline (PrOH in the figure) (C), and tryptophan (D). The Amadori compounds Ala–Fru, Leu–Fru, Hyp–Fru (PrOH–Fru in the figure), and Trp–Fru are recovered as indicated on the shaded portions of the chromatograms. Chromatographic conditions: semipreparative column packed with $\mu$Bondapak $NH_2$; mobile phase, methanol–water (80:20); flow rate, 2 mL/min. A.U. = arbitrary units.

was extracted with 150 mL of anhydrous methanol and refluxed for another 3–8 h, removing water as its benzene azeotrope. The reactions were followed by TLC [eluent: pure methanol or butanol–acetic acid–water (4:1:5 v/v) or chloroform–acetic acid–water (47:44:3 %/%/v)] and stopped when it was estimated that the spot of the Amadori compound on the TLC plate no longer increased. After being cooled, the mixture was evaporated to dryness and the residue taken off by a small amount of the eluent for the HPLC analysis. The crude samples were injected directly into the liquid chromatograph.

**HPLC Apparatus and Procedures.** The chromatographic system (Waters Associates, Milford, MA) included a Model 720 system controller, a Model 6000 A pump, a Model Wisp 710 B injector, and a Model R-401 differential refractometer. The fraction collector was a Model Microcol TDC 220 A, Gilson (95400 Villiers Le Bel, France). The semipreparative column (300 × 7.8 mm i.d.) was packed with 9 $\mu$m of $\mu$Bondapak $NH_2$ (Waters Associates).

The precolumn (25 × 3.9 mm i.d.) was packed with 30 $\mu$m of Rsil $C_{18}$ HL (R.S.L., Eke, Belgium). Aliquots of the Maillard crude extracts (400–1500 $\mu$L) were separated at a methanol–water (80:20 v/v) flow rate of 2 mL/min.

**Spectrometric Apparatus and Procedures.** The IR spectrophotometer was a Model PE 580 E (Perkin-Elmer). The Cameca NMR spectrometer was operated at 250 MHz. The $^{13}C$ NMR spectra were conducted in $^2H_2O$ as the solvent with DSS (sodium trimethylsilylpropionate-$d_4$) as the internal standard [$\delta$(TMS) = $\delta$(DSS)]. The Ribermag R 10/10 quadrupole mass spectrometer coupled

Table I. $^{13}C$ NMR Assignments for Ala-Fru (Figure 1)[a]

| δ(Me$_4$Si) | fructose C atom | fructose configuration | alanine, C atom |
|---|---|---|---|
| 177.39 | | | COOH |
| 104.57 | 2 | α-p, β-f, β-p | |
| 98.15 | | | |
| 95.36 | | | |
| 85.44 | 3 | α-f | |
| 85.23 | 4 | α-f, β-f | |
| 83.67 | | | |
| 80.67 | 3 | α-f, β-f | |
| 78.88 | 5 | | |
| 76.94 | | | |
| 73.11 | 3 | α-p, β-p | |
| 72.67 | 4 | | |
| 72.14 | 5 | | |
| 71.70 | | | |
| 69.88 | 1 | α-p, α-f | |
| 69.35 | 6 | β-p | |
| 68.90 | | | |
| 66.67 | | | |
| 64.67 | | | |
| 63.90 | | | |
| 63.70 | | | |
| 61.22 | | | |
| 58.46 | | | |
| 54.63 | | | |
| 54.31 | | | |
| 51.72 | | | Cα |
| 17.62 | | | Cβ |

[a] p = pyranose; f = furanose.

Table II. $^{13}C$ NMR Assignments for Leu-Fru (Figure 1)[a]

| δ(Me$_4$Si) | fructose C atom | fructose configuration | leucine, C atom |
|---|---|---|---|
| 178.27 | | | COOH |
| 98.71 | 2 | β-p | |
| 72.52 | 3, 4, 5 | α-p, β-p | |
| 72.20 | | | |
| 71.73 | | | |
| 66.46 | 1 | α-p, α-f, β-f | |
| 65.14 | 6 | | |
| 56.28 | | | Cα |
| 42.72 | | | Cβ |
| 27.36 | | | Cγ |
| 24.83 | | | Cδ |
| 24.30 | | | |

[a] p = pyranose; f = furanose.

Table III. $^{13}C$ NMR Assignments for Hyp-Fru (Figure 1)[a]

| δ(Me$_4$Si) | fructose C atom | fructose configuration | hydroxyproline, C atom |
|---|---|---|---|
| 176.21 | | | COOH |
| 104.62 | 2 | α-p, β-f, β-p | |
| 98.62 | | | |
| 98.56 | | | |
| 85.65 | 3 | α-f, | |
| 85.20 | 4 | α-f, β-f | |
| 83.56 | | | |
| 80.61 | 3 | β-f | |
| 78.85 | 5 | α-f, β-f | |
| 76.44 | | | |
| 73.44 | | | Cγ |
| 73.29 | | | |
| 73.05 | | | |
| 72.67 | 3, 4, 5 | α-p, β-p | |
| 72.61 | | | |
| 72.46 | | | |
| 72.17 | | | |
| 71.67 | | | |
| 66.52 | 1, 6 | α-p, α-f, β-f | |
| 66.02 | | | |
| 65.64 | | | |
| 65.58 | | | |
| 65.34 | | | |
| 64.55 | | | |
| 64.17 | | | |
| 63.87 | | | Cα |
| 59.96 | | | Cδ |
| 40.33 | | | Cβ |
| 40.28 | | | |
| 40.10 | | | |

[a] p = pyranose; f = furanose.

to the Sidar 111 A data processing system was purchased from Nermag. The analyses were carried out according to the ionization–desorption (CI/D) technique (Arpino and Devant, 1979). The reactant gas used was $NH_3$. In this case the CI/D analyses give three ions corresponding to M, $(M + H)^+$ (M + 1), and $(M + NH_4)^+$ (M + 18). Conditions were as follows: desorption, 40–500 mA; speed, 7 mA/s; desorption point, 378 mA; source temperature, 70 °C. For the desorption process, 1 μL of a methanolic solution of the sample (5 mg in 5 mL) was deposited on the filament.

## RESULTS AND DISCUSSION

The analytical HPLC profiles of the crude Maillard reactions between glucose and alanine (Ala), leucine (Leu), hydroxyproline (Hyp), and tryptophan (Trp) are shown respectively in parts A, B, C, and D of Figure 2. The chromatograms show that the sugar generally eluted together with the solvent, separated or not from the amino acid. The fractions containing the Amadori compounds Ala–Fru, Leu–Fru, Hyp–Fru and Trp–Fru could be recovered (shaded portions on the chromatograms) and evaporated to dryness. The residue was dissolved in a small amount of solvent and chromatographed a second time for further purification under the same conditions. Two purifications were generally sufficient to obtain the pure Amadori compound. The residue obtained after evaporation of the last purified fractions was crystallized from anhydrous methanol. The yields of the products, calculated from the parent amino acid, were as follows: Ala–Fru, 15%; Leu–Fru, 20%; Hyp–Fru, 22%; Trp–Fru, 18%. These yields, including the yields of the reaction itself and the recoveries of the HPLC procedure, can be optimized.

The purity of the Amadori compounds was examined by TLC and HPLC.

Their structures were confirmed as follows. Ala–Fru: IR (KBr) ν (C=O) 1620 $cm^{-1}$; mass spectrum (Figure 3A), parent = Ala–Fru, 252 (M + 1), fragments = fructose, 180 (M), and alanine, 107 (M + 18); $^{13}C$ NMR, the chemical shifts with respect to tetramethylsilane $Me_4Si$) (Table I) were compared with literature data (Breitmaier and Voelter, 1978; Doddrell and Allerhand, 1971; Funcke and Klemer, 1976). Leu–Fru: IR (KBr) ν (C=O) 1620 $cm^{-1}$; mass spectrum (Figure 3B), parent = Leu–Fru, 294 (M + 1), fragment = fructose, 180 (M); $^{13}C$ NMR, the chemical shifts with respect to $Me_4Si$ (Table II) were compared with literature data (see above). Hyp–Fru: IR (KBr) ν (C=O) 1630 $cm^{-1}$; mass spectrum (Figure 3C), parent = Hyp–Fru, 294 (M + 1), fragments = fructose, 180 (M), and hydroxyproline, 132 (M + 1) and 149 (M + 18); $^{13}C$ NMR, the chemical shifts with respect to $Me_4Si$ (Table III) were compared with literature data (see above). Trp–Fru: IR (KBr) ν (C=O) 1550 $cm^{-1}$; mass spectrum (Figure 3D), parent = Trp–Fru, 367 (M + 1), fragments = fructose, 180 (M), and tryptophan, 205 (M + 1) and 222 (M + 18); $^{13}C$ NMR, the chemical shifts with respect to $Me_4Si$ (Table IV) were compared with literature data (see above).

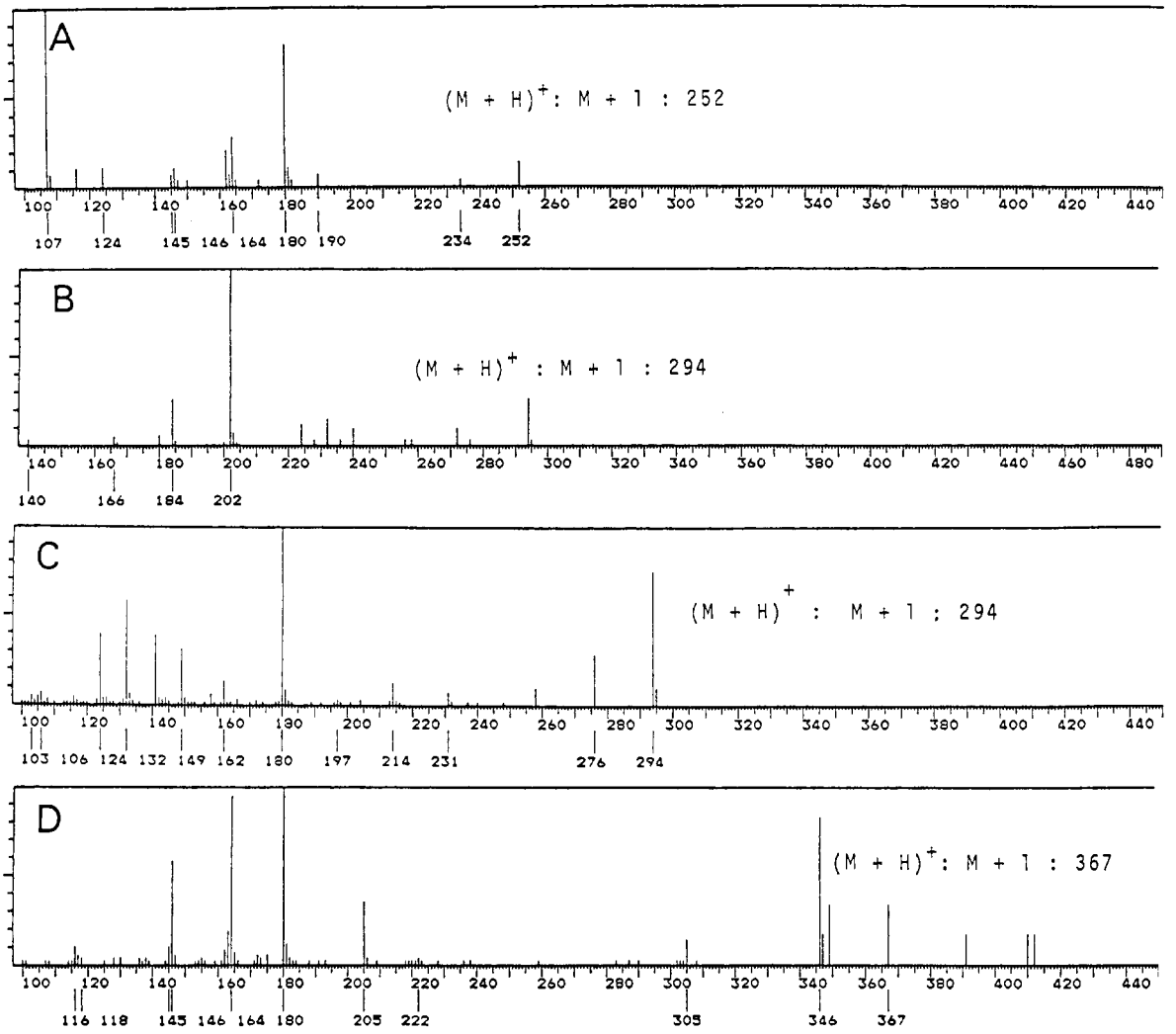


**Figure 3.** Mass spectra of the peak due to the Amadori compounds: Ala–Fru (A), Leu–Fru (B), Hyp–Fru (C), and Trp–Fru (D) (by chemical ionization with ammonia).

Table IV. $^{13}C$ NMR Assignments for Trp-Fru (Figure 1)[a]

| | fructose | | |
|---|---|---|---|
| δ(Me$_4$Si) | C atom | configuration | tryptophan, C atom |
| 177.24 | | | COOH |
| 139.17 | | | |
| 129.49 | | | |
| 127.84 | | | |
| 125.13 | | | |
| 124.99 | | | aromatic C |
| 122.31 | | | |
| 121.37 | | | |
| 114.78 | | | |
| 110.07 | | | |
| 97.97 | 2 | α-p, α-f, β-p, β-f | |
| 85.29 | 3 | α-f, β-f | |
| 85.23 | 4 | | |
| 73.05 | 3 | | |
| 72.11 | 4 | α-p, β-p | |
| 71.61 | 5 | | |
| 66.55 | 1 | α-p, α-f, β-f | |
| 66.62 | 6 | | |
| 55.72 | | | Cα |
| 28.60 | | | Cβ |

[a] p = pyranose; f = furanose.

On the basis of the results we have presented, we consider that our chromatographic procedure shows a greatly improved method for the isolation of the Amadori compounds from Maillard reactions. The products are, in each case, well separated from the sugar and the parent amino acid by a phenomenon of both ion-exchange chromatography and adsorption chromatography. The new separation procedure presents a distinct advantage over the earlier one cited (Moll and Gross, 1981); it is a more general method which can be applied to several Maillard model systems; three Amadori compounds cited (Leu–Fru, Hyp–Fru, and Ala–Fru) could be bad or not resolved on the $C_{18}$ column. The much better resolution of the Amadori compound from the starting materials needed two purifications of each product by HPLC vs. three purifications in the earlier work.

Both chromatographic procedures on the $C_{18}$ and $NH_2$ column present an enormous advantage toward classical multiple ion-exchange chromatographic separations: they avoid the use of eluents such as strong acids and bases or buffers. After removal of water, or methanol–water, the Amadori compounds are directly obtained as free products, which makes their crystallization easier. All of them are obtained in the crystalline form and are generally stable. If not, they can be rapidly purified for further studies by HPLC under the same conditions. The complete automation of the chromatographic system has resolved the problem of the time-consuming classical separations of these compounds and should permit, in the future, larger amounts of them to be made available for further specific biological studies.

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# Identification of Some Volatile Constituents of *Aspergillus clavatus*

The vacuum steam volatile concentrates from three strains of *Aspergillus clavatus* grown on standard media have been analyzed by capillary gas–liquid chromatogrphy–mass spectrometry. Fifty compounds (eight tentatively) were identified from all the strains. Aliphatic alcohols and ketones accounted for about half of the identified compounds while the rest were mainly aromatic compounds. The major compounds were oct-1-en-3-ol (30–52% in NRRL 2 and 11–21% in the other strains), 4-methylbenzaldehyde (34–40% in NRRL 5199), phenylacetaldehyde (10–27% in NRRL 5199 and NRRL 6320), and 2-methylphenol with lesser amounts of the other isomers (25–57% in NRRL 6320 and 13% in NRRL 5199). The chromatotographic pattern and known volatile composition were distinctive for each strain and could be used to characterize these fungi.

Recently gas–liquid chromatography (GLC) of fungal volatiles has been used to study the differentiate various fungi. Vincent and Kulik (1970, 1973) and Kulik and Vincent (1973) classified a number of common lower fungi using a mathematical analysis of their pyrolysis–gas–liquid chromatography (PGLC) patterns without identification of any volatiles. The method relies on exacting standard conditions and the assumption that the composition will always be indentical for the conditions used. Gunasekaran and Hughes (1980) used the GLC pattern of methylated fatty acids to distinguish several species of *Candida*.

Other researchers have identified fungal volatiles for characterization of fungi rather than relying completely on GLC pattern differences. Collins (1979) reviewing some of the literature of odor producing fungi discussed the identification of some major compounds and the techniques which were used for their isolation. A successful approach was the isolation of volatiles by vacuum steam distillation followed by GLC–mass spectrometry (MS). For example, Kaminski et al. (1972, 1974) identified the major volatiles from several fungal strains and Freeman et al. (1976) identified 22 compounds associated with spoilage of chicken using these techniques. However, other techniques have been used with assays for different areas of research. Halim et al. (1975) extracted odorous contituents of *Pencillium decumbens* directly and used column chromatography and GLC to separate major compounds for identification by their infrared spectra. Norrman (1977), applying headspace chromatography (HSGC) to one organism, suggested using it as a general method for following metabolic changes in fungi. Repke et al. (1978) extracted mushroom toxins with methanol and assayed the trimethylsilyl derivatives by GLC–MS.

Some microbial odorants are common pollutants of food and water. Gerber (1979) reviewed the most important odorants, particularly geosmin, which is produced by the bacterium *Streptomyces*, some algae, and a fungus.

Some of the best examples of comprehensive assays using a variety of successful techniques are the investigations of mushroom volatiles by Yajima et al. (1981, Thomas (1973), and Pyysalo (1976). Individually they used different but fairly mild techniques (headspace, extraction–vacuum distillation, continuous extraction ion–vacuum distillation) to obtain and identify 50–70 mushroom volatiles per species by GLC–MS. The characteristic and desirable aromas of edible mushroom were also investigated by Cronin and Ward (1971), Pyysalo and Suihko (1976), and Picardi and Issenberg (1973).