A Method for the Determination of α-Dicarbonyl Compounds[1]

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ABSTRACT

A method is reported for determining the α-dicarbonyls glyoxal, methylglyoxal, and diacetyl. The carbonyls were reacted at pH 8 with .05% aqueous solution of *o*-phenylenediamine for 4 h at 25°C to form quinoxalines. The derivatives were extracted with chloroform, transferred to methanol, and separated by HPLC on a Supelcosil LC-18 column with methanol-water as the mobile phase. The method was applied to several dairy cultures and cheese varieties. The amounts of glyoxal, methylglyoxal, and diacetyl in the cultures varied from 2 to 227, 0 to 7, and 1 to 11 μg/ml, respectively, depending on the species, strain, and culture medium.

INTRODUCTION

Glyoxal and methylglyoxal play a role in generating the characteristic flavor of some fermented dairy products (4, 5, 17). These carbonyls have little flavor impact themselves but are able to interact with amino acids to form potent flavor compounds at ambient temperatures. Methylglyoxal is metabolized by a number of microorganisms (3), and glyoxal and methylglyoxal are known to be produced by lactobacilli (5, 17, 19). Diacetyl, which also is an α-dicarbonyl, is a well-known dairy flavor compound, and it is produced by such cultures as *Streptococcus diacetylactis* and *Leuconostoc cremoris* (2, 5, 10, 13, 18, 19).

Although diacetyl analyses often are applied to dairy products (1, 7, 8, 9, 12, 14, 15, 18), there are few methods for the determination of glyoxal and methylglyoxal. Methylglyoxal has been determined qualitatively in coffee and cigarette smoke as a quinoxaline derivative formed by reacting the carbonyl with *o*-phenylenediamine (16). Methylglyoxal also has been determined as a 2-acetylthiazolidine derivative after reaction with cysteinamine (6). This report describes a method for the quantitative determination of glyoxal, methylglyoxal, and diacetyl based on the HPLC separation of their quinoxaline derivatives and the application of this technique to several dairy cultures and cheeses.

MATERIALS AND METHODS

Glyoxal, methylglyoxal, and diacetyl were purchased from Sigma Chemical Company (St. Louis, MO) and used without further purification.

Carbonyl Analysis

Samples containing 5 to 450 μg of the dicarbonyls in about .4 ml of water were mixed with .34 ml of an .05% aqueous solution of *o*-phenylenediamine, and skatole, typically 340 μg in .34 ml of methanol, was added as an internal standard. The pH of the reaction mixture was adjusted to 8.0 with 1 N sodium hydroxide, and the reaction mixture was held 4 h at 25°C. Next, the pH was adjusted to 3.0 with 1 N hydrochloric acid, and 2 ml of chloroform were mixed vigorously with the sample by using a Vortex mixer. The chloroform was removed by centrifugation at 12,000 × *g* for 15 min and collected. The extraction with chloroform was repeated two additional times, and the chloroform, which contained the quinoxaline derivatives, was allowed to evaporate at ambient temperature from an open container. One milliliter of methanol was added to the residue, and after 1 h, the methanol was filtered through a nylon filter (1MS1,CAMEO, Westboro, MA) and the quinoxalines were analyzed by HPLC.

The samples were analyzed by a Waters Associates (Milford, MA) HPLC instrument

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Model ALC-201 that was fitted with a Varian (Varian Instrument Group, Palo Alto, CA) Model 2050 UV variable-wavelength detector. The detector was operated at 254 nm, and the output was integrated with a Hewlett-Packard (Palo Alto, CA) Model 3392A integrator. The concentrations of carbonyls were calculated by reference to the internal standard. For the separation of quinoxalines, a Supelcosil LC-18 (Supelco, Bellefonte, PA) column, 250 × 4.6 mm, with 20 mm Supelguard LC-18 cartridge column, was used. The mobile phase was methanol-water (68% vol/vol) delivered at .6 to .7 ml/min.

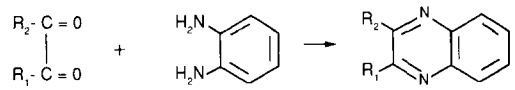

Figure 1. The reaction used for the derivatization of α-dicarbonyls. R_1 and R_2 are H and CH_3.

Cheese Analysis

Samples of Swiss, Cheddar, and Mozzarella cheeses were obtained from a local grocery. Approximately 10 g of each was homogenized with 20 ml of distilled water and transferred to a centrifuge tube with an additional 10 ml of water. The samples were centrifuged at 30,000 × *g* for 20 min at 1°C, and the aqueous layer was collected and adjusted to pH 8.0 with 1 N sodium hydroxide. The aqueous layer was centrifuged again at 20,000 × *g* for 15 min. The volume of the aqueous phase was measured, .34 ml of a methanolic solution of skatole (1 mg/ml) was added, and sufficient 1% aqueous solution of *o*-phenylenediamine was added to give a final concentration of .05% in the reaction mixture. The analysis was then completed as described previously.

Culture Conditions

Lactobacillus bulgaricus LB-3 and AR-2, *Lactobacillus casei* C-9, *Streptococcus thermophilus* AC-2, and *Propionibacterium shermanii* P-19 were obtained from the culture collection in the Department of Food Technology, Iowa State University. The media used were MRS (Difco, Detroit, MI) or permeate from cottage cheese whey produced by ultrafiltration through an Amicon (Danvers, MA) filter with a nominal cut-off of 30,000 daltons. The permeate was fortified with 2% Caseamino acids (Difco) and adjusted to pH 7.0 with 1 N sodium hydroxide. The media were autoclaved at 121°C for 15 min and inoculated with 2% of a 18-h culture having an absorbance of .21 at 600 nm. Cultures were incubated at 37°C for 7 d. After incubation, the cultures were adjusted to pH 8.0 with 1 N sodium hydroxide and centrifuged at 20,000 × *g* for 15 min. The supernatant solution was used for carbonyl analysis.

RESULTS AND DISCUSSION

Factors Affecting the Analysis

Figure 1 shows the formation of the quinoxaline derivatives of the α-dicarbonyls, and Figure 2 shows the separation of the derivatives of glyoxal, methylglyoxal, and diacetyl in the HPLC system. Interference by the reagent, *o*-phenylenediamine, was avoided by extracting the reaction mixtures under acid conditions. Skatole proved to be a satisfactory internal standard that separated well from the carbonyl derivatives.

Considerable effort was made to analyze the quinoxaline derivatives by gas chromatography since they are fairly volatile compounds. Although resolution was good, it was not possible

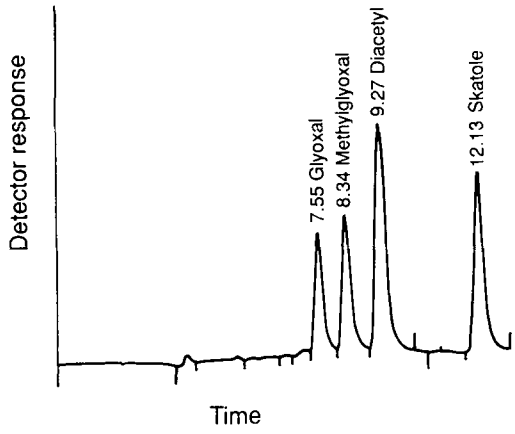

Figure 2. A typical chromatogram of the HPLC separation of the quinoxaline derivatives of the α-dicarbonyl standards. Retention times are in minutes.

TABLE 1. Dicarbonyl content of some cheese varieties.

Variety	Carbonyl (μg/g)					
	Glyoxal		Methylglyoxal		Diacetyl	
		SD		SD		SD
Cheddar	4.29	.05	10.89	.93	.70	.10
Swiss	41.14	2.28	1.98	.11	.59	.08
Mozzarella	6.06	.27	4.06	.13	2.87	.06

to obtain satisfactory quantitative data because of the very polar nature of the derivatives and their variable adsorption on the surfaces of the gas chromatograph with which they came in contact. This problem was overcome by using HPLC. With HPLC, replicate injections were quite reproducible, and peak areas varied linearly with the amount of sample. On the basis of the carbonyls as received from the supplier, the peak areas of glyoxal, methylglyoxal, and diacetyl per unit area of skatole should be multiplied by 1.07, .463, and .399 to obtain the correct weight of carbonyl. Seemingly, the methyl groups on the ring cause the carbonyl to be overestimated. The carbonyls being investigated are all subject to polymerization, and no satisfactory method of purification was discovered. Polymerization of the standards was ignored in calculating the results.

The yield of the reaction of glyoxal and methylglyoxal with *o*-phenylenediamine increased with the pH from pH 4 to 7 and was at an optimum at pH 8. Reaction times longer than 4 h did not improve the yield. Three extractions of the reaction mixture with chloroform gave quantitative recovery. Each extraction recovered about 80% of the derivatives remaining in the reaction mixture.

The carbonyls being investigated are known to react with amino acids (4), so the effect of amino acids in the reaction mixture on the recovery of the carbonyls was investigated. The recovery of carbonyls relative to the internal standard did not change significantly during incubation at 25°C for up to 144 h in the presence of Casamino acids. For reasons that are not clear, the recovery of glyoxal was increased about 12 to 24% by the presence of amino acids. The casein hydrolyzate alone contained no glyoxal, so evidently, the amino acids increase the yield of the derivatization reaction.

TABLE 2. Dicarbonyl content (μg/ml) of various cultures grown on MRS broth and whey permeate (perm) supplemented with 2% Casamino acids. Each value is the mean of three separate cultures.

Carbonyls	Media[1]		*Lactobacillus bulgaricus* LB-3		*Lactobacillus bulgaricus* AR-2		*Lactobacillus casei* C-9		*Streptococcus thermophilus* AC-2		*Propionibacterium shermanii* P-19	
	MRS	Perm	MRS	Perm	MRS	Perm	MRS	Perm	MRS	Perm	MRS	Perm
Glyoxal	8.78	3.05	27.69	3.39	236.42	230.45	26.75	5.50	39.37	21.19	32.78	8.45
Methylglyoxal	7.23	2.64	14.78	3.28	1.76	.97	4.51	5.21	6.37	2.91	3.58	6.80
Diacetyl	3.03	.64	4.59	.74	4.07	3.46	4.95	5.41	12.66	3.86	14.33	1.93

[1]Autoclaved but not inoculated.

The yields of other carbonyls were unaffected.

Table 1 shows the results for the carbonyl analyses of the three cheese varieties and the standard deviation of the analyses on the same cheese sample. Replicate analyses agreed quite well. The coefficient of variation of the analyses averaged 6.4.

Glyoxal was found at the greatest concentration, 41 μg/g, in Swiss, possibly reflecting the growth of *L. bulgaricus* strains that are good glyoxal generators. Methylglyoxal was found at the greatest concentration, 11 μg/g, in the Cheddar sample. Diacetyl was found at the greatest concentration, 3 μg/ml, in Mozzarella. The levels of diacetyl reported in these cheeses are similar to those reported by others: 11.3 μg/g in 5-day Cheddar to .9 in 200-d samples (7); .2 in 3-mo Swiss (14), and .9 to 2.5 in 60-d kefalotyri cheese (12).

Production of Carbonyls by Cultures

Table 2 shows the amounts of glyoxal, methylglyoxal, and diacetyl found in cultures grown on two media after 7 d of incubation. As reported previously by Reps et al. (17), these carbonyls are formed to some extent by autoclaving of typical culture media. Uninoculated MRS media produced more of the carbonyls on autoclaving than did the fortified permeate. Organisms grown on MRS media also produced more of the dicarbonyls in nearly every instance.

Glyoxal production as a result of microbial growth ranged from about 2 to 227 μg/ml. Of the strains investigated, *L. bulgaricus* AR-2 was notable for its production of glyoxal. Obviously there is considerable strain variation because *L. bulgaricus* LB-3 produced much less glyoxal. Reps et al. (17) reported that LB-3 produced less carbonyl than AR-2 on the basis of their qualitative analysis of these carbonyls as 2,4-dinitrophenylhydrazones. Methyl glyoxal production as a result of microbial growth ranged from negative values in the instance of *L. bulgaricus* AR-2 to about 7 μg/ml for *L. bulgaricus* LB-3. Diacetyl production was greatest for *P. shermanii* P-19 and *S. thermophilus* AC-2 grown on MRS medium, about 10 μg/ml. These values are similar to the results reported by others for various dairy cultures: .07 to 4.05 μg/ml for *L. casei*, and 17-68 for *Streptococcus diacetylactis* and 1.01 to 7.8 for mixed strain butter cultures (9, 10, 11).

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