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Determination of Acetaldehyde in *Streptococcus lactis* Cultures as 2,4-Dinitrophenylhydrazone by High-Performance Liquid Chromatography

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A method is described for acetaldehyde analysis in *Streptococcus lactis* cultures that involves distillation and formation of acetaldehyde 2,4-dinitrophenylhydrazone with separation by high-performance liquid chromatography (HPLC). Relative standard deviations for data obtained from HPLC analysis were slightly lower than for data obtained by a conventional spectrophotometric method for *S. lactis* broth cultures. Acetaldehyde recovery data were comparable for both techniques. Acetaldehyde data for two strains of *S. lactis* incubated at 32 °C are compared.

The importance of acetaldehyde as a flavor contributor in cultured dairy products has been well documented (Lindsay et al., 1965; Keenan and Bills, 1968; Lees and Jago, 1978). However, a wide variation in levels of acetaldehyde production and metabolism has been reported for different strains of lactic acid bacteria. The detectable acetaldehyde levels in microbial cultures may also vary with culturing conditions. Much of the variability in reported acetaldehyde data may relate to variation in method of analysis used since acetaldehyde is a highly volatile and reactive compound (Lees and Jago 1969). The most commonly employed methods involve distillation and subsequent reaction of acetaldehyde with reagents such as 3-methyl-2-benzothiozolone hydrazone (MBTH; Lindsay and Day, 1965) or 2,4-dinitrophenylhydrazine (DNPH; Harvey, 1960) followed by spectrophotometric analysis. These techniques are nonspecific and, therefore, are subject to background interference. Direct gas chromatographic (GC) analysis requires specialized sample handling because of the high volatility of acetaldehyde. While this problem can be overcome by hydrazone derivatization, difficulties have been reported for GC analysis of carbonyl 2,4-dinitrophenylhydrazone (DNP) derivatives because of formation of isomers with heating (Papa and Turner, 1972a,b). High-performance liquid chromatography (HPLC) has been reported as an acceptable method for carbonyl DNP separation and analysis (Fung and Grosjean, 1981). HPLC analysis techniques involving DNPH derivatization have been reported for formaldehyde in shrimp (Radford and Dalsis, 1982) and for methyl ketones in dry food model systems (Reinedccius et al., 1978). The objective of this investigation was to evaulate the use of DNPH derivatization and HPLC separation for estimation of acetaldehyde levels in cultures of lactic acid bacteria.

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MATERIALS AND METHODS

Microorganisms and Growth Conditions. The organisms, *Streptococcus lactis* 60 (Hansen's Laboratories, Milwaukee, WI) and *S. lactis* C2 (University of Minnesota, St. Paul, MN) were subcultured (12 h at 32° C) 3 successive times in Elliker Broth (Difco). In experimental trials, 3 L of Elliker broth was inoculated with *S. lactis* at 1.0%. Incubation was in a New Brunswick Model 19 fermentor (New Brunswick Scientific Co., Edison, NJ). with constant agitation (200 rpm) at 32 °C. Duplicate samples were removed from the fermentor at incubation times of 0, 3, 6, and 9 h.

Acetaldehyde Analysis. *Reagents and Standards.* Carbonyl-free hexane was prepared by elution of nanograde hexane through a 2,4-DNPH–Celite column followed by reaction with 1.0% activated charcoal and filtering. The DNPH reagent mixture was prepared as a mixture of 2,4-DNPH (Aldrich Chemical Co., Milwaukee, WI), H_3PO_4, and Celite (Schwartz and Parks, 1961). Distilled water, used as a diluent or in chromatography, and the chromatographic solvent (HPLC-grade CH_3CN; Fisher Scientific Co., Fairlawn, NJ) were further purified through Porapak Q (Supelco, Inc., Bellefonte, PA). Acetaldehyde stock standard solution was prepared by weighing cold acetaldehyde (Aldrich Chemical Co., Milwaukee, WI) into cold water. The stock solution was calibrated by iodometric titration (McCloskey and Mahaney, 1981) and appropriately diluted for standard curve preparation.

Distillation. Two distillation procedures were compared. A low-temperature purge system was assembled as described by Pack et al. (1964) and used by Lindsay and Day (1965). The system involved nitrogen purging of tubes containing culture in a 65 °C water bath into a collection trap containing derivatizing reagents. For steam distillation, a micro-Kjeldahl apparatus fitted with a cold water condenser and graduated collection trap containing derivatizing reagents was used.

3-Methyl-2-benzothiozolone Hydrazone (MBTH) Spectrophotometric Procedure. The MBTH procedure

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according to Lindsay and Day (1965) was used with minor modifications. In the low-temperature purge system, 5.0 mL of diluted culture (1:10 with water) was purged into the MBTH reagent mixture for 1 h. For steam distillation, 5.0 mL of diluted culture (1:10) was placed into the distillation flask wtih 20 mL of water and distilled into the MBTH reagents to a final distillate volume of 10 mL. Blanks were prepared by distilling and reacting uninoculated Elliker broth. Absorbance values at 666 nm were corrected by subtraction of blank absorbance and compared to a similarly reacted acetaldehyde standard curve. Standard curves were linear in the acetaldehyde concentration range used (0–24 $\mu g\ mL^{-1}$). Results are reported as micrograms of acetaldehyde per milliliter of broth culture.

2,4-Dinitrophenylhydrazine High-Performance Liquid Chromatography (DNPH-HPLC) Procedure. A 10-mL sample of culture was placed into the distillation flask with 20 mL of water and steam distilled into distillate collection tubes containing 200 mg of DNPH–Cellite reagent and H_3PO_4 (Schwartz and Parks, 1961). Distillation was continued to a final distillate volume of 10 mL. After a postdistillation reaction time of 30 min, the DNP carbonyls were extracted from the Celite by mixing (Vortex mixer, 15 s with three successive 10-mL aliquots of carbonyl-free hexane. The hydrazone fraction was extracted with three successive 3.0-mL fractions of CH_3CN, and the pooled fractions were brought to a volume of 10 mL in CH_3CN.

The HPLC analysis system consisted of an Altex Model 153 ultraviolet detector (365 nm), a Model 110A delivery pump, a Rheodyne Model 7125 injector, and an Altex 25 cm × 4.6 mm i.d. column packed with 5-μm Ultrasphere–octadecylsilane (ODS, C_{18}) (Altex Scientific Inc., Berkely CA). A 20-μL injection was isocraticaly eluted at a flow rate of 1.5 mL of CH_3CN–H_2O (60:40)/min. Peak heights, corrected by subtraction of values for a distilled and reacted Elliker broth blank, were compared to a similarly reacted acetaldehyde DNP standard curve. Standard cures were linear in the acetaldehyde concentration range used (0–30 $\mu g\ mL^{-1}$). Relative standard deviations (RSD) for mean peak heights from triplicate injections were <3%. Data are reported as micrograms of acetaldehyde per milliliter of broth culture.

RESULTS AND DISCUSSION

HPLC Separation of Carbonyl 2,4-Dinitrophenylhydrazones (DNP). Under the experimental conditions described, baseline resolution was attained in less than 15 min for a standard mixture composed of formaldehyde, acetaldehyde, acetone, propionaldehyde, butyraldehyde, valeraldehyde, and hexanal which were reacted with DNPH as described. Elution order increased according to chain length. Separation of aliphatic carbonyl DNP's by reversed-phase HPLC has been reported previously (Fung and Grosjean, 1981).

Typical HPLC chromatograms for an acetaldehyde DNP standard, a reagent blank, and a *S. lactis* culture distillate are presented in Figure 1. Peaks were assigned based on retention time (1 is 2,4-DNPH; 2 is acetaldehyde DNP).

Comparison of HPLC and Spectrophotometric Procedures. To assess effects of distillation conditions on acetaldehyde recovery Elliker broth was fortified at levels ranging from 3 to 30 μg of acetaldehyde mL^{-1}. In preliminary investigations it was observed that steam distillation resulted in higher acetaldehyde recovery than did the low-temperature purge system. In addition, data obtained by DNPH-HPLC analysis of low-temperature purge distillates were highly variable. This is probably due to a combination of factors such as more efficient condensation in the water-cooled system, higher driving force during distillation, codistillation of water to aid in trapping into the DNPH–Celite mixture, and a more airtight system. Therefore, the low-temperature purge system was not used in further investigation. Recovery data obtained by DNPH-HPLC analysis of steam distillates were comparable to those for the conventional MBTH spectrophotometric procedure (Table I).

Data for effects of distillation conditions on acetaldehyde analysis of a single growth trial for the *S. lactis* 60 culture are presented in Table II. By correction of the data through subtraction of initial or zero time mean values, similar data trends were observed with increased incubation times for the two analytical methods compared. Data

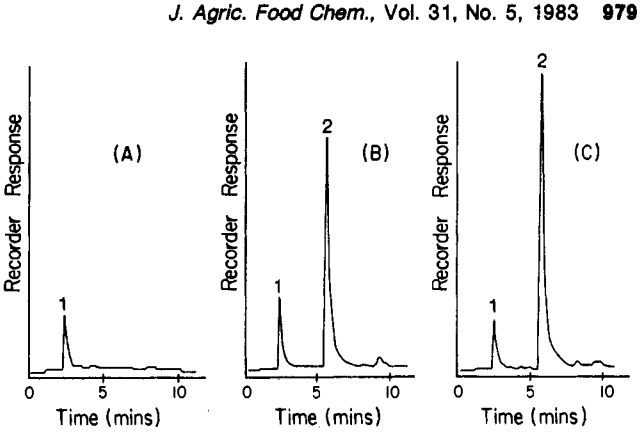

Figure 1. High-performance liquid chromatography of acetaldehyde DNP in *S. lactis* culture distillate. (A) Reagent blank; (B) acetaldehyde DNP standard at 2.36 $\mu g\ mL^{-1}$; (C) *S. lactis* 60 culture distillate from 3-h incubation at 32 °C (peak 1 is 2,4-DNPH reagent; peak 2 is acetaldehyde DNP).

Table I. Acetaldehyde Recovery Data Obtained from Steam Distillates by Spectrophotometric and High-Performance Liquid Chromatographic (HPLC) Methods

analysis method[a]	acetaldehyde recovery[b]
spectrophotometric	104.2 ± 11.6
HPLC	104.5 ± 6.3

[a] Spectrophotometric analysis with 3-methyl-2-benzothiozolinone (MBTH; Lindsay and Day, 1965); HPLC analysis of acetaldehyde 2,4-dinitrophenylhydrazone (DNP). [b] Mean percent recovery (±standard deviation) for Elliker broth fortified at 3–30 μg of acetaldehyde mL^{-1}.

Table II. Comparison of Acetaldehyde Data for Steam Distillates of *S. lactis* Broth Cultures by Spectrophotometric and by High-Performance Liquid Chromatography (HPLC) Procedures

incubation time[a]	acetaldehyde data[b]			
	spectrophotometric		HPLC	
	raw	corrected	raw	corrected
0	1.39 ± 0.07		0.55 ± 0.02	
3	2.42 ± 0.14	1.03	1.79 ± 0.03	1.24
6	4.79 ± 0.15	3.40	4.33 ± 0.08	3.96

[a] Incubation of *S. lactis* 60 in Elliker broth (h at 32 °C). [b] Raw data are means (±standard deviation) of triplicate distillate analyses (micrograms of acetaldehyde per milliliter): spectrophotometric analysis with 3-methyl-2-benzothiozolinone hydrazone (MBTH; Lindsay and Day, 1965); HPLC separation of aldehyde 2,4-dinitrophenylhydrazones (DNP). Corrected data are corrected by subtraction of initial (0 time) acetaldehyde level.

Table III. Comparison of Acetaldehyde Data Obtained by by High-Performance Liquid Chromatography (HPLC) for Two *S. lactis* Strains

organism	incubation time[a]	acetaldehyde[b] mean	RSD
S. lactis 60	3	1.29	10.4
	6	3.99	8.1
	9	4.53	7.9
S. lactis C2	3	0.56	15.4
	6	2.97	5.7
	9	2.93	4.6

[a] Incubation in Elliker broth (h at 32 °C). [b] Corrected for initial (0 time) data; means from triplicate steam distillates from duplicate growth trials (micrograms of acetaldehyde per milliliter); relative standard deviation (RSD, percent).

obtained by DNPH-HPLC had slightly higher precision than did MBTH data. The mean RSD (across incubation times) for DNPH-HPLC data was 2.4% compared to 4.6% for MBTH data from steam distillates.

Comparison of acetaldehyde data for two strains of *S. lactis* are presented in Table III. Data presented are means from triplicate steam distillates for duplicate growth trials. Slightly higher acetaldehyde levels were detected in *S. lactis* 60 compared to *S. lactis* C2. The RSD for these data ranged from 4.6 to 15.4%, which included error associated with distillation and DNPH reaction as well as between trial error.

The steam distillation and DNPH trapping and reaction techniques appear to be acceptable for acetaldehyde analysis in *S. lactis* cultures. It has been suggested that steam distillation could result in artifacts from alehyde formations due to Strecker degradation of amino acids (Lees and Jago, 1969). However, low data variability between distillates for cultures or for Elliker broth blanks would suggest that heating effects are minimal. Any background produced by heating may also be minimized by correction for broth blank data and for zero time values. The advantages of high recovery and speed of analysis by steam distillation compared to low-temperature purging may overcome any potential data variability induced by artifacts resulting from steam distillation. When one distillation apparatus and manual HPLC injection were used, an approximate rate of six samples per h was attained. This could be greatly improved with more automated modifications.

Registry No. Acetaldehyde, 75-07-0; acetaldehyde 2,4-dinitrophenylhydrazone, 1019-57-4.

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Protection of Vitamin C by Sugars and Their Hydrogenated Derivatives

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The oxidation of vitamin C was studied in unaerated solutions at 100 °C and aerated solutions at 20 °C, with and without the presence of sugars or their hydrogenated derivatives. All sugars and derivatives protected the vitamin under certain conditions, but there was no obvious difference between hydrogenated and unhydrogenated carbohydrates. In copper-catalyzed, rapidly oxidizing systems, the protective effect of carbohydrate was marked. These results are interpretable on the basis of complexing of copper by carbohydrates or their hydrogenated counterparts by which means the catalytic destruction of the vitamin may be prevented.

The oxidation of vitamin C in food systems is well-known (Barron et al., 1936; Birch and Parker, 1974; Dekker and Dickinson, 1940; Selman, 1976) to be catalyzed by copper and other metals with loss of vitamin potency. The reactions involved are the sequential formation of dehydroascorbic acid (DHA), diketogulonic acid (DKA), and ultimately oxalic acid. The biological effectiveness of the vitamin is lost with the disappearance of the initial L-xyloascorbic acid and DHA. The oxidation is also evidently pH dependent because most studies report greatest stability of the vitamin at low pH.

Foods containing vitamin C are usually characterized by a high carbohydrate content. It is therefore important to study the role of sugars, as natural ingredients of foods, on vitamin C stability.

Carbohydrates, both mono- and polysaccharides, are well-known to form complexes with minerals and other food components (Angyal, 1973; Gallali et al., 1978; Ren-

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