inant proportion, as in other leaf glycolipids (Mudd and Garcia, 1975). The chief fatty acid was linolenic in cardiolipin and phosphatidylglycerol and palmitic in the other phospholipid classes. Significant proportions of lauric acid were also found in many of the lipid classes. Triacylglycerols, phosphatidylglycerol, cardiolipin, and phosphatidylethanolamine contained appreciable proportions of palmitoleic acid. The occurrence of this acid in a number of leaves is known (Hitchcock and Nichols, 1971). Phosphatidylglycerol contained *trans*-3-hexadecenoic acid. This acid is frequently present in phosphatidylglycerols of photosynthetic tissues (Hitchcock and Nichols, 1971).

ACKNOWLEDGMENT

Thanks are due to the Council of Scientific and Industrial Research, New delhi, for the award of a Research Fellowship to K.S.R. Lipid standards were kindly given by Prof. K. Subba Rao, Hyderabad University, Dr. N. Patnaik, Centre for Cellular and Molecular Biology, Hyderabad, and colleagues.

Registry No. Lauric acid, 143-07-7; myristic acid, 544-63-8; myristoleic acid, 544-64-9; palmitic acid, 57-10-3; palmitoleic acid, 373-49-9; stearic acid, 57-11-4; oleic acid, 112-80-1; linoleic acid, 60-33-3; linolenic acid, 463-40-1; arachidic acid, 506-30-9; *trans*-3-hexadecenoic acid, 1686-10-8.

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Received for review November 17, 1983. Revised manuscript received April 12, 1984. Accepted July 9, 1984.

Determination of Volatile Fatty Acids in Molasses by Gas–Liquid Chromatography of Their Benzyl Esters

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The determination of formic, acetic, propionic, and *n*-butyric acids in cane molasses by gas–liquid chromatography is described. The acids were isolated by selective elution from silicic acid, using dichloromethane/ethanol (95/5). The benzyl esters, prepared by reaction of the tetrabutylammonium salts of the acids with benzyl bromide in acetone, were analyzed on a column of 20% DEGS on Chromosorb W by using benzyl *n*-valerate as the internal standard. Recovery of these acids (0.25–4 mg) from molasses averaged 99.7 ± 3.6%. The method applied to the analysis of a range of commercial molasses samples showed that formic (1.1–4.1 mg/g) and acetic (3.1–3.7 mg/g) acids were predominant, with traces (<0.1 mg/g) of propionic and *n*-butyric acids.

Molasses is widely used as a growth medium in the fermentation industry, due to its high content of fermentable sugars. Typically these include sucrose (30–40%), glucose (4–9%), and fructose (5–12%) (Paturau, 1982; Baker, 1980). In addition, the molasses contributes significant amounts of other nutrients, such as amino acids, minerals, and vitamins (Paturau, 1982; Baker, 1980; Schiweck, 1980). However, it may also contain small amounts of volatile carboxylic acids, which have been found to have an inhibitory action in fermentation (Labendzinski, 1980; Baker, 1980; Dierssen et al., 1956). It has been previously shown that these can occur in molasses at concentrations up to 0.4% (formic acid), 1% (acetic acid), 0.3% (propionic acid), and 0.6% (*n*-butyric acid) (Dierssen et al., 1956; Baker, 1980). Since the inhibitory level of these materials may be quite low (about 0.2% for formic acid) (Schiweck and Harberl, 1973), a convenient method for their estimation is required.

Previously reported methods for estimation of carboxylic acids in molasses have included paper chromatography (Dierssen et al., 1956), solvent extraction followed by either

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0021-8561/84/1432-1363$01.50/0

thin-layer chromatography (Skala and Friml, 1977) or gas–liquid chromatography (GLC) (Mee et al., 1979), ion-exchange followed by GLC (Oldfield et al., 1973; Reinefeld et al., 1979; Saito et al., 1983), and steam distillation followed by titration (Diersson et al., 1956). These methods generally use inconvenient and time-consuming sample preparation procedures that are not selective for the volatile fatty acids. Further, emulsification is commonly encountered in solvent extraction of high-sugar products, while steam distillation of such samples can lead to formation of formic acid (Joslyn, 1970).

GLC of volatile acids is well established and offers a convenient method for simultaneous estimation of mixtures of acids. Due to their polarity and poor FID response (particularly formic acid), the acids are normally analyzed as esters. A number of esters have been described (Staruszkiewicz et al., 1978) including the benzyl esters, which are stable and conveniently prepared (Monseur et al., 1981; Jones and Kay, 1976).

This paper describes a simple method for estimation of formic, acetic, propionic, and *n*-butyric acids in molasses. The acids were isolated by selective elution from silicic acid, followed by GLC as their benzyl esters using benzyl *n*-valerate as the internal standard.

MATERIALS AND METHODS

Apparatus. A Packard 7400 series gas chromatograph equipped with flame ionisation detector was used. The column was a 4 mm × 2 m silanized glass column packed with 20% DEGS on acid-washed Chromosorb W (80–100 mesh). The column oven temperature was 150 °C isothermal; inlet and detector temperatures were 220 and 240 °C, respectively. The nitrogen (carrier gas) flow rate was 60 mL/min. Quantitation was achieved by measurement of peak heights, referenced to the internal standard (benzyl *n*-valerate).

Reagents. Formic (90%), acetic, propionic, and *n*-butyric acids and tetrabutylammonium hydroxide were obtained from BDH Chemicals, Ltd., Poole, England. The acids were assayed acidimetrically and results were corrected accordingly. *n*-Valeric acid was obtained from Sigma Chemical Corp., St. Louis, MO, and benzyl bromide was obtained from Koch-Light Laboratories, Ltd., Colnbrook, England. Silicic acid (100 mesh) was obtained from Mallinckrodt Chemical Works, St. Louis, MO. Samples of commercial cane molasses, originating from Australia, Indonesia, and the Philipines, were obtained from Mauri Foods Division, Burns Philp and Co., Pty. Ltd., Sydney, Australia.

Determination of Relative Response Ratio. To mixtures of formic, acetic, propionic, and *n*-butyric acids (0.5, 1, 2, and 4 mg of each acid) (in duplicate) was added *n*-valeric acid (3 mg) plus a few small crystals of phenolphthalein. The mixtures were titrated with tetrabutylammonium hydroxide solution (0.05 N) and then evaporated to dryness with a rotary evaporator at 50 °C. To the residue, dissolved in acetone (2.5 mL), was added benzyl bromide (equivalent to the acidity of the sample, plus 10 μL), and the mixture was allowed to stand for 1 h at room temperature prior to GLC analysis (2-μL aliquots). Each solution was analyzed in triplicate.

Analysis of Molasses. Molasses (1 g) in a 50-mL beaker was mixed with sulfuric acid (1/1, 2 drops). The sample was then thoroughly mixed with silicic acid/anhydrous sodium sulfate (1/1, 6 g), and transferred to a glass chromatography column (22 mm × 25 cm) containing silicic acid (10 g) over anhydrous sodium sulfate (5 g), held in place with a plug of cotton wool. The beaker was dry-washed with silicic acid/anhydrous sodium sulfate (1/1, 2 g) with the washings added to the top of the column. The acids were eluted with dichloromethane/95% ethanol (95/5) at a flow rate of 2 mL/min. To the first 50 mL of eluate, collected in a 150-mL beaker, was added *n*-valeric acid (2 mL of 0.15% aqueous solution) and water (20 mL). The aqueous phase was titrated potentiometrically, with vigorous stirring, to pH 9.3 by using tetrabutylammonium hydroxide solution (0.05 N). The bulk of the aqueous phase was transferred to a 100-mL pear-shaped flask, evaporated to dryness, and derivatized as given under Determination of Relative Response Ratio.

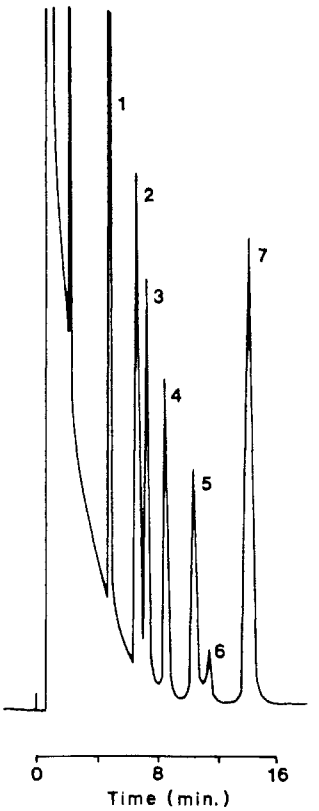

Figure 1. Gas chromatogram of derivatized acid mixture (1 mg of C1–C4 acids, 2.75 mg of C5 acid). 1 = benzyl bromide (excess). 2 = benzyl formate. 3 = benzyl acetate. 4 = benzyl propionate. 5 = benzyl *n*-butyrate. 6 = benzyl alcohol (byproduct). 7 = benzyl *n*-valerate (internal standard).

Recovery Test. To sucrose (0.7 g) was added formic acid (2 mg) in water (0.3 mL) and sulfuric acid (1/1, 2 drops). The mixture was chromatographed on silica gel as described under Analysis of Molasses, and the eluate was titrated to pH 8.3 with sodium hydroxide solution (0.01 N). The procedure was then repeated using a mixture of formic, acetic, propionic, and *n*-butyric acids (0.5 mg each) in place of formic acid. These procedures were carried out in duplicate.

To Australian molasses (0.8 g) was added various amounts of formic, acetic, propionic, and butyric acids (Table I) in water (0.2 mL). The resultant samples were analyzed as described under Analysis of Molasses. The procedure was carried out in triplicate.

RESULTS AND DISCUSSION

It was found that separation of the benzyl esters could be achieved using a number of polar stationary phases, such as SP2330 and SP2340. However, their use led to problems such as a lack of separation from benzyl alcohol (byproduct of derivatization reaction) and severe tailing of the solvent peak with molasses extracts. These problems were overcome by use of a 20% DEGS column, giving the separation shown in Figure 1. Peaks were well resolved,

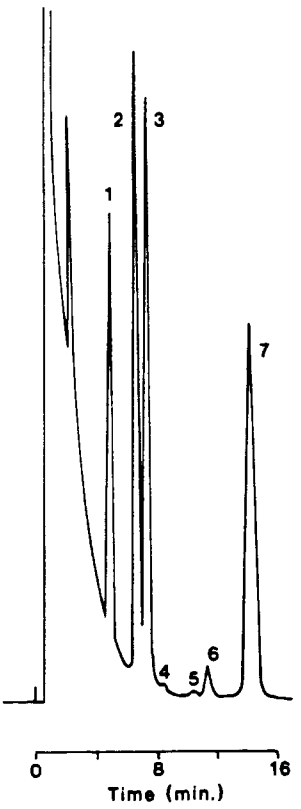

Figure 2. Gas chromatographic profile of Australian Molasses. 1 = benzyl bromide (excess). 2 = benzyl formate. 3 = benzyl acetate. 4 = benzyl propionate. 5 = benzyl *n*-butyrate. 6 = benzyl alcohol (byproduct). 7 = *n*-valerate (internal standard).

with near baseline separation of the formate and acetate esters. Retention times of 6.7, 7.3, 8.5, 10.5, and 14.3 min were obtained for the benzyl esters of formic, acetic, propionic, *n*-butyric, and *n*-valeric acids, respectively. The relative response ratios were found to be constant in the range tested, giving values (mean and SD) of 3.05 ± 0.05, 2.43 ± 0.08, 1.88 ± 0.02, and 1.39 ± 0.03 for benzyl esters of formic, acetic, propionic, and *n*-butyric acids, respectively. Quantitation was by peak height measurement, both for simplicity and to avoid problems that may occur with integration by the tangent skimming method. Estimated limits of detection were typically less than 0.1 mg/g of molasses.

A chromatogram showing the volatile fatty acid profile of a typical Australian molasses is shown in Figure 2. No *n*-valeric acid was detected in any of the samples tested, although it has been previously reported to be present in trace quantities in some unspecified European samples (Dierssen et al., 1956). No significant interference was noted with any of the peaks of interest, indicating the efficacy of the extraction procedure used, which was based on the column chromatographic sample cleanup step described by the Association of Official Analytical Chemists (1980). It was chosen for its simplicity and its avoidance of previously discussed problems encountered with alternate procedures. The selectivity of the extraction procedure was demonstrated by the observation that the volatile fatty acids accounted for 90–95% of the acidity of the molasses extracts, for all samples tested. The efficiency of the extraction procedure was established by titrimetric recovery studies. Recovery of formic acid (2 mg) and a mixture of formic, acetic, propionic, and *n*-butyric acids (0.5 mg each), added to a model sample (sucrose/water mixture), averaged 102.3% and 100.7%, respectively. The validity of the whole procedure, including derivatization, was established by GLC estimation of recovery of acids,

Table I. Recovery of Acids from Australian Cane Molasses

acid	amount added, mg	av % recovery	SD (n = 3)
formic	0.508	99.5	2.8
	1.016	99.4	0.2
	2.032	104.5	3.0
acetic	1.515	96.1	0.1
	3.030	97.4	2.5
	6.060	101.4	2.1
propionic	0.258	93.8	0.5
	0.516	95.5	0.8
	1.075	98.9	0.7
n-butyric	0.269	103.5	1.8
	0.538	100.4	2.5
	1.075	105.4	3.0

Table II. Results of Analysis of Cane Molasses Samples

sample source	acid, mg/g			
	formic	acetic	propionic	*n*-butyric
Australia	1.1	3.0	<0.05	0.1
Indonesia	4.1	3.6	<0.05	0.1
Indonesia	4.1	3.7	<0.05	<0.05
Indonesia	2.7	3.2	<0.05	<0.05
Indonesia	2.6	3.1	<0.05	<0.05
Philippines	1.8	3.0	<0.05	<0.05

covering a range of concentrations, added to molasses. The results, in Table I, show that quantitative recoveries were obtained for each acid, with the overall recovery being 99.7% with an RSD of 3.6%.

The procedure was then applied to analysis of a range of commercial molasses samples, originating from Australia, Indonesia, and the Philippines. Results, given in Table II, show that propionic and *n*-butyric acids were present in only trace quantities. Formic and acetic acids were present in higher amounts, with the formic acid level (1.1–4.1 mg/g) varying more than the acetic acid level (3.0–3.7 mg/g). The levels of formic acid in the Indonesian and Philippino molasses were in the range likely to affect microbial growth rates.

ACKNOWLEDGMENT

We thank Mauri Foods Division for their permission to publish this work.

Registry No. Benzyl formate, 104-57-4; benzyl acetate, 140-11-4; benzyl propionate, 122-63-4; benzyl butyrate, 103-37-7; formic acid, 64-18-6; acetic acid, 64-19-7; propionic acid, 79-09-4; butyric acid, 107-92-6.

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Received for review March 16, 1984. Accepted August 6, 1984.

Amino Acid Analysis of Feedstuffs: Determination of Methionine and Cystine after Oxidation with Performic Acid and Hydrolysis[1]

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Whereas the cystine content of feedstuffs is usually analyzed after oxidation of cystine to cysteic acid and subsequent hydrolysis of the protein, methionine is analyzed in many cases from the unoxidized hydrolysate. With corn, wheat, soybean meal, and feather meal, such values for methionine are 11–15% lower compared to the recovery obtained from oxidized hydrolysates. Only meat and bone meal and poultry byproduct meal showed smaller losses for methionine after direct hydrolysis. For complete oxidation an amount of 5 mL of performic acid/10 mg of nitrogen is sufficient. From multiple analyses of broiler feeds the repeatability (relative standard deviation) was determined to be 2.1% for methionine and 2.7% for cystine.

When analyzing the amino acid content of feedstuffs after protein hydrolysis, methionine and cystine present special problems due to their instability during hydrolysis conditions. Values obtained after direct hydrolysis of feedstuffs would lead to an underestimation of the requirement for methionine and cystine in animal nutrition. The oxidation of cystine to cysteic acid with performic acid before hydrolyzing the protein is widely accepted in order to determine the cystine content of feedstuffs (Moore, 1963; de Belsunce and Pion, 1963; Heese et al., 1971; Davies and Thomas, 1973; Wall and Gehrke, 1976; Mason et al., 1979). Concerning methionine, however, a number of laboratories use the direct hydrolysis of feedstuffs under nitrogen atmosphere (Jamalian and Pellett, 1967; Mondino and Bongiovanni, 1970; Hackler, 1971; Wall and Gehrke, 1976; Moodie et al., 1982). Attempts have been made to reduce losses of methionine during hydrolysis by increasing the temperature and reducing the time of hydrolysis (Kaiser et al., 1974; Wall and Gehrke, 1976; Lucas and Sotelo, 1982; Philipps, 1983). Other reports, however, indicate that for maximum recoveries methionine needs to be oxidized with performic acid to its sulfone (Jennings and Lewis, 1969; Slump, 1969; Heese et al., 1971; Beck et al., 1978; Mason et al., 1979, 1980b; Sarwar et al., 1983). Part of the discrepancies described in the literature may be due to the fact that the analysis of amino acids in food and feed presents problems that are not encountered when analyzing pure proteins (Blackburn, 1978). Various collaborative studies have shown that methionine and cystine usually give the highest variation of the results, compared with other amino acids (Porter et al., 1968; Knipfel et al., 1971; Kwolek and Cavins, 1971; Cavins et al., 1972; Williams et al., 1979; Chavana et al., 1980; Kreienbring, 1981; Sarwar et al., 1983). Considering the economic importance of amino acid levels for feed formulation, a standardization of the method to analyze amino acids in feedstuffs is necessary. Since neither in the United States nor in the European Community (Andersen et al., 1984) such a standard method has been established, further work is necessary.

The objectives of the present investigation were to determine the reliability of the partially contradicting results from literature on the necessity to protect methionine by oxidation before hydrolysis, to investigate oxidation conditions, and to check the accuracy of a standard oxidation procedure for the simultaneous determination of methionine and cystine, as well as other amino acids in feedstuffs and complete feeds.

MATERIALS AND METHODS

Chemicals and sources were as follows: formic acid, 88% (Merck, prepared from 98 to 100%, p.a.); hydrogen peroxide, 30%, p.a. (Merck); phenol, p.a. (Merck); hydrobromic acid, minimum 47%, p.a. (Merck); hydrochloric acid, 6 N, prepared by dilution (1:1) of 37%, p.a. (Merck); trisodium citrate·$2H_2O$, p.a. (Merck); sodium chloride, p.a. (Merck); thiodiglycol, ca. 98% (Serva); Brij 35 solution, prepared from Brij 35, practical quality (Serva), (Brij–water, 1:3); 1-propanol, p.a. (Merck); amino acids for calibration (Serva, Degussa).

Amino acid analysis conditions were as follows: pH Meter CG 803 (Schott); Biotronik Amino Acid Analyzer LC 2000, resin type Durrum DC-6A; resin height 27 cm; column diameter 0.6 cm; flow rate of buffer 35 mL/h; flow rate of ninhydrin 20 mL/h. The buffer composition and chromatographic program were as follows: A, sodium citrate, 0.173 N, pH 3.35, run time 15 min; B, sodium citrate, 0.2 N, pH 4.35, run time 40 min; C, sodium citrate, 0.2 N, sodium chloride, 0.5 N, pH 6.41, run time 33 min; D, sodium citrate, 0.2 N, sodium chloride, 1.4 N, pH 7.16, run time 29 min. Each buffer contained 0.1% of Brij 35–detergent solution and 0.01% of phenol. Buffer A also contained 0.5% of thiodiglycol as antioxidant and 2% of 1-propanol (Atkin and Ferdinand, 1970).

Other conditions were as follows: temperature T_1 = 48.5 °C (48 min) and T_2 = 60 °C (69 min); regeneration with 0.4 N NaOH solution, 10 min at 60 °C; equilibration with

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[1]Dedicated to Dr. Wilhelm A. Schuler in honor of his 70th birthday.

0021-8561/84/1432-1366$01.50/0