APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1989, p. 1617–1623
0099-2240/89/061617-07$02.00/0

Vol. 55, No. 6

Freon 11 Extraction of Volatile Metabolites Formed by Certain Lactic Acid Bacteria

RALPH P. TRACEY[1]†* AND TREVOR J. BRITZ[2]

Viticultural and Oenological Research Institute, Stellenbosch,[1] and Department of Microbiology, University of the Orange Free State, 9300 Bloemfontein,[2] South Africa

Received 7 November 1988/Accepted 3 March 1989

The volatile metabolites formed by 18 lactic acid bacteria, representing three genera, were extracted from a complex medium by using a Freon 11 extraction method. The Freon extracts were then analyzed by capillary gas chromatography, and certain extracts were analyzed by gas chromatography-mass spectrometry. A total of 35 major peaks, of which 20 were positively identified, were used to differentiate between the various strains. On the basis of the results obtained, it was possible to differentiate between the members of the genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc*, as well as between various species within the genus *Leuconostoc*. Of the 10 *Leuconostoc oenos* strains included in this study, 9 yielded similar results, but it was still possible to differentiate between the various strains. *L. oenos* B66 differed from the other *L. oenos* strains. Use of the Freon 11 extraction technique to determine volatile metabolites formed by lactic acid bacteria was shown to be highly reproducible and of great value. Furthermore, certain compounds not previously known to be formed by lactic acid bacteria were found.

The use of end product analysis by gas-liquid chromatography for the characterization and identification of bacteria is well documented. In the eighth edition of *Bergey's Manual of Determinative Bacteriology* (3), as well as in *Bergey's Manual of Systematic Bacteriology* (7, 13), a large number of generic descriptions include metabolic end products as criteria for the inclusion of an organism in a particular genus.

Most gas-liquid chromatography methods suffer from the disadvantage that a pretreatment of the sample is often necessary and that this is sometimes followed by a derivatization procedure (14). Even though Thornhill and Cogan (14) regard pretreatment and derivatization as a disadvantage, it must be borne in mind that pretreatment or derivatization of samples can contribute greatly to the analyses and, in so doing, can increase the value of the analyses and subsequent grouping or classification of species or strains. Direct injection of samples to measure the fermentation products formed by bacteria has only a limited application, because in most cases only a few compounds are determined. Because only the main compounds of fermentation are determined, organisms may be grouped together when, in reality, their relationship to each other is not very high. It is therefore necessary that additional tests be carried out to confirm the relationships.

Recently a reproducible technique, involving the use of a Freon 11 extraction method, was published to determine the amounts and identities of terpenes in grape juice and wine by capillary gas chromatography (10). The use of Freon 11 for the extraction of volatiles has also gained wide acceptance in the field of wine flavor research. It has been used to determine the amounts and identities of volatile yeast metabolites in fermenting grape musts, for which the gas-chromatographic analysis of Freon 11 extracts revealed over 72 esters and other metabolites (1). Furthermore, Freon 11 has various other advantages: it is relatively cheap, easily obtainable, noninflammable, and nontoxic (11). Two disadvantages, however, are that Freon 11 does not extract ethanol, which is an important metabolite formed by some bacteria, and that because Freon has a boiling point of 23.6°C, all the work must be carried out below this temperature in air-conditioned rooms.

Since ethanol is not one of the major metabolites formed during glucose catabolism by lactic acid bacteria, it is possible that the Freon 11 metabolite extraction technique will be of value in the differentiation of species or even strains of the lactic acid bacteria. The purpose of this study was to determine whether this metabolite extraction technique could be used in the differentiation of lactic acid bacteria and, more specifically, strains of *Leuconostoc oenos* which are of importance to the wine industry.

MATERIALS AND METHODS

Bacterial strains. The strains used in this study include the type strains *Lactobacillus casei* NCIB 8010, *Pediococcus damnosus* DSM 20331, *Leuconostoc cremoris* DSM 20346, *L. Dextranicum* DSM 20484, *L. lactis* DSM 20202, *L. mesenteroides* DSM 20343, *L. paramesenteroides* DSM 20288, and *L. oenos* DSM 20252, as well as reference strains *Lactobacillus plantarum* NCIB 6376, *L. oenos* DSM 20255, and *L. oenos* DMS 20257. *L. oenos* B66, 37D, 19, and 36 were selected as representative centrotype strains of four clusters obtained during a numerical taxonomic study on malolactic bacteria from the main wine-producing areas in the world (15). Three *L. oenos* strains (Lc5x, 30, and 7B) were selected on the basis of having the lowest similarity to the centrotype strain (15) of each of the three particular clusters. The strains were preserved by lyophilization on filter paper disks (6).

Culture conditions. For each strain, 50 ml of enriched tomato juice broth (15) was inoculated with a filter paper disk and incubated at 25°C for 48 h. A 10-ml sample of this culture was then used to inoculate 300 ml of the same medium. Excellent growth was found after incubation at 25°C for 4 days, whereafter the cells were removed by centrifugation for 20 min at 18,000 × *g*. The supernatant was recovered and

* Corresponding author.
† Present address: Department of Microbiology, University of the Orange Free State, P.O. Box 339, 9300 Bloemfontein, South Africa.

immediately cooled to 0°C for extraction with Freon 11 and subsequent gas-chromatographic analysis.

Extraction procedure and concentration of the extract. The procedure and extraction apparatus as described by Marais (10) were used. The internal standard, 2-ethyl-1-hexanol (80 μg/liter) was added to 250 ml of the cooled sample in a measuring flask and carefully mixed. A 20-ml portion of Freon 11 and the cooled sample containing the internal standard was carefully poured into the extraction apparatus described previously (10). This was then installed with its bottom in 50 mm of ice to prevent the formation of an emulsion. A collecting funnel and condenser, through which ethanol (at −5°C) was circulated, were fitted to the extraction unit. A 25-ml pear-shaped flask containing 20 ml of Freon was fitted to the extraction unit, and the fitted flask was then immersed in a water bath at 35°C. The extraction was performed for 20 h at a controlled temperature of 20°C. To concentrate the extract, a Vigreux fractioning column (270 by 20 mm) and an air condenser (550 by 13 mm) were fixed onto the flask containing the extract to facilitate reflux. The flask was placed in a water bath at 35°C, with the room temperature at about 20°C, and the extract was concentrated to 2 ml. The flask was then placed in solid CO_2 to freeze out any traces of water from the extract. The concentrated extract was then transferred to a 3-ml pear-shaped flask with a Pasteur pipette. A small condenser (220 by 8 mm), fitted with a spiral made from Teflon, was fitted to the flask, and concentration was continued under partial reflux to approximately 0.1 ml. The extracts were then stored at −12°C prior to analysis.

Gas chromatography conditions. The gas-chromatographic analyses were carried out on a Hewlett Packard 5880A gas chromatograph equipped with a hydrogen flame ionization detector and dual integrators. A Carbowax 20M fused-silica capillary column (50 m by 0.31 mm [inner diameter]) was used under the following operating conditions: injection temperature, 200°C; detector temperature, 250°C; column temperature, 60°C for 10 min, then increased by 1°C/min to 190°C and held at 190°C for 30 min; flow rate of carrier gas (helium), 1.5 ml/min; split flow rate, 120 ml/min; split ratio, 90:1; septum purge, 6 ml/min; hydrogen flow rate, 30 ml/min; air flow rate, 300 ml/min; injection volume, 1 μl.

Identification of the major peaks. A Finnigan gas chromatograph-mass spectrometer (model 4500) equipped with a Carbowax 20M fused-silica column (50 m by 0.31 mm [inner diameter]) and gas-chromatographic operating conditions, as described above, were used to identify the major peaks formed by the *L. oenos* strains on the basis of retention time and mass spectra. The identities of the various chemical compounds were confirmed by comparing their mass spectra with those of authentic standards analyzed under similar conditions. The following operating conditions were used: interface temperature, 190°C; manifold temperature, 100°C; column flow rate, 1.4 ml/min; split ratio, 48:1, electron multiplier voltage, 1,600 V; scanning rate, 35 to 450 amu/ehs with a 0.1-s delay between scans.

Determination of the relative concentrations. The relative concentrations of the major compounds were calculated by using the internal standard calibration method: relative concentration (*b*) = [area (*b*) x concentration (*a*)]/area (*a*), where *a* is the internal standard and *b* is an unknown compound.

Reproducibility. The reproducibility of the technique was determined by analyzing the Freon 11 extracts of four separately grown cultures of each of *Lactobacillus casei* NCIB 8010 and *L. oenos* DMS 20252. The mean, standard deviation, and coefficient of variation for each separate peak was calculated from the relative concentrations of each peak.

RESULTS AND DISCUSSION

Reproducibility. The data for the four separately grown cultures of *Lactobacillus casei* NCIB 8010 and *L. oenos* DSM 20252 are given in Table 1. The coefficients of variation were very low, and it was therefore possible that all the peaks could be used as an aid in the differentiation of the strains. The high level of reproducibility also indicates that this technique is reproducible when determining volatile metabolites formed by lactic acid bacteria.

Basal medium. The composition of the Freon extract of the basal medium (enriched tomato juice broth) is given in Table 2. The relative amounts of volatile products were found to be similar for each batch of basal medium used. A chromatogram showing the volatiles present in the basal medium is presented in Fig. 1A. With the use of the Freon 11 extraction method, 12 compounds were found in the basal medium. This complex medium was specifically used because it was previously found to contain all the required amino acids and other growth factors (15).

Metabolites produced by the various lactic acid bacteria. The relative concentrations of the major peaks of all the strains are given in Table 2. A total of 35 major volatiles could be differentiated, of which 20 were positively identified. By using the Freon 11 extraction technique, it is possible to identify several previously unknown compounds formed by lactic acid bacteria. No previous literature on the formation of these compounds by lactic acid bacteria could be found. These compounds include 3-methyl-2-butanol, ethyl-2-hydroxypropionate, benzaldehyde, 3-(methylthio)-1-propanol, α,α-dimethylbenzenemethanol, and benzenemethanol. Of these compounds, two were present in the basal medium but at much lower concentrations than produced by the organisms used in the study. It is possible that by applying this technique to other bacteria, even more compounds formed by bacteria will be discovered. These unique metabolites could play a vital role in differentiating between bacteria and thus possibly contribute to an even better classification of bacteria. A fact that must be taken into consideration is that a rich complex basal medium was used in the study, and this will greatly influence the type and concentration of metabolites produced.

Differentiation between the various lactic and bacteria. After visual comparison of the chromatograms of the various strains on the basis of the presence or absence and/or the relative concentrations of the compounds, we used the following compounds to differentiate between the various strains (peak numbers also given): isobutanol (peak 1), isoamyl alcohol (peak 2), acetoin (peak 3), 3-methyl-2-butanol (peak 4), ethyl-2-hydroxypropionate (peak 5), acetic acid (peak 6), butanoic acid (peak 9), α,α-dimethylbenzenemethanol (peak 13), hexanoic acid (peak 14), heptanoic acid (peak 17), octanoic acid (peak 19), nonanoic acid (peak 21), decanoic acid (peak 22), dodecanoic acid (peak 25), tetradecanoic acid (peak 31), and the unidentified compounds in peaks 8, 10, 18, 23, 24, 27, 28, 33, and 34.

The various strains of lactic acid bacteria (Table 2; Fig. 1B) could easily be differentiated. *Lactobacillus casei* (Fig. 1B) could be separated from the other strains on the basis that it formed the largest amount of ethyl-2-hydroxypropionate (peak 5) of all the strains studied (Table 2) and also produced large amounts of acetic acid (peak 6), α,α-dimethylbenzenemethanol (peak 13), benzenemethanol (peak 15),

TABLE 1. Relative concentrations, means, standard deviations, and coefficients of variation for the four Freon 11 extracts of each of *Lactobacillus casei* NICB 8010 and *L. oenos* DSM 20252

Compound	Peak no.	Retention time (min)	*Lactobacillus casei*		*L. oenos*	
			Mean concn ± SD	Coefficient of variation[a] (%)	Mean concn ± SD	Coefficient of variation (%)
Isobutanol	1	7.26	152.80 ± 3.08	2.01	133.58 ± 1.10	0.83
Isoamyl alcohol	2	10.20	252.83 ± 5.92	2.34	248.05 ± 2.90	1.17
Acetoin	3	14.81	7.10 ± 0.20	2.82		
3-Methyl-2-butanol	4	18.31	25.53 ± 0.43	1.67	24.18 ± 0.22	0.92
Ethyl-2-hydroxypropionate	5	20.23	1,122.05 ± 24.81	2.21	482.12 ± 8.69	1.80
Acetic acid	6	29.95	599.53 ± 12.87	2.15	601.13 ± 14.76	2.45
Benzaldehyde	7	43.52	88.50 ± 1.09	1.23	86.00 ± 0.78	0.91
Unidentified	8	48.53	118.23 ± 1.57	1.33		
Butanoic acid	9	50.07	203.23 ± 1.73	0.85	217.93 ± 2.59	1.19
Unidentified	10	53.92	95.83 ± 0.35	0.37		
Pentanoic acid	11	54.92	121.85 ± 0.72	0.59	126.40 ± 1.97	1.56
3-(Methylthio)-1-propanol	12	59.41	233.83 ± 1.56	0.67	192.90 ± 4.62	2.39
α,α-Dimethylbenzenemethanol	13	64.59	86.35 ± 0.76	0.88	37.35 ± 1.12	3.01
Hexanoic acid	14	74.16	41.45 ± 0.52	1.25	37.55 ± 1.46	3.88
Benzenemethanol	15	76.15	233.23 ± 0.81	0.35	211.38 ± 6.24	2.95
2-Phenylethanol	16	79.63	375.25 ± 0.97	0.26	342.50 ± 9.19	2.68
Heptanoic acid	17	85.32	52.40 ± 0.14	0.27	54.78 ± 1.74	3.17
Unidentified	18	92.02	24.30 ± 0.22	0.89	42.13 ± 0.28	0.65
Octanoic acid	19	96.12	120.70 ± 0.65	0.54	101.88 ± 2.28	2.23
Unidentified	20	99.22	44.75 ± 0.34	0.76	32.13 ± 1.08	3.37
Nonanoic acid	21	106.40	20.88 ± 0.26	1.26	10.63 ± 0.25	2.35
Decanoic acid	22	116.21	119.00 ± 1.60	1.34	46.03 ± 0.59	1.27
Unidentified	24	127.60	46.90 ± 0.42	0.90		
Dodecanoic acid	25	134.71	233.95 ± 0.82	0.35	58.50 ± 2.10	3.59
Unidentified	27	138.37	30.18 ± 0.39	1.28		
Tetradecanoic acid	31	154.85	229.70 ± 5.71	2.49	98.15 ± 3.73	3.80
Unidentified	34	160.40	143.90 ± 2.90	2.04	65.98 ± 0.25	0.38

[a] Coefficient of variation = (standard deviation/mean) × 100.

nonanoic acid (peak 21), decanoic acid (peak 22), and tetradecanoic acid (peak 31).

The formation of the highest concentrations of hexanoic acid (peak 14) and dodecanoic acid (peak 25) by the *Lactobacillus plantarum* strain and the formation of high concentrations of acetoin (peak 3), octanoic acid (peak 19), and decanoic acid (peak 22) and a small amount of acetic acid (peak 6), as well as the absence of two unidentified compounds (peaks 8 and 10) and nonanoic acid (peak 21), could be used to differentiate this strain from the other strains included in this study (Table 2).

The *P. damnosus* strain was differentiated from the others on the basis that it formed the largest amount of isoamyl alcohol (peak 2) of all the strains studied; it also formed high concentrations of ethyl-2-hydroxypropionate (peak 5), and it formed two unidentified compounds (peaks 8 and 10). The above-mentioned differences for these organisms can also be complemented with the use of morphology and cell wall composition as previously described (15).

When the various *Leuconostoc* species were compared with each other, it was found that although they generally yielded similar profiles, they could still be separated (Table 2). *L. cremoris* was separated mainly on the basis of the high concentrations of 2-phenylethanol (peak 16) and dodecanoic acid (peak 25) formed and the presence of high concentrations of the unidentified peaks 26 and 33. This strain was the only organism that produced the unidentified compound in peak 33.

The *L. dextranicum* strain could be differentiated on the basis of the amounts of butanoic acid (peak 9), nonanoic acid (peak 21), decanoic acid (peak 22), and an unidentified compound (peak 34) formed.

The high concentration of 3-(methylthio)-1-propanol (peak 12) and the low concentrations of α,α-dimethylbenzenemethanol (peak 13) and heptanoic acid (peak 17) formed were used to separate *L. lactis* from the other strains. According to Cogan et al. (4), *L. lactis* forms acetoin (a fact that was confirmed in this study), even though only at low concentrations (Table 2).

L. mesenteroides was differentiated from the other strains on the basis of the formation of the largest amount of acetoin (peak 3), as well as the relatively high concentrations of 3-methyl-2-butanol (peak 4), the presence of the unidentified compounds in peaks 18 and 24, and the absence of acetic acid (peak 5) and the unidentified compound in peak 10 (Table 2).

The formation of the largest amounts of α,α-dimethylbenzenemethanol (peak 13), heptanoic acid (peak 17), and the unidentified compounds in peaks 18, 20, 27, and 28 made it possible to separate *L. paramesenteroides* from all the other strains. This was the only strain to form the unidentified compound 28, no ethyl-2-hydroxypropionate (peak 5), no acetic acid (peak 6), and no unidentified compounds 8 and 10, and it characteristically produced only low concentrations of butanoic acid (peak 9), hexanoic acid (peak 14), octanoic acid (peak 19), and tetradecanoic acid (peak 31). Garvie (5) found that it was difficult to separate *L. paramesenteroides* from the non-dextran-forming strains of *L. mesenteroides*, whereas this study (Table 2) showed that these species can easily be distinguished. The formation of acetic acid (peak 6) can also be used to separate them from various other *Leuconostoc* species, since the *L. mesenteroides* and *L. paramesenteroides* cultures used in this study did not form any acetic acid (peak 6).

TABLE 2. Relative concentrations of the volatile metabolites produced by various strains of lactic acid bacteria

Compound	Peak no.	Retention time (min)	Composition of test medium	Relative concn of peaks in following organisms:																
				Lactobacillus casei	*Lactobacillus plantarum*	*Pediococcus damnosus*	*L. cremoris*	*L. dextranicum*	*L. lactis*	*L. mesenteroides*	*L. paramesenteroides*	*L. oenos*								
												DSM 20252	DSM 20255	DSM 20257	B66	Lc5x	37D	30	19	36	7B
Isobutanol	1	7.26		156.0	76.6	133.2	140.4	97.5	135.5	118.0	96.4	134.6	152.1	72.9	163.8	100.0	114.7	106.8	99.1	61.5	59.6
Isoamyl alcohol	2	10.20		259.8	173.8	415.5	259.0	185.1	266.1	207.9	242.0	251.1	270.9	140.6	324.8	402.6	173.6	206.0	251.3	148.8	133.3
Acetoin	3	14.81		7.0	1,192.3	14.0	3.0	10.0	5.4	1,810.0	129.1		7.8	1.0	18.3		6.8	14.3	325.1	11.1	
3-Methyl-2-butanol	4	18.31		25.0	22.2	27.1	19.6	14.1	23.2	32.0	24.2	24.4	22.6	10.9	40.4	77.5	31.3	20.8	54.3	27.0	37.0
Ethyl-2-hydroxypropionate	5	20.23		1,148.1	16.1	783.5	507.0	541.0	603.6	46.8		492.9	727.2	94.4	491.6	904.0	398.6	246.4		737.8	886.8
Acetic acid	6	29.95		612.6	21.5	523.1	350.3	257.9	370.6			614.1	448.0	357.0	385.5	594.0	782.5	590.9	8.3	310.0	427.8
Benzaldehyde	7	43.52	14.1	89.2	40.7	92.7	63.4	57.7	74.1	52.2	57.4	87.0	67.8	58.9	41.5	97.5	88.1	53.8	41.6	70.2	68.6
Unidentified	8	48.53		119.7		249.0	70.6	159.3	160.5	4.3			118.8	12.8	3,908.2		73.3	159.2		129.7	
Butanoic acid	9	50.07	47.7	205.4	146.2	207.7	167.8	223.8	183.8	180.0	54.6	220.7	155.3	158.7	151.3	241.5	244.1	154.0	107.0	200.0	170.9
Unidentified	10	53.92	15.6	96.0		197.6	40.6	112.5	125.0				89.4		3,637.4	34.5	55.7	85.8		107.2	
Pentanoic acid	11	54.92	29.8	122.5	63.4	131.6	96.5	93.2	106.5	104.5	173.3	128.5	98.3	93.1	198.3	160.6	133.3	99.6	106.8	111.7	94.3
3-(Methylthio)-1-propanol	12	59.41		235.4	81.2	169.4	130.3	185.2	231.6	137.8	81.3	197.9	201.0	145.4	273.4	182.6	102.9	152.7	64.7	226.0	142.1
α-α-Dimethylbenzenemethanol	13	64.59		87.3	11.6	20.5	18.5	20.1	4.6	2.3	88.5	38.8	36.7	4.3	65.8	67.8	4.3	27.3	2.4	31.5	15.2
Hexanoic acid	14	74.16		41.7	168.1	45.8	34.9	37.5	33.7	46.4	14.8	39.3	34.4	43.4	38.1	30.4	32.2	45.8	20.0	44.3	30.9
Benzenemethanol	15	76.15	31.2	234.3	138.3	198.1	175.4	184.1	201.7	161.2	84.7	217.1	178.7	167.9	204.9	222.5	133.2	149.3	164.3	207.6	179.6
2-Phenylethanol	16	79.63		376.2	229.8	313.1	406.6	398.2	342.4	291.1	119.4	355.3	369.6	340.1	350.7	371.6	345.6	337.7	252.3	341.4	361.0
Heptanoic acid	17	85.32	85.7	52.3	84.0	83.6	58.3	72.0	41.5	89.1	273.3	56.4	43.1	77.7	89.2	79.3	64.6	95.6	86.0	57.3	45.8
Unidentified	18	92.02		24.1	23.0	20.0	21.4	17.3	20.9	65.4	74.3	41.8	31.8	20.4	18.1	34.7	16.5	16.0	5.7	26.7	33.0
Octanoic acid	19	96.12	12.0	120.2	299.0	127.8	98.1	115.0	101.8	116.9	18.9	104.3	111.6	125.3	153.8	104.8	44.8	140.6	17.8	130.6	114.1
Unidentified	20	99.22	3.5	44.3	12.7	24.0	61.3	43.8	35.5	28.0	173.6	33.4	42.4	30.8	37.0	26.1	23.7	16.1	3.9	33.7	34.6
Nonanoic acid	21	106.40	8.9	20.5		16.2	16.3	20.7	14.5	17.4	7.3	10.9	17.9	16.4	22.6	17.5	15.9	7.8	8.7	13.5	13.9
Decanoic acid	22	116.21	6.2	117.0	140.7	92.5	80.4	110.8	83.8	65.2	36.6	46.7	89.4	74.4	112.5	18.9	71.4	14.2	42.9	44.7	56.4
Unidentified	23	124.85																	113.0		
Unidentified	24	127.60		47.5	41.1	39.1		35.2		52.2			38.5	34.5	28.7	51.0	47.5	34.6		39.3	
Dodecanoic acid	25	134.71	24.3	233.0	384.2	194.5	296.1	192.5	167.3	163.5	105.9	60.8	174.1	98.1	205.6	216.1	65.3	58.7	254.1	58.7	56.2
Unidentified	26	135.85					439.6								160.0						
Unidentified	27	138.37		29.9				17.1			227.5		15.9	14.0	19.2		21.6		20.9	20.5	
Unidentified	28	145.65									442.8										
Unidentified	29	148.43																1736.6			
Unidentified	30	152.00																	60.3	65.1	76.7
Tetradecanoic acid	31	154.85	29.6	223.0	131.0	197.7	161.8	192.8	158.6	180.0	52.0	101.9	153.3	113.1	153.6	78.1	68.9	129.2	15.1	63.7	43.7
Unidentified	32	156.17				343.1															
Unidentified	33	156.69					705.1														
Unidentified	34	160.40		140.7	35.9	100.4	84.0	130.3	100.6	46.8		65.7	107.6	60.8	211.7	91.5	170.6	25.0	21.4	59.1	162.5
Unidentified	35	161.24													675.5	3.0					

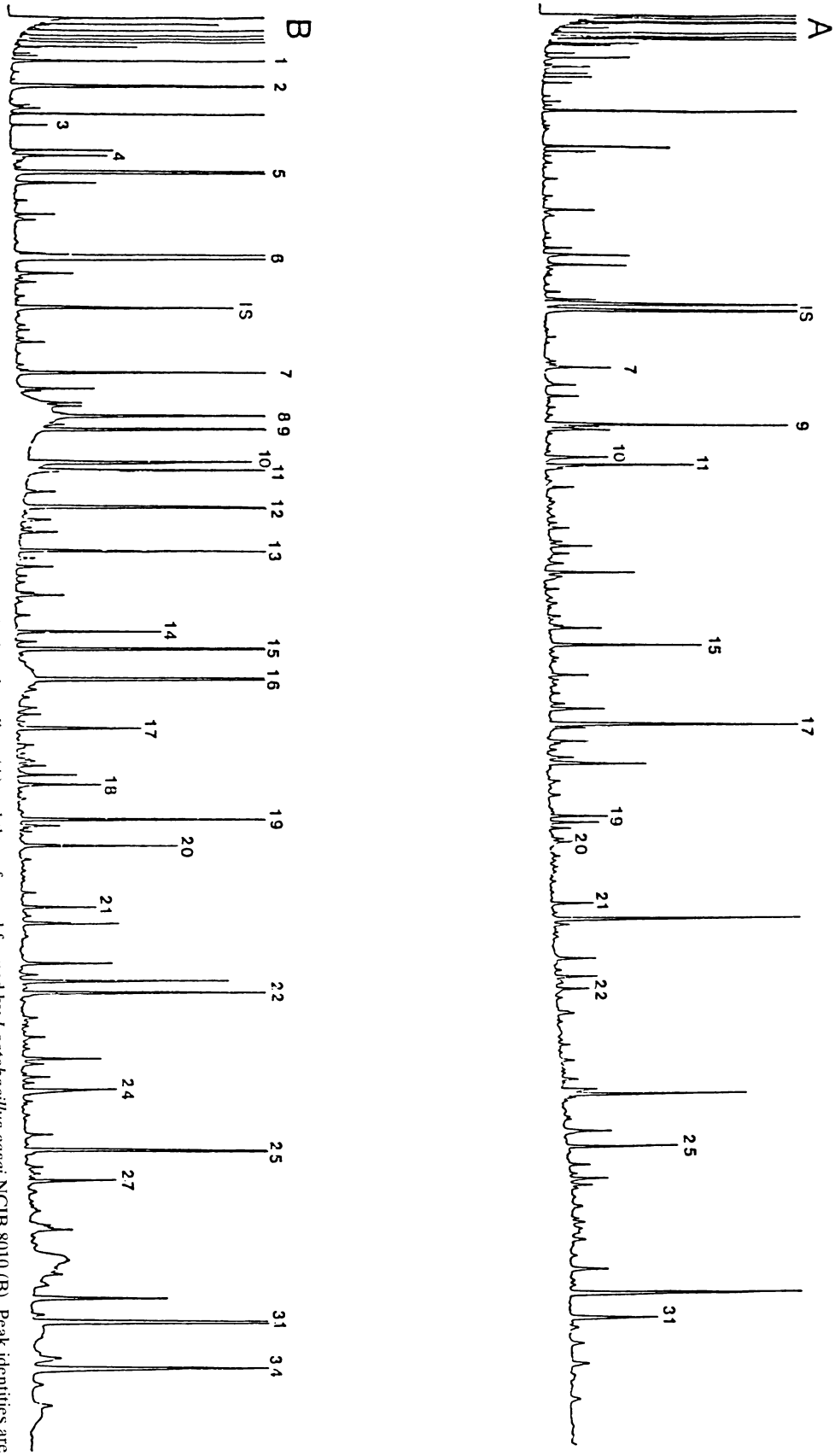

FIG. 1. Chromatogram of the Freon 11 extract of the volatiles present in the basal medium (A) and those formed formed by *Lactobacillus casei* NCIB 8010 (B). Peak identities are listed in Table 1. IS, Internal standard.

TABLE 3. Peaks used to differentiate between the various *L. oenos* strains

Strain	Cluster[a]	Relative concns[b] of peak no.[c]:												
		2	3	5	6	8	10	23	24	26	27	29	30	35
DSM 20252		4+	−	6+	7+	−	−	−	−	−	−	−	−	−
DSM 20255		4+	+	7+	6+	3+	2+	−	+	−	+	−	−	−
DSM 20257		3+	+	2+	5+	+	−	−	+	−	+	−	−	−
B66	A (centrotype)	5+	+	6+	5+	8+	8+	−	+	3+	+	−	−	7+
Lc5x	A	6+	−	8+	7+	−	+	−	2+	−	−	−	−	+
37D	B (centrotype)	3+	+	5+	8+	2+	2+	−	+	−	+	−	−	−
30	B	4+	+	4+	7+	3+	2+	−	+	−	−	8+	−	−
19	C (centrotype)	4+	5+	−	+	−	−	3+	−	−	+	−	1+	−
36	D (centrotype)	3+	+	7+	5+	3+	3+	−	+	−	+	−	2+	−
7B	D	3+	−	8+	6+	−	−	−	−	−	−	−	2+	−

[a] Strains not designated as centrotype strains are the strains selected as having the lowest similarity to the centrotype strain.
[b] Relative concentrations are expressed as follows: −, 0; +, 1 to 50; 2+, 51 to 100; 3+, 101 to 200; 4+, 201 to 300; 5+, 301 to 400; 6+, 401 to 500; 7+, 501 to 750; 8+, >750.
[c] Peak identities are given in Table 2.

Garvie (5) reduced the species in the genus *Leuconostoc* from six to four by including *L. dextranicum* and *L. cremoris* as subspecies of *L. mesenteroides*. The results from this study (Table 2) generally confirm that *L. cremoris* and *L. dextranicum* are closely related. However, *L. mesenteroides* does not appear to be closely related to them. The *L. mesenteroides* strain appeared to be more closely related to the *L. paramesenteroides* strain than to the *L. dextranicum* and *L. cremoris* strains. More representative strains from these four species (or subspecies, as proposed by Garvie [5]) will have to be studied to confirm the above observations.

In a previous study (15), it was shown that the differentiation between the leuconostocs and pediococci can be problematic and that cell wall analysis and morphology are important criteria in differentiating the leuconostocs from other genera. Morphologically, *Lactobacillus* can easily be distinguished from the coccoid genera *Leuconostoc* and *Pediococcus*. Separation of the three genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc* by using volatile metabolites was comparatively easy, especially when comparing the differences in the amounts of isoamyl alcohol (peak 2), acetoin (peak 3), ethyl-2-hydroxypropionate (peak 5), acetic acid (peak 6), and hexanoic acid (peak 14) formed. The presence or absence of several other metabolites is also of value. However, the results obtained in this study, with the exception of results for the *L. oenos* species, were based on only a single representative of each species and must therefore be carefully interpreted before any general conclusions or recommendations can be made. One positive conclusion that can be made is that the Freon 11 technique can be of great value in differentiating between lactic acid bacteria.

Differentiation of the *L. oenos* strains. Overall, the majority of *L. oenos* strains yielded similar metabolic profiles, but the relative amounts of each compound formed differed markedly. Of the 35 important compounds detected in this study, only 13 yielded major differences that could be used to differentiate between the *L. oenos* strains (Tables 2 and 3). The use of these 13 compounds in the separation of the *L. oenos* strains used in this study is shown in Table 3. These include acetoin (peak 3), ethyl-2-hydroxypropionate (peak 5), and the unidentified compounds in peaks 8, 10, 24, and 27 (Table 3). Although the *L. oenos* strains compared favorably with the *L. oenos* reference strains regarding the presence or absence of compounds 1 to 22, marked differences were found for the remaining compounds.

Strain B66 (Table 2) was separated from the other strains since this organism formed the highest concentrations of the unidentified compounds in peaks 8, 10, 34, and 35 of all the strains studied. Several other differences are also evident. Strain B66 is the centrotype strain of cluster A (15), and the volatile metabolites formed by this strain is a further confirmation that it differs from the other strains included in this study. In contrast to this, strain Lc5x did not form or formed much less of the unidentified compounds in peaks 8, 10, 34, and 35 (Table 3).

L. oenos 37D and 30 were selected as representatives of the largest phenotypic cluster (cluster B) from a previous taxonomic study (15). The volatile-metabolite profiles of these strains differed to a certain degree, and they could be separated on the basis of the presence or absence and/or concentrations of compounds 5, 6, 9, 27, 29, 31, and 34 (Tables 2 and 3).

Strain 19 could be separated, mainly because it was the only *L. oenos* strain to form large concentrations of acetoin (peak 3) and was the only strain to form the unidentified compound in peak 23. Further profile characteristics of this organism were the absence of ethyl-2-hydroxypropionate (peak 5) and the unidentified compounds in peaks 8 and 10 (Table 3). This strain had a different overall volatile-metabolite profile from those of the other *L. oenos* strains. It was the centrotype strain of cluster C and was phenotypically fairly closely related to cluster B (15).

The two representative strains from cluster D (36 and 7B) were separated from the other strains on the basis of the formation of less isobutanol (peak 1) and isoamyl alcohol (peak 2). They could also be separated from each other by using acetoin (peak 3) and the unidentified compounds in peaks 8, 10, 24, and 27 (Table 3). The overall volatile-metabolite profiles of these two strains compared favorably with those of the other *L. oenos* strains.

Once again, it must be stressed that the data obtained and used in the differentiation of the different *L. oenos* strains are based on single representatives of the phenotypic clusters (15) and that any conclusions made must be seen in this light. Further work is also required on these and other strains that are to be used to induce malolactic fermentation. It has been shown that *L. oenos* strains are the most reliable organisms which can be used for inducing malolactic fermentation (2, 8, 9). Therefore, to find the *L. oenos* strain that is best suited for inducing malolactic fermentation and that will have the least negative effect on the wine, the strains will have to be tested individually in wine, and these wines must subsequently then undergo a sensory evaluation and analysis by the Freon extraction method.

Utility and value of the Freon 11 extraction technique. Although the Freon extraction technique is more time-consuming and elaborate than previously published gas-chromatographic techniques for the study of volatile metabolites produced by bacteria, we believe that the amount of work and time taken for the extraction are duly rewarded in the results obtained, since a wider range of metabolites are determined than by conventional direct-injection chromatography. Furthermore, it is possible to differentiate between species of a particular genus and between strains of a particular species.

When organisms are used industrially, as with the lactic acid bacteria, this technique could help to determine the contribution of the organisms to the flavor of the various products. In 1965, Pilone and Kunkee (12) showed that flavor differences in wines in which malolactic fermentation had been induced by bacteria were small. The use of different and newer strains thus warrants a new look at the possible contribution of the microorganisms to the flavor of wine. It is also important to try and correlate the sensory evaluation of wines which have undergone malolactic fermentation with chemical analyses by the technique described in this paper.

ACKNOWLEDGMENTS

The technical assistance of Helena Kritzinger and Isabeau Brönn is acknowledged. Johan Marais is thanked for his critical review of the paper.

LITERATURE CITED

1. **Akhtar, M., R. E. Subden, J. D. Cunningham, C. Fyfe, and A. Meiering.** 1985. Production of volatile yeast metabolites in fermenting grape musts. Can. Inst. Food Sci. Technol. J. **18**:280–283.
2. **Beelman, R. B., A. Gavin, and R. M. Keen.** 1977. A new strain of *Leuconostoc oenos* for induced malo-lactic fermentation in Eastern wines. Am. J. Enol. Vitic. **28**:159–165.
3. **Buchanan, R. E., and N. E. Gibbons (ed.).** 1974. Bergey's manual of determinative bacteriology. The Williams & Wilkins Co., Baltimore.
4. **Cogan, T. M., M. O'Dowd, and D. Mellerick.** 1981. Effects of pH and sugar on acetoin production from citrate by *Leuconostoc lactis*. Appl. Environ. Microbiol. **41**:1–8.
5. **Garvie, E. I.** 1986. Genus *Leuconosoc*. p. 1071–1075. *In* P. H. A. Sneath, N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.), Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
6. **Joubert, W. A., and T. J. Britz.** 1987. A simple and inexpensive method for the long-term preservation of microbial cultures. J. Microbiol. Methods **7**:73–76.
7. **Krieg, N. R., & J. G. Holt (ed.).** 1984. Bergey's manual of systematic bacteriology, vol. 1. The Williams & Wilkins Co., Baltimore.
8. **Kunkee, R. E.** 1967. Malo-lactic fermentation. Adv. Appl. Microbiol. **9**:235–279.
9. **Lafon-Lafourcade, S., E. Carre, and P. Ribéreau-Gayon.** 1983. Occurrence of lactic acid bacteria during the different stages of vinification and conservation of wines. Appl. Environ. Microbiol. **46**:874–880.
10. **Marais, J.** 1986. A reproducible capillary gas chromatographic technique for the determination of specific terpenes in grape juice and wine. S. Afr. J. Enol. Vitic. **7**:21–25.
11. **Marais, J., and A. C. Houtman.** 1979. Quantitative gas chromatographic determination of specific esters and higher alcohols in wine using Freon extraction. Am. J. Enol. Vitic. **30**:250–252.
12. **Pilone, G. J., and R. E. Kunkee.** 1965. Sensory characterization of wines fermented with several malo-lactic strains of bacteria. Am. J. Enol. Vitic. **16**:224–230.
13. **Sneath, P. H. A., N. S. Mair, M. E. Sharpe, and J. G. Holt (ed.).** 1986. Bergey's manual of systematic bacteriology, vol. 2. The Williams & Wilkins Co., Baltimore.
14. **Thornhill, P. J., and T. M. Cogan.** 1984. Use of gas-liquid chromatography to determine the end products of growth of lactic acid bacteria. Appl. Environ. Microbiol. **47**:1250–1254.
15. **Tracey, R. P., and T. J. Britz.** 1987. A numerical taxonomic study of *Leuconostoc oenos* strains from wine. J. Appl. Bacteriol. **63**:523–532.