Z Lebensm Unters Forsch (1981) 172: 377–381

Zeitschrift für
Lebensmittel-Untersuchung und -Forschung
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The Gas Chromatographic-Mass Spectrometric Examination of the Volatiles Produced by the Fermentation of a Sucrose Solution

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Gaschromatographisch-massenspektrometrische Untersuchung der durch Gärung einer Saccharoselösung gebildeten flüchtigen Bestandteile

Zusammenfassung. Gaschromatographisch-massenspektrometrische Analyse der während der Gärung einer Saccharoselösung gebildeten flüchtigen Substanzen hat zur endgültigen Identifizierung von 8 Alkoholen, 6 Carbonylverbindungen, 9 Acetalen, 25 Estern und 17 Säuren geführt. Es werden auch vorläufige Beweise für die Anwesenheit von 15 anderen Komponenten erbracht.

Summary. Gas chromatographic-mass spectrometric examination of the volatiles produced during the fermentation of a sucrose solution has led to the definite identification of 8 alcohols, 6 carbonyls, 9 acetals, 25 esters, and 17 acids, tentative evidence being presented for a further 15 components.

The volatiles produced during the fermentation of synthetic media have been investigated a number of times since the advent of gas chromatography both in connection with alcoholic beverages [1–5] and bread fermentations [6, 7]. Although recent papers have investigated the results of using many different sugars for the primary carbon source [3], and of different yeast strains [4] for carrying out the fermentation, only a few compounds have been identified by techniques other than gas chromatographic [4] retention times.

As part of a programme of research into the factors influencing the formation of the volatile components of ciders, in particular the effect of the various constituents of the apple, a coupled gas chromatographic-mass spectrometric investigation into the volatiles produced during the fermentation of a solution in which sucrose was the carbon source, other nutrients being kept to a minimum compatible with reasonable yeast growth, was carried out. The following paper reports on the results of such an examination.

Materials and Methods

1. Solvents and Reagents

All solvents and reagents used in large quantities were purified prior to use as described previously [8]. Sucrose was of normal commercial grade obtained from Tate & Lyle Ltd.

2. Fermentation

A sucrose solution (38 litres) of specific gravity 1.050, containing 1% of Yeast Nitrogen Base [9] (Difco Laboratories), pasteurized by passing through a tube pasteurizer, was inoculated with 12×10^4 cells of *Saccharomyces cerevisiae* (AWY 350R) and fermented at 25 °C over a period of three weeks until all the sucrose had been metabolized. After fermentation was completed the supernatant liquid was centrifuged free of the yeast residues and stored at 0 °C until stripped of volatiles.

3. Isolation of Neutral Volatile Constituents

The volatiles were removed from a portion of the fermented liquor (30 litres) under vacuum (6,300 N/m^2) using a Kestner climbing-film evaporator [10]. The aroma distillate (7.75 litres) was saturated with sodium chloride (300 g/litre) and extracted in batches of 2 litres with trichlorofluoromethane (300 ml per batch) using a continuous extractor. The bulked extracts were dried over anhydrous sodium sulphate (300 g/litre), concentrated to approximately 150 ml using a 91×2.5 cm fractionating column packed with Fenske helices, and washed with saturated sodium bicarbonate solution (2×75 ml). The aqueous layer was washed with further trichlorofluoromethane (2×75 ml), the organic layer was bulked with the original extract, dried over anhydrous sodium sulphate, and concentrated to approximately 2 ml, the final stage of concentration being performed via a 25×1 cm Vigreux fractionating column.

Quantitative information on major components was obtained both by direct injection of a sample of the fermented sucrose solution, and by simple extraction of 100 ml of the liquid with 10 ml of ether and concentrating the extract to 1 ml. External standards were used in the former case and an internal standard (2-ethylhexanol) in the latter. Amounts of minor components that would only be detected in the large-scale examination were estimated by comparison

0044-3026/81/0172/0377/$1.00

of their peak heights with the chromatographically nearest major component of similar chemical class, whose concentration had been estimated by direct injection or simple extraction.

4. Isolation of Volatile Acids

The sodium bicarbonate solution obtained from washing the trichlorofluoromethane extract from the large scale distillation was evaporated to dryness on a rotary evaporator, the last traces of volatile material being removed by applying vacuum from a rotary oil pump. The solid material was dissolved in 100 ml of distilled water and acidified to pH 6.0 with 4 N hydrochloric acid. Sodium chloride (30 g) was added and the aqueous solution of acids was extracted with ethyl ether ($1 \times 100 + 2 \times 50$ ml). The extracts were bulked, dried over anhydrous sodium sulphate, and concentrated to approximately 0.5 ml using the fractionating columns previously described for concentrating the neutral fraction.

Quantitative information on acids and further confirmation of identity was obtained by distilling 1 litre of the fermented liquor in an apparatus similar to, but larger than, described for the determination of fusel alcohols [10]. Once 500 ml had been distilled, distilled water (500 ml) was added and a further 500 ml was removed. The distillate was made alkaline (pH 11) with N sodium hydroxide (Analar grade) and evaporated to dryness as previously described. Free acids were either regenerated as outlined above, or by using the minimum quantity of 30% hydrochloric acid (B.D.H. Analar grade) in redistilled dioxan.

Portions of the ether extracts obtained by both the large and small scale distillations were converted to methyl esters by reaction with diazomethane saturated ether (3 ml) as described by De Boer [11], the final solution being concentrated to 1 ml before examination.

Sodium salts from the small scale distillation were also converted directly to methyl esters by refluxing for 5 min with sufficient 14% boron trifluoride in methanol (B.D.H.) to ensure solution (5 ml). The resultant mixture was extracted with pentane (3×2 ml), and the extract was concentrated to approximately 0.5 ml and made up to 1 ml with hexane. In all cases amounts of components were estimated by comparison with external standards.

5. Examination of Extracts

Gas chromatography was accomplished using either a FM 810, a Hewlett Packard 7620, 5710 or 5830 gas chromatograph fitted with flame ionisation detectors and using the following columns:

a) Stainless steel capillary column 150 m × 0.76 mm i.d. coated with Carbowax 20 M, held isothermally for 15 min and then programmed from 65–110 °C at 2 °C/min, 110–145 °C at 4 °C/min and 145–210 °C at 2 °C/min. Injection port and detector temperatures were held at 250 °C and the nitrogen flow maintained at 3 ml/min.

b) Stainless steel capillary column (150 m × 0.76 mm i.d. coated with SP 1200 (Supelco Ltd.) and operated under similar conditions to column (a) except that the programme rate was maintained at 3 °C/min throughout the run.

c) Stainless steel capillary column 150 m × 0.76 mm i.d. coated with SE 30 and operating under similar conditions to column (b).

d) Glass column (2 m × 3.2 mm o.d. packed with 10% SP 1200 (Supelco Ltd.) and 1% phosphoric acid on Chromosorb W 60–80 mesh and programmed from 65–200 °C at 6 °C/min. The injection port was held at 250 °C and fitted with glass liners packed with glass wool prewashed in phosphoric acid. The detector was held at 250 °C and the nitrogen flow was maintained at 30 ml/min.

e) Glass column (3.6 m × 3.2 mm o.d.) packed with 10% Carbowax 20 M and 1% potassium hydrogen sulphate on Chromosorb W (60–80 mesh) and programmed from 100–210 °C at 8 °C/min. The injection port was held at 250 °C and fitted with a glass-wool liner prewashed in potassium hydrogen sulphate solution. The detector was held at 250 °C and the nitrogen flow was maintained at 30 ml/min.

f) Copper column (3.6 m × 3.2 mm o.d.) packed with 10% Carbowax 400 on Chromosorb W (60–80 mesh) held isothermal for 4 min and then programmed from 65–100 °C at 4 °C/min. Injection port and detector temperature were held at 150 °C and the nitrogen flow was maintained at 20 ml/min.

g) Glass column (6.1 m × 3.2 mm o.d.) packed with 10% Carbowax 20 M on Chromosorb W (60–80 mesh) held isothermally for 4 min and then programmed from 65–210 °C at 8 °C/min. The injection port and detector were held at 250 °C and the nitrogen flow was maintained at 30 ml/min.

h) Stainless-steel column (51 cm × 3.2 mm o.d.) packed with CCW 982 on 80–100 mesh acid washed, dimethyl dichlorosilane treated Chromosorb W (Hewlett Packard Ltd.) and programmed from 70–240 °C at 8 °C/min. Nitrogen flow was maintained at 30 ml/min.

The neutral extract was examined using columns (a), (b), and (c), free acids on columns (d) and (e) and methyl esters on columns (f), (g), and (h).

Mass spectra were obtained using an LKB 9000 coupled gas chromatograph mass spectrometer operating at 70 eV and fitted with similar columns and operating under similar conditions to those used for analytical gas chromatography. The length of capillary columns, however, had to the reduced to 90 m, with a consequential slight reduction in resolution, as this was the maximum length that could be accommodated in the oven. Spectra were recorded both manually and on magnetic tape for processing off-line at the Food Research Institute, Norwich [12]. Compounds in the extracts were identified by comparison of their mass spectra and retention times with published data, and with those obtained from authentic compounds as previously described in connection with cider.

Results and Discussion

136 components were detected in the neutral extract on the SP-1200 capillary column, similar examinations on the Carbowax 20 M and SE-30 columns revealing 98 compounds on both phases. As a consequence of coupled gas chromatographic mass spectroscopic examination on all three phases, 62 neutral components have been identified. Good evidence was found for eight alcohols, six carbonyls, nine acetals, 25 esters and one chlorinated compound (probably derived from the extracting solvent) and tentative evidence for a further two alcohols, one carbonyl and ten esters.

Examination of the acid extracts, both in their own right and as methyl esters, gave definite evidence for 17 acids (Table 2). Conversion of the acids to methyl esters enabled heptanoic and nonanoic acids to be detected conclusively, and provided evidence for the presence of C_{14}, C_{16}, C_{18}, and C_{20} acids.

Of the 79 compounds found, 45 have not previously been reported as a result of a sucrose fermentation, although they have been described in connection with fermented beverages [13]. Confirmation has also been obtained for many compounds whose identities have previously only been based on retention data.

As expected, alcohols constituted the major part of the neutral extract. The presence of *n*-butanol and *n*-pentanol was a little surprising since, despite the former

Table 1. Neutral volatile components identified in a fermented sucrose solution

Component	Identified by GC-MS and retention times on following columns			Amount ppm	Reported in literature
	Carbowax 20 M	SP 1200	SE 30		
Alcohols					
Methanol		***		1	
Ethanol	***	**	***	Not estimated	[1[a], 2[a], 6[a]]
n-Propanol	***	***	***	80	[2[a], 6[a]]
Isobutanol	***	***	***	8	[1[a], 2[a], 3[a], 4[a], 6[a]]
n-Butanol	***	***		0.5	[2[a]]
2 and/or 3-Methylbutanol	***	***	***	81	[1[a], 2[a], 3[a], 4[b], 5[a], 6[a]]
Pentanol		**	***	tr	
A hexanol	*		*	tr	
An octanol		*	*	tr	
2-Phenethanol	***	***	***	9	[1[a], 2[a], 3[a], 4[a,b], 5[a], 6[a]]
Carbonyls					
Acetaldehyde			***	6	[6[a]]
Propanal	***		**	2	
Acetone	***	**	***	1	[6[a]]
Butanal	***			0.02	
A butanone			**	tr	
A pentanone			*	tr	
Benzaldehyde	***			0.02	
Acetals					
Ethoxy-3-methylbutoxyethane	***	***	***	0.4	
Ethoxy-2-methylbutoxyethane		***		tr	
Dibutoxyethane			**	tr	
Isobutoxy-2-methylbutoxyethane	***	***	***	tr	
Isobutoxy-3-methylbutoxyethane		***	**	0.03	
Di-2-methylbutoxyethane	***	***	***	0.01	
2-Methylbutoxy-3-methylbutoxyethane	**			1	
Di-3-methylbutoxyethane	***	***	***	6	
1,3,4-Trimethyl-2,5-dioxolane	***			1	
Esters					
Ethyl acetate	***	***	***	15	[1[a], 2[a], 3[a]]
Propyl acetate	***	***	***	0.1	
Isobutyl acetate	***	*	***	0.02	
n-Butyl acetate		**		tr	
2 and/or 3-Methylbutyl acetate	***	***	***	1	[1[a], 2[a], 3[a], 4[a,b], 5[a]]
2-Phenethyl acetate	***	***	***	0.4	[4[a], 5[a]]
Ethyl propionate	*			tr	
2 and/or 3-Methylbutyl propionate		***		0.03	
Ethyl butyrate	***			0.3	[4[a]]
Isobutyl butyrate		*		tr	
2 and/or 3-Methylbutyl butyrate		***	**	tr	
2-Phenethyl butyrate	*	**	*	0.02	
Ethyl hexanoate	***		***	2	[1[a], 2[a], 3[a], 4[a,b], 5[a]]
Isobutyl hexanoate	*		**	tr	
2 and/or 3-Methylbutyl hexanoate	*	***	***	0.01	[1[a], 2[a]]
Ethyl octanoate	***	***	***	2	[1[a], 2[a], 3[a], 4[a,b], 5[a]]
Propyl octanoate	*			0.01	
Isobutyl octanoate	*	***	***	0.005	
n-Butyl octanoate			*	tr	
2 and/or 3-Methylbutyl octanoate	***	***	***	0.1	[1[a], 2[a]]
Ethyl decanoate	***	***	***	0.4	[1[a], 2[a], 4[a,b,c], 5[a]]
Isobutyl decanoate	***	***	***	tr	
n-Butyl decanoate			***	tr	
2 and/or 3-Methylbutyl decanoate	***	***	***	0.1	[1[a], 2[a]]
Ethyl 9-decenoate	***		*	0.2	
2 and/or 3-Methylbutyl 9-decenoate			*	tr	
Ethyl dodecanoate	***	***	***	0.04	[1[a], 2[a], 5[a]]

Table 1 (continued)

Component	Identified by GC-MS and retention times on following columns			Amount ppm	Reported in literature
	Carbowax 20 M	SP 1200	SE 30		
2 and/or 3-Methylbutyl dodecanoate		***	***	tr	
Ethyl lactate	***			0.04	[3[a]]
2 and/or 3-Methylbutyl lactate		*		tr	
Dimethyl phthalate	*			0.005	
Diethyl phthalate	*			0.01	
Di-isobutyl phthalate		*		0.01	
Diethyl succinate	**	**		0.01	
Di-2 and/or 3-Methylbutyl succinate			*	tr	
Others					
Trichlorofluoromethane	***	***	***	Solvent	
Chloroform	***	***	***	0.01	

* = tentative
** = good evidence but not as conclusive as***
*** = definite identification
tr = less than 0.005 ppm
[a] = identity based on retention index
[b] = identity based on infra red spectrum
[c] = identity based on mass spectrum

Table 2. Acidic volatile components identified in a fermented sucrose solution

Component	Identified by gas chromatographic-mass spectrometry and retention times				Amount ppm	Reported in literature
	As free acid on $SP\ 1200/H_3PO_4$ or Carbowax $20\ M/KHSO_4$	As methyl esters on				
		Carbowax 400	Carbowax 20 M	UCCW 982		
Acetic	**	**	***		124	[2[a], 7[a]]
Propionic	**	***	*		2	[2[a], 7[a]]
Isobutyric	**	***	**		0.5	[2[a], 7[a]]
Butyric	**	***	***	*	1	[2[a], 7[a]]
2 and/or 3-Methylbutyric	**	***	***	*	1	[2[a], 7[a]]
Pentanoic	**	**		*	0.5	[2[a], 7[a]]
Hexanoic	***	***	***	***	3	[2[a], 7[a]]
Heptanoic	*		***	*	0.25	[2[a], 7[a]]
Octanoic	***		***	***	4	[2[a], 3[a], 7[a]]
Nonanoic			***	*	0.1	[7[a]]
Decanoic	***		***	***	2.5	[2[a], 3[a], 7[a]]
9-Decenoic	***		***	***	0.1	
Dodecanoic	***		***	***	0.75	[2[a]]
Tetradecanoic			*	***	0.02	[2[a]]
Hexadecanoic				***	0.02	[2[a]]
Octadecanoic				***	0.06	[2[a]]
Eicosanoic				***	0.002	

* = tentative
** = good evidence but not as conclusive as ***
*** = definite identification
[a] = identity based on retention index

having been reported previously [2], these alcohols are not normally considered as fermentation alcohols and most probably arise as by-products of fatty acid biosynthesis, rather than leakage products from the biosynthesis of amino acids.

Esters were also present in abundance, these reflecting in composition and amounts the acids found in the uncombined state. Acids reached a maximum concentration at octanoic acid and each series of esters correspondingly at their octanoates.

No evidence could be found for esters based on acids with an odd number of carbon atoms, or with more than twelve carbon atoms, as reported in the literature [1, 2, 7]. Even carbon number acids up to C_{20} were, however, detected, so it is possible that esters based on acids with more than twelve carbon atoms were present but were not removed by the vacuum distillation step in sufficient quantities to enable them to be detected in the distillate. Trace compounds were detected during the examination of acids at retention times corresponding to methyl esters of unsaturated C_{16} and C_{18} acids, but their mass spectra gave little indication of their being methyl esters. The use of trichlorofluoromethane as the main extracting solvent could be the reason why no propanediol or butanediol esters were found in this examination.

Despite the "sulphury" odour of the fermented liquor, no sulphur compounds could be detected. Benzthiazole has been reported in sucrose fermentations and an ion at *m/e* 135, the base peak of benzthiazole, was found in mass spectra taken at the retention time of this compound on the Carbowax 20 M column, but was not borne out by other ions. An unidentified methyl ester, also from its mass spectra, could have been methylthiopropionate, but no reference compound was available for confirmation.

Comparison of the volatile components of this sucrose fermentation with those obtained for ciders [8, 14–16] indicates that the major differences are the lack of *n*-hexanol, *cis*-3-hexenol and the esters derived from these two alcohols, all of which are significant in apple fermentations. Not surprisingly, there is also no indication of monomeric phenolic compounds derived from such compounds as p-coumaryl, quinic and chlorogenic acids and believed to be important in ciders made from bittersweet and bittersharp apple cultivars. Apart from these, all the major esters of cider were found, clearly indicating the contribution of the fermentation stage and of carbohydrates in particular in influencing the aroma of ciders.

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Received December 16, 1980