# Polar Lipids of Brewer's Yeasts

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**ABSTRACT**

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Both traditional and DNA-based methods sometimes fail to differentiate between closely related strains of commercial interest in the brewing industry. The aim of this study was to compare species and sub species of *Saccharomyces cerevisiae* on the basis of their polar lipid chemistry using chromatographic methods. Six isolates were studied after propagation under batch conditions. Polar lipids were then extracted from lyophilised cultures and analysed by TLC in order to separate phospholipid families. TLC showed that the major phospholipid classes present were PC > PE > PG. Two unidentified phospholipids were found, one only in strain 34/70. The major peaks detected by GLC were identified as methyl esters of palmitic acid and palmitoleic acid. The fatty acid composition of PC varied between strains and novel data on lecithin acyl constituents were observed. The polar lipid method succeeded in differentiating strain 34/70 – one of the most commonly used brewer's lager yeast – from strain 34/78 and other species tested. The presence of unusual polar lipids in *Saccharomyces sensu stricto* yeasts may be useful in distinguishing between other closely related strains.

**Key words:** Chemotaxonomy, fatty acid, GLC, lipid, *Saccharomyces cerevisiae,* TLC.

## INTRODUCTION

The approach to yeast identification has changed significantly over the past decade, due to technological developments and a requirement for quality assurance in research and industry. In addition to traditional methods advanced techniques have been developed[10,28]. Although it is possible to distinguish between some genera and species using molecular techniques, success is not guaranteed when modern genotyping is used[8,27]. Differentiation between yeast species of *Saccharomyces sensu stricto* remains problematic and controversial. A number of strains, in particular *Saccharomyces carlsbergensis* can only be distinguished on the basis of one or two physiological characteristics. These yeasts have shown an identical genotype when compared by PCR-, PFGE-, and AFLP-techniques (unpublished observation, Prof. E. Geiger). However, it is of great importance to check the identity of pure culture yeast before pitching to exclude contaminants which could cause technical problems such as stuck fermentations.

Chemical analysis has been applied to the taxonomy of microorganisms, and has been reviewed elsewhere by Drucker[6]. Such studies which include analysis of fatty acid and lipid profiles have been reported in the beverage industry. In 1976, Kaneko *et al.*[15] showed that lipids extracted from various yeasts could assist chemotaxonomy. The use of cellular fatty acid analysis has proved to be successful in distinguishing between *Saccharomyces* yeasts and other genera[3]. Earlier studies have shown difficulty in differentiating between sub species of yeasts[25], although a more recent study by Morakile and co-workers[24] proved cellular fatty acid and sterol analysis to be a useful tool for differentiating between brewing strains and related yeasts. However, the methods failed to distinguish brewing strains from a derived variant. In the same year, Mahmoudabadi *et al.*[20] successfully applied phospholipid fingerprinting to clinical isolates of medically important *Candida* species. *Candida dubliniensis* and *C. albicans* are genetically and phenotypically very closely related to each other which has resulted in their misidentification in the past. However, phospholipids and their acyl constituents have not previously been used as strain specific chemical markers for yeast identification in the brewing industry. The aim of this work was to find differences between *S. cerevisiae* isolates of commercial importance, in particular of closely related strains for lager beer production, on the basis of their polar lipid chemistry. Such differences were studied between species using thin-layer chromatography (TLC) and gas-liquid chromatography (GLC).

## MATERIALS AND METHODS

### Microorganisms

Six strains of *S. cerevisiae* were examined (Table I). The source of microorganisms was the strain collection of the Hefebank Weihenstephan, Freising, Germany. The inocula were prepared from pure yeast cultures maintained on wort agar slants. Cultures were grown in 100 mL of wort medium (fluid-bed spray-dried wort granules of unhopped, original gravity wort, re-dissolved in sterile water, 12°Plato) (PlatoTec GbR, Munich, Germany) under standard conditions for 4 days at 25°C. Subsequently the pre-cultured strains were transferred into a 2.5 L batch of prepared medium and incubated 10 days at 25°C, stirring occasionally every second day. All cultures were harvested from stationary phase by centrifugation (5000 *g*, 10 min, at 0°C). The cell pellets were washed with 0.9% saline and then distilled water at 5000 *g* for 10 min at 0°C.

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**Table I.** *Saccharomyces cerevisiae* isolates studied.

| Organism | Strain no. |
|---|---|
| *Saccharomyces carlsbergensis* | 34/70 |
| *Saccharomyces carlsbergensis* | 34/78 |
| *Saccharomyces carlsbergensis* | 66 |
| *Saccharomyces carlsbergensis* | 128 |
| *Saccharomyces cerevisiae* | 68 |
| *Saccharomyces cerevisiae* | 184 |

After freezing to –20°C, cells were lyophilised at –70°C and $10^{-2}$ to $10^{-3}$ Torr for 48 h, with a Beta freeze drier (Christ Gefriertrocknungsanlagen GmbH, Osterode, Germany).

### Polar lipid extraction

Lipids were extracted by the technique of Aluyi *et al.*[1] Approximately 100 mg lyophilised cells were extracted with 2 mL of freshly prepared methanol-chloroform (2:1 v/v) (BDH Chemicals, Poole, UK) for 4 h at 25°C, with intermittent vortexing. After repeating the procedure, pooled solvent extracts were evaporated to dryness in a vacuum desiccator. One mL chloroform was added to the dried extract, gently mixed, and the re-dissolved residues washed with one mL distilled water to remove hydrophilic impurities. After phase separation, the lower, chloroform phase was retrieved, dried *in vacuo* over silica gel and extracts stored at –20°C prior to lipid analysis. Analar-grade solvents were used throughout the study. The usage of non-solvent proof plastic ware and rubber was avoided, as well as human lipid contamination.

### Polar lipid analysis

**Analytical TLC.** The polar lipids were separated by one-dimensional TLC on silica gel 60 $F_{254}$ plates (10 cm × 10 cm) (Merck, Darmstadt, Germany), using the following solvent system: chloroform-methanol-acetic acid-distilled water (55:43:3:4, v/v/v/v)[23]. Lipids were detected using following spray reagents: molybdenum blue[16] (1:1 with 4.2 M sulphuric acid), *p*-anisaldehyde-sulphuric acid[9], ninhydrin[5] (0.2% in ethanol). For identification, visualised lipid spots were compared with standards (Larodan Fine Chemicals, Malmo, Sweden; Sigma, Poole, UK) within each band. Additionally $R_f$ values were calculated. TLC was performed at +8°C to avoid temperature variation effects and to enhance resolution.

**Preparative TLC.** Using the same system as for analytical TLC, the lipid extract was applied as a line rather than spotted onto TLC plates. Thus it was possible to recover whole bands of phospholipids for further GLC analysis.

### Fatty acid analysis

The fatty acid composition of PC was determined after saponification and methylation of re-extracted samples according to Drucker *et al.*[7]. A High Resolution Gas Chromatograph (HRGC) equipped with flame ionisation detector, FID (Carlo Erba Instruments, Milan, Italy) was used throughout the study. Peak areas and retention times were measured and recorded by the Summit Data Collection Program. For separation of fatty acid methyl esters, a flexible fused non-polar silica capillary column BP 1 (50 m × 0.32 mm × 0.25 μm) (SGE Europe Ltd., Milton Keynes, UK) was employed. The column was set at an initial temperature of 50°C and was raised to a final temperature of 320°C at the rate of 10°C/min. All samples and standards analysed by GLC were run for 200 min. Hydrogen was used as the carrier gas with the split ratio of 10:1 and the flow rate through the column of 2 mL/min. All peaks were identified by comparing their relative retention times with those of known peaks. Authentic standard mixtures (ME 100, Qualmix BR1) were supplied by Larodan Fine Chemicals (Malmo, Sweden). Both standards were run before, and in between sets of samples to increase comparability of retention times.

### Data analysis

The $R_f$ values of phospholipids were calculated and the ten most abundant peaks of fatty acid methyl esters of PC per isolate observed in the GLC chromatogram were selected for data analysis. Because not all isolates had the same ten most intense peaks, the total peaks measured per sample were well in excess of ten. Data were entered into a spreadsheet (Excel, version 2002 for Windows XP) normalised ($\sum$% peaks = 100), copied and pasted into SPSS (version 11.5 for Windows XP) to perform statistical analyses. The Pearson Coefficient of Linear Correlation (r) was used for comparing all pairs of strains. Applying nearest neighbour cluster analysis of the coefficients, the relationship of strains was presented in the form of dendrograms.

## RESULTS

### Analytical TLC

Analyses are presented in the form of $R_f$ values (Table II) for all strains investigated. Among the six strains of yeast, eight phospholipid classes were found, of which six were identified as PA (phosphatidic acid), PE (phosphatidylethanolamine), PG (phosphatidylglycerol), PC (phosphatidylcholine), PS (phosphatidylserine), and PI (phosphatidylinositol). The latter corresponded to the $R_f$-values of standards and were present in each strain. In addition, all six yeast samples developed distinct spots with matching $R_f$-values of 0.82. This was seen between the spots of standard PG and standard CL (cardiolipin), and named unidentified phospholipid (UL1). Another unidentified phospholipid (UL2) was found in strain 34/70. With strain 34/70, as little as 1 μL lipid extract was sufficient to clearly detect this lipid whereas with extracts of all other yeast isolates the same volume of lipid extract did not lead to the detection of this unknown lipid. The proportions of different phospholipids present varied between strains. However, the major phospholipid analogues detected were PC > PE > PG > UL1 whereas PS > PI, PA > UL2 were minor components.

Comparison between strains is shown in Fig. 1 which is a dendrogram, based on nearest neighbour clustering of correlation coefficients of phospholipid distributions.

No alteration was observed in between the individual phospholipid classes of set 1 and its repeat set 2. The results under batch conditions achieved were identical. All TLC analyses were performed in triplicate.

**Table II.** $R_f$ values of phospholipid standards and of polar lipids of *Saccharomyces* species.

| Standards analysed | | | | | | | | Samples analysed | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **M71** | **M73** | **PG** | **PE** | **PA** | **PI** | **PS** | **PC** | **184** | **68** | **128** | **66** | **34/70** | **34/78** |
| 0.85 | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| ** | ** | ** | ** | ** | ** | ** | ** | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 | 0.82 |
| 0.75 | 0.75 | 0.75 | 0.75 | ** | ** | ** | ** | 0.75 | 0.75 | 0.75 | 0.75 | 0.74 | 0.75 |
| ** | ** | ** | ** | 0.68 | ** | ** | ** | 0.67 | 0.68 | 0.68 | 0.67 | 0.68 | 0.67 |
| 0.58 | ** | ** | ** | ** | 0.59 | 0.58 | ** | 0.57 | 0.58 | 0.58 | 0.57 | 0.58 | 0.57 |
| 0.25 | 0.25 | ** | ** | ** | ** | ** | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 | 0.25 |
| ** | 0.11 | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** |
| ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | ** | 0.07 | ** |

PA = phosphatidic acid; PI = phosphatidylinositol; PS = phosphatidylserine; PG = phosphatidylglycerol; PE = phosphatidylethanolamine; PC = phosphatidylcholine; M73 = Pol Mix 73 standard mixture – containing: sphingomyelin, phosphatidylcholine, and phosphatidylglycerol; M71 = Pol Mix 71 standard mixture – containing: phosphatidylcholine, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphatidylethanolamine, and cardiolipin; ** = no spot detected. (See text for details.)

### Preparative TLC

For further GLC analysis preparative TLC were carried out for all six yeast samples. PC was separated and recovered for further analysis. Other polar lipid classes comigrated and overlapped due to overloading of the TLC plate by the high concentration of lipid extract and therefore were not selected for further analysis. Heavy loading was essential to separate enough sample for methylation of fatty acids.

### Fatty acid analysis

Fig. 2 shows a typical chromatogram produced by GLC analysis of fatty acids of PC, in this case of *S. carlsbergensis,* strain 34/70. The analysis was carried out for PC of all six isolates of culture set 1 and a repeat set from freshly grown cultures. Of the peaks observed, 22 were considered to be major peaks within the sets. Of these 22, 18 were identified using standard mixtures and 4 remained unidentified. Peak UP1 fell between the retention times of undecanoic acid ($C_{11:0}$) and lauric acid ($C_{12:0}$) while peak UP2 eluted between the retention times of erucic acid ($C_{17:1}$) and margaric acid ($C_{17:0}$). UP3 fell between eicosapentaenoic acid ($C_{20:5(n-3)}$) and eicosatrienoic acid ($C_{20:3(n-6)}$). Peak UP4 was found to occur between arachidic acid ($C_{20:0}$) and heneicosanoic acid ($C_{21:0}$). In Table III identified fatty acids and unidentified peaks are presented in their hierarchical order of the ten major peaks for each strain. In addition the proportions of the 3 most abundant fatty acids are listed in Table III. Carboxylic methyl ester profiles displayed quantitative as well as qualitative differences. Applying single linkage cluster analysis of the Pearson coefficients, the relationship of strains was presented in the form of a dendrogram (data not shown). Differences were also seen between repeat sets of the same species. Therefore all strains were scattered randomly in between cluster 1 and 2. All GLC-analyses were performed in triplicate.

## DISCUSSION

### Polar lipids

Most of the individual phospholipid classes found in this study have been described previously by Kaneko *et al.*[15], except for the content of PG where CL rather than PG was reported. Mahmoudabadi *et al.*[19] observed PE as the major phospholipid analogue in *S. cerevisiae* using FAB-MS analysis. However, in negative-ion FAB-MS spectra PC would be seen as its demethylated fragmentation product, PE. All strains were grown under batch conditions which resulted in a change from an aerobic to an anaerobic environment and eventually a decreasing growth rate. Higher amounts of PC, accompanied by lower contents of PE, have been observed by Hunter and Rose[12] in strains of *S. cerevisiae* when growth rate decreases. Furthermore, a partial or total loss of CL and its replacement by PC or by an unidentified phospholipid have been reported for anaerobically grown *S. cerevisiae*[4,14]. Also Peña and Sandra[26] reported PC > PE as the main polar lipid

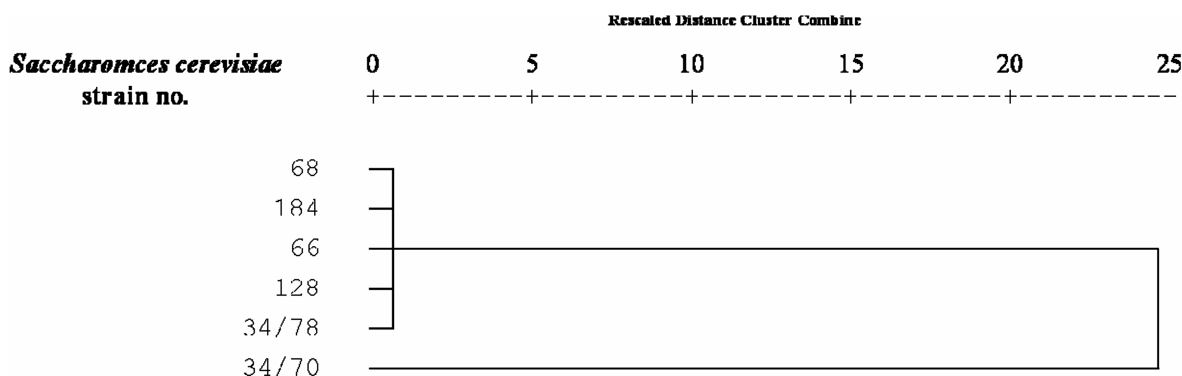


**Fig. 1.** Dendrogram showing relationship of brewing yeast species calculated by single linkage cluster analysis of r-values (Pearson coefficient of Linear Correlation), using phospholipid distributions.

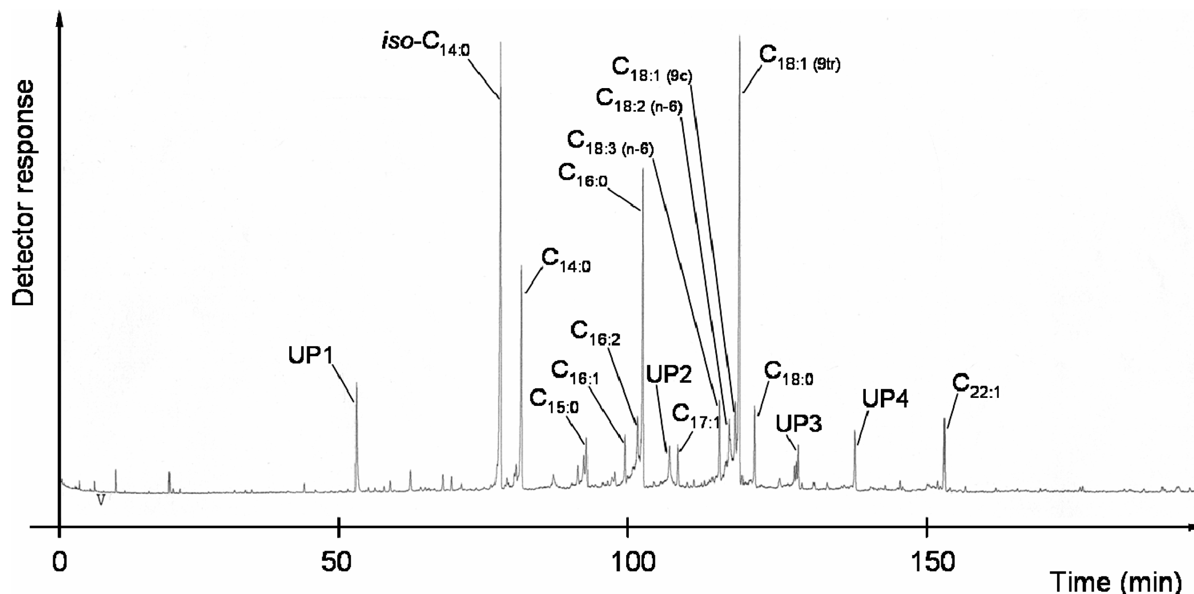


**Fig. 2.** Gas liquid chromatogram for fatty acids from phosphatidylcholine of *Saccharomyces carlsbergensis,* strain 34/70, analysed after methylation. UP = Unidentified peak; Retention time of UP1 = 54 min 34 s (± 30 s), UP2 = 105 min 49 s (± 30 s), UP3 = 126 min 52 s (± 30 s), UP4 = 136 min 12 s (± 30 s).

compounds of baker's and brewer's yeast. However, we do not accept their conclusion that differentiation on the basis of phospholipid profiling is impossible. An unidentified lipid (UL1) was found in all our isolates. Its position between PG and CL standards and its negative response to ninhydrin indicates that it is not an amino lipid. Neither is it PG or CL. Phospholipids present varied amongst strains in a highly reproducible manner. To determine precisely there proportions, quantitative analysis of separated lipid families will need to be performed, using HPLC. Nevertheless, all brewing strains investigated of *S. cerevisiae* possessed qualitatively similar phospholipid classes not withstanding whether isolates were "bottom-fermenting", "top-fermenting", "flocculant" or "non-flocculant" – except for isolate 34/70. This strain is the most commonly used brewer's yeast in the production of lager beer in Germany and was the only isolate containing additional unidentified lipid (UL2). This unidentified polar lipid corresponded to the $R_f$ value of an impurity detected in 'Pol Mix standard mixture 73' using molybdenum blue and *p*-anisaldehyde-sulphuric acid as detection reagents. Its high polarity, close migration to sphingomyelin, and manufacturer's response (personal communication) regarding the impurity, confirms that it could possibly be a phosphosphingolipid derivate. Using phospholipid distributions, Fig. 1 shows the relationships between brewing yeast species studied, in the form of a dendrogram. The finding of UL2 may be useful in the brewing industry since it has been difficult to distinguish strain 34/70 from other isolates investigated, in a simple way, prior to actual fermentation. For example differentiation between strain 34/70 and its putative mutant, 34/78, entirely relies on the appearance of sedimentation after the main fermentation; 34/70 is less flocculant than 34/78 (unpublished observation, Prof. E. Geiger).

## Phosphatidylcholine acyl constituents

The structure of PC of *S. cerevisiae* has not been extensively investigated in the microbiological literature. Thus, the fatty acid distribution within PC *per se* is still poorly understood and only few data are available on possible fatty acid constituents of this polar lipid class. However, the results of this study (Table III) were consistent with the results of Longley *et al.*[18] and Watson and Rose[29] who observed $C_{10:0}$, $C_{12:0}$, $C_{14:0}$, $C_{15:0}$, $C_{16:0}$, $C_{16:1}$, $C_{18:0}$, and $C_{18:1}$ to be the major fatty acid component of PC. $C_{16:2}$ and $C_{17:1}$ have been found in *S. kluyveri*[2]; dienoic acids of n-$C_{17}$, n-$C_{18}$ and polyenoic fatty acids of n-$C_{18}$ have been previously identified in PC of other *Ascomycetes* for example in species of *Candida*[13,22]; $C_{18:2}$, and $C_{18:3}$ isomers

**Table III.** Fatty acid composition of phosphatidylcholine of *Saccharomyces cerevisiae* isolates.

| Strain no. | Hierarchy of the 10 major peaks | 3 most abundant fatty acids* |
|---|---|---|
| 34/70 | *iso*-$C_{14:0} > C_{18:1(9tr)} > C_{16:0} > C_{14:0}$ > UP1 > $C_{18:3(n-6)} > C_{18:0} > C_{22:1} > C_{16:2}$ > UP4 | 22.9 > 19.3 > 14.7 |
| 34/78 | $C_{16:0} > C_{18:1(9tr)}$ > *iso*-$C_{14:0} > C_{18:0} > C_{9:1} > C_{18:2(n-6)} > C_{10:0} > C_{18:1(9c)} > C_{8:0} > C_{16:1}$ | 34.7 > 13.9 > 7.7 |
| 66 | $C_{16:0}$ > *iso*-$C_{14:0} > C_{18:0} > C_{18:1(9c)} > C_{18:3(n-6)}$ > UP2 > $C_{22:1}$ > UP3 > $C_{18:2(n-6)} > C_{14:0}$ | 33.6 > 16.6 > 14.7 |
| 128 | $C_{18:1(9tr)} > C_{16:0}$ > *iso*-$C_{14:0} > C_{14:0}$ > UP4 > $C_{18:0} > C_{18:3(n-6)}$ > UP3 > $C_{15:0}$ > UP2 | 21.3 > 19.8 > 13.4 |
| 184 | $C_{16:1} > C_{18:1(9c)} > C_{16:0} > C_{18:0}$ > UP2 > $C_{22:1} > C_{16:2} > C_{9:0} > C_{6:0} > C_{18:1(9tr)}$ | 27.3 > 26.2 > 24.0 |
| 68 | *iso*-$C_{14:0} > C_{16:0} > C_{14:0} > C_{18:1(9tr)}$ > UP2 > $C_{18:0} > C_{22:1} > C_{16:2}$ > UP3 > $C_{18:3(n-6)}$ | 24.3 > 17.6 > 13.4 |

PC = phosphatidylcholine; UP = Unidentified peak; Retention time of UP1 = 54 min 34 s (± 30 s), UP2 = 105 min 49 s (± 30 s), UP3 = 126 min 52 s (± 30 s), UP4 = 136 min 12 s (± 30 s); * value calculated as (peak area)/∑(peak areas) as %.

have also been reported by Peña and Sandra[26] to be a component of total lipid distribution of *S. cerevisiae;* $C_{18:2}$ have only been found in bacterial PC[17]. However, they have not previously been found to be present in PC of brewer's yeast using GLC analysis. Unfortunately studies differ in the cultivation procedures used, which, according to the literature, significantly influence cellular fatty acid compositions[1]. Therefore comparisons between previous studies are problematic. In this study each GLC analysis lasted 200 min which had the disadvantage of requiring much longer use of the gas chromatograph per sample yet the advantage of improved separations (Fig. 2). In our hands, the GLC technique would have overlooked several fatty acids if a short analysis time had been used. This may explain why some fatty acids have not been reported previously in *S. cerevisiae* lipids. "Brewing" conditions were employed to grow strains in order to simulate practical conditions of pure yeast culturing. The idea was to test the suitability of the analytical techniques to distinguish and fingerprint strains in the brewing industry. Care was taken to conduct the analysis of the strains from set 1 and the repeat set under precisely the same conditions. However, the dendrogram based on the analysis of phosphatidylcholine acyl constituents (data not shown) showed no significant clustering of strains into species. Nevertheless, it was obvious that strains tend to group together either into set 1 or set 2. This gives support to the generalisation made by Holub & Lands[11] and McCammon *et al.*[21], that fatty acid composition of phospholipids is more susceptible to modification than is the class composition. Thus, in this study, inter-strain variation was less than inter-batch variation because strains were so similar, except for the differences described above.

## CONCLUSION

Batch propagation for culturing strains is not ideal for the chemotaxonomic analysis of fatty acids by GLC. Greater reproducibility of GLC analyses should be possible by use of continuous culture, controlling culture age, aeration and oxygen levels, dilution rate, pH, and the use of a chemically defined minimal medium. Nevertheless, it was possible to differentiate the *S. cerevisiae* strain 34/70 from other yeasts investigated and it may be possible to differentiate between other strains on the basis of their lipid chemistry. Further work is in progress to extend the present findings, to identify unknown lipids and acyl constituents, in order to improve our understanding of the significance of individual polar lipid analogues in *Saccharomyces.*

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