LONG-CHAIN FATTY ACID COMPOSITIONS AND VOLATILE METABOLITE PATTERNS OF YEASTS ASSOCIATED WITH WINE

by

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GOD LEFT THE CHALLENGE IN THE EARTH

When God made the Earth, He could have finished it, but He didn't. Instead, He left it as raw material - to tease us, to tantalize us, to set us thinking and experimenting and risking and adventuring, and therein we find our supreme interest in living.

God gave us the world unfinished so that we might share in the joys and satisfactions of creation - He left the oil in the rock, He left the forests un-felled and the cities un-built, He left the music un-sung and the dramas un-played, He left the poetry un-dreamed in order that men and women might not become bored but engage in stimulating, exciting and experiencing all the joys and durable satisfactions of achievement. He gave us the challenge of raw materials, not the satisfaction of perfect finished things. Works, thought, creation - these give life its stimulus, its real satisfaction, its intriguing value.

- Dr. Allen A. Stockdale

This thesis is dedicated to my wife

LOUIZE

C O N T E N T S

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CHAPTER 1

INTRODUCTION

1.1 THE NEED FOR A YEAST IDENTIFICATION SYSTEM IN THE WINE INDUSTRY

The South African wine industry annually ferments approximately 10 million hectolitres of grape must with a total value of 243 million rands (KWV annual report, 1985). This fermentation relies almost exclusively on the inoculation of selected *Saccharomyces cerevisiae* strains. The inoculation with these strains (supplied as active dried wine yeast) should ensure swift fermentations and the production of quality wines minimizing negative qualities such as off-odours and -tastes.

The practice of inoculating grape must, instead of allowing spontaneous fermentation by the natural flora, prevails in many of the newer wine producing countries such as Australia and South Africa and is also gaining ground in the traditional wine producing areas of France and Germany (Rankine, 1968).

The presence of contaminants in the inoculum and later during the fermentation may hamper the fermentation process and reduce the quality of the wine (Rankine, 1968; Radler, 1973; Heard and Fleet, 1985). *Sacch. cerevisiae* strains are selected for their fermentative capability and ability to produce wines without off-odours such as H_2S or ethylacetate. Contaminants in the inoculum can produce these off-flavours in such quantities that the quality of the wine is severely affected. Since these contaminants, so-called wild yeasts, are almost always of lesser fermentative capability (Rankine, 1968), they can reduce the rate of fermentation - leading to sluggish or stuck fermentations which again leads to a reduction in wine quality.

Stuck or lagging fermentations have lately become a major problem, causing concern in the local wine industry (Tromp, 1980). Although this is a multi-faceted problem, one of the causes could be related to contaminated inocula or the domination, during fermentation, of the selected yeast strain by another that does not possess the ability to conduct a satisfactory fermentation.

Consequently, in order to conduct a satisfactory fermentation, there is a need for a yeast identification system which can detect contamination by wild yeasts. In order to accomplish this, it is important that yeast

species and strains are well defined and that an appropriate yeast identification and classification system exists.

1.2 DEFINITION OF YEASTS

In order to give a definition of the term "yeast" it is important to retrace some aspects of the historical development.

Antonie van Leeuwenhoek (1680) defined yeasts as globular to spherical bodies which were found in beer and are able to multiply by budding (Phaff *et al.*, 1978). Eventually these yeasts were termed "zuckerpilz" or "sugar fungus" from which the name *Saccharomyces* originates (Brock, 1961).

Since these definitions were unsatisfactory, yeasts were described by other investigators as unicellular organisms that reproduce asexually by budding, fission or both and produce ascospores under suitable conditions within a naked ascus, originating either from a zygote or parthenogenically from a single cell (Alexopoulos and Mims, 1979; Gorin and Spencer, 1970; Kreger-van Rij, 1969 and Phaff *et al.*, 1966). In the above definitions, mainly morphological aspects were taken into consideration.

Flegel (1977), on the other hand, defined yeasts as assimilative growth forms which are unicellular and reproduce by budding or fission. Finally, in 1985, van der Walt and von Arx stated that "yeasts are hyaline microfungi which, with numerous exceptions, reproduce asexually by budding, ferment at least glucose and form naked asci".

1.3 DEVELOPMENT OF YEAST TAXONOMY

The techniques currently in use for the classification of yeasts to species level are based upon morphological, physiological, sexual and biochemical characteristics (Barnett *et al.*, 1983). This system has evolved through the years as a result of the work of several authors.

Reess (1870) observed endospores in different yeasts and described their shapes and mode of germination. He also suggested the name *Saccharomyces* for the spore-forming yeasts and they were included in the Ascomycetes. De Bary stated in 1884 that yeast spores and ascospores were produced by "free cell formation". These spores were free from attachment to the cell wall in contrast with spore formation in other classes of fungi. Hansen perfected Pasteur's methods for obtaining pure cultures and also studied some morphological and physiological aspects of these cultures. He also attempted the first comprehensive system of yeast taxonomy in 1896. Many of the species differentiated by Hansen are still recognized today

(Phaff *et al.*, 1978).

From 1920 to 1928 Guilliermond expanded the field of taxonomy with additional information on physiology, sexuality and phylogenetic relations. He also devised various dichotomous keys for identifying yeast species (Guilliermond, 1920; 1928).

The Delft school of taxonomists under the inspiration of Kluyver produced mainly six leading contributions on taxonomy from 1931 to 1984:

1931: Stelling-Dekker produces a scheme of classification for the sporulating yeasts.

1934: Lodder publishes a volume on non-sporeforming yeasts.

1942: Diddens and Lodder publishes a second volume on non-sporeforming yeasts.

1952: Lodder and Kreger-van Rij produces a comprehensive classification of both sporogenous and asporogenous yeasts.

1970: Lodder edits a comprehensive volume on yeasts.

1984: Kreger-van Rij edits a comprehensive volume on yeasts.

There were also other important contributions to yeast taxonomy. Wickerham (1951) introduced new techniques and principles e.g. synthetic media for the study of morphology and assimilation tests with more carbon compounds and vitamins. He also put greater emphasis on the chromosomal state of the yeast in nature and the existence of heterothallic mating types.

Kudrjawzew (1954) classified the yeasts in a new order, the Unicellomycetales, later changed to Saccharomycetales (Kudrjawzew, 1960). The Saccharomycetales was divided into three families according to their mode of vegetative reproduction, i.e. Saccharomycetaceae (budding), Schizosaccharomycetaceae (fission) and Saccharomycodaceae (bud-fission).

In 1956, Wickerham and Burton proposed the genus *Dekkeromyces*, which is similar to the genera *Fabospora* and *Zygofabospora*. These three genera are now merged into the genus *Kluyveromyces* (van der Walt *emend.* van der Walt).

Phaff *et al.* (1978) proposed three yeast families which contained the known ascosporogenous yeasts namely the Saccharomycetaceae, Sporobolomycetaceae and Cryptococcaceae. The first group was divided into six subfamilies: Schizosaccharomycetoideae, Endomycetoideae, Uptomycetoideae, Nematosporoideae, Saccharomycetoideae and Eremascoideae.

In a more recent classification system (Von Arx, 1981), the ascosporogenous yeasts were divided into six families (Table 1).

The yeasts associated with the wine industry (Barnett *et al.*, 1983) are given in Table 2.

TABLE 1 The classification of the ascomycetous yeasts as proposed by Von Arx (1981)

Dipodascaceae	Endomycetaceae	Saccharomycodaceae	Saccharomycetaceae	Metchnikowiaceae	Schizosaccharomycetaceae
Dipodascus	*Endomyces*	*Saccharomycodes*	*Saccharomyces*	*Metchnikowia*	*Schizosaccharomyces*
	Ascoidea	*Hanseniaspora*	*Zygosaccharomyces*	*Nematospora*	
	Cephaloascus	*Nadsonia*	*Torulaspora*	*Asbya*	
	Ambrosiozyma	*Wickerhamia*	*Debaryozyma*	*Crebrothecium*	
	Hormoascus		*Issatchenkia*	*Eremothecium*	
	Botryoascus		*Pachytichospora*		
	Hyphopichia		*Sporopachyderma*		
	Stephanoascus		*Kluyveromyces*		
	Pichia		*Lodderomyces*		
	Hansenula		*Clavispora*		
	Pachysolen		*Wickerhamiella*		
	Dekkera		*Citeromyces*		
			Wingea		
			Williopsis		
			Schwanniomyces		
			Endomycopsella		
			Saccharomycopsis		
			Arthroascus		
			Cyniclomyces		
			Lipomyces		

Table 2: Yeasts associated with wine and wine-making (Barnett *et al.*, 1983)

Brettanomyces claussenii Custers
Brettanomyces custersii Florenzano
Brettanomyces lambicus Kufferath & van Laer
**Candida albicans* (Robin) Berkhout
Candida apicola (Hajsig) Meyer & Yarrow
Candida boidinii Ramirez
Candida cantarellii (van der Walt & van Kerken) Meyer & Yarrow
Candida catenulata Diddens& Lodder
Candida diversa Ohara *et al. ex* van Uden & Buckley
Candida glabrata (Anderson) Meyer & Yarrow
Candida incommunis Ohara *et al.*
Candida inconspicua (Lodder & Kreger-van Rij) Meyer & Yarrow
Candida intermedia (Cifferri & Ashford) Langeron & Guerra
Candida norvegica (Reiersøl) Meyer & Yarrow
Candida parapsilosis (Ashford) Langeron & Talice
**Candida rugosa* (Anderson) Diddens & Lodder
Candida sake (Saito & Ota) van Uden & Buckley
Candida solani Lodder & Kreger-van Rij
**Candida steatolytica* Yarrow
Candida stellata (Kroemer & Krumbholz) Meyer & Yarrow
**Candida tenuis* Diddens & Lodder
Candida tropicalis (Castellani) Berkhout
Candida vanderwaltii (Vidal-Leiria) Meyer & Yarrow
Candida veronae Florenzano *ex* van Uden & Buckley
Candida versatilis (Etchells & Bell) Meyer & Yarrow
Candida vini (Desmazières) van Uden & Buckley
Candida zeylanoides (Castellani) Langeron & Guerra
Citeromyces matritensis (Santa Maria) Santa Maria
**Cryptococcus albidus* (Saito) Skinner
Cryptcoccus humicolus (Daszewska) Golubev
Cryptococcus laurentii (Kufferath) Skinner
Cryptococcus luteolus (Saito) Skinner
**Debaryomyces hansenii* (Zopf) Lodder & Kreger-van Rij
Debaryomyces polymorphus (Klöcker) Price & Phaff
Dekkera bruxellensis van der Walt
Dekkera intermedia van der Walt
**Endomyces fibuliger* Lindner

Filobasidiella neoformans Kwon-Chung
**Filobasidium capsuligenum* Rodrigues de Miranda
Geotrichum fermentans (Diddens & Lodder) von Arx
Hanseniaspora occidentalis Smith
Hanseniaspora osmophila (Niehaus) Phaff *et al.*
**Hanseniaspora uvarum* (Niehaus) Shehata *et al.*
**Hanseniaspora valbyensis* Klöcker
Hanseniaspora vineae van der Walt & Tscheuschner
**Hyphopichia burtonii* (Boidin *et al.*) von Arx & van der Walt
**Issatchenkia orientalis* Kudrjawzew
**Kluyveromyces marxianus* (Hansen) van der Walt
**Kluyveromyces thermotolerans* (Phillippov) Yarrow
Leucosporidium scottii Fell *et al.*
Lipomyces starkeyi Lodder & Kreger-van Rij
**Lodderomyces elongisporus* (Recca & Mrak) van der Walt
**Metschnikowia reukauffii* Pitt & Miller
Nadsonia elongata Konokotina
Pachytispora transvaalensis (van der Walt) van der Walt
**Pichia anomala* (Hansen) Kurtzman comb. nov.
**Pichia canadensis* (Wickerham) Kurtzman comb. nov.
Pichia carsonii Phaff & Knapp
**Pichia etchellsii* Kreger-van Rij
Pichia farinosa (Lindner) Hansen
**Pichia fermentans* Lodder
**Pichia guilliermondii* Wickerham
Pichia humboldtii Rodrigues de Miranda & Török
Pichia jadinii (A. *et* R. Sartory, Weill *et* Meyer) Kurtzman comb. nov.
**Pichia membranaefaciens* (Hansen) Hansen
Pichia silvicola (Wickerham) Kurtzman comb. nov.
**Pichia subpelliculosa* Kurtzman sp. nov.
Rhodotorula aurantiaca (Saito) Lodder
Rhodotorula bogoriensis (Deinema) von Arx & Weijman
Rhodotorula glutinis (Fresenius) Harrison
Rhodotorula minuta (Saito) Harrison
**Rhodotorula mucilaginosa* (Jörgenson) Harrison
Rhodotorula pallida Lodder
**Saccharomyces cerevisiae* Meyen *ex* Hansen
**Saccharomyces exiguus* Reess *ex* Hansen
**Saccharomyces kluyveri* Phaff *et al.*
**Saccharomyces unisporus* Jörgenson

Saccharomycodes ludwigii (Hansen) Hansen
Schizosaccharomyces japonicus Yukawa & Maki
**Schizosaccharomyces malidevorans* Rankine & Fornachon
**Schizosaccharomyces octosporus* Beijerinck
**Schizosaccharomyces pombe* Lindner
Sporidiobolus pararoseus Fell & Tallman
Sporidiobolus salmonicolor Fell & Tallman
Sporobolomyces roseus Kluyver & van Niel
**Torulaspora delbrueckii* (Lindner) Lindner
Torulaspora globosa (Klöcker) van der Walt & Johannsen
Trichosporon beigelii (Küchenmeister & Rabenhorst) Vuillemin
Trichosporon pullulans (Lindner) Diddens & Lodder
**Wickerhamiella domercqiae* van der Walt & Liebenberg
Williopsis californica (Lodder) von Arx
**Williopsis saturnus* (Klöcker) Zender
**Zygosaccharomyces baillii* (Lindner) Guilliermond
Zygosaccharomyces bisporus Naganishi
Zygosaccharomyces florentinus Castelli *ex* Kudrjawzew
**Zygosaccharomyces microellipsoides* (Osterwalder) Yarrow
**Zygosaccharomyces rouxii* (Boutroux) Yarrow

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* indicates yeast species analyzed in this study

The family Saccharomycetaceae comprises yeasts which are associated with the wine industry and include species of the genera *Saccharomyces, Zygosaccharomyces, Torulaspora, Issatchenkia, Kluyveromyces, Lodderomyces, Lipomyces, Wickerhamiella* and *Williopsis*.

This family also includes 11 other genera which are separated on the basis of the shape, number and mode of ascospore formation (Kreger-van Rij, 1984).

The family Endomycetaceae contains the wine associated species *Dekkera, Endomyces, Hyphopichia, Pichia* and *Hansenula*. The latter two genera are now combined in *Pichia* Hansen *emend*. Kurtzman (1984). This family also contains 7 other genera and differs from the Dipodascaceae in producing a small and generally definite number of ascospores (one to eight) in each ascus. The mycelium is composed of well-developed, typical hyphae. Asexual reproduction is by means of arthrospores or blastospores.

The family Dipodascaceae comprises of only one genus, *Dipodascus*, characterized by elongated asci, borne singly from two mating hyphae and containing a large number of single cell hyaline ascospores surrounded by a sheath (von Arx, 1972).

The family Saccharomycodaceae comprises 4 genera of which species of *Saccharomycodes* and *Hanseniaspora* are associated with wine. The genera of this family are characterized by bipolar budding (von Arx, 1972) and the formation of occasional pseudomycelium (Kreger-van Rij, 1984).

In the family Metchnikowiaceae only one of the five genera, namely *Metchnikowia* is associated with the wine environment (Barnett *et al.*, 1983). The genera of this family are generally characterized by non-septate hyphae and multilateral budding and were originally placed in the family Spermophthoraceae by Lodder (1970) and Phaff *et al.* (1978).

Schizosaccharomyces, a wine associated yeast and only genus belonging to the family Schizosaccharomycetaceae, is mainly characterized by fission of the vegetative cells and the formation of true hyphae and arthrospores.

1.4 PROBLEMS ENCOUNTERED WITH THE CONVENTIONAL TAXONOMIC SYSTEM

The conventional system of species differentiation is based upon morphological, physiological, sexual and biochemical characteristics and (Barnett *et al.*, 1983) has certain limitations. The ascospore shape of a species, long considered to be a constant character, proved to be variable when Wickerham and Burton (1954) reported the formation of both spherical and hat-shaped ascospores by strains of *Pichia ohmeri*.

Furthermore, *Candida* was separated from *Torulopsis* solely on the ability

of the former to produce pseudohyphae (Lodder *et al.*, 1958). It was, however, observed that the same species might produce two or more forms simultaneously or at different growth stages (Gorin and Spencer, 1970). It is thus evident that different strains of the same species may differ in their ability to produce pseudohyphae, making this characteristic invalid as a differentiating criterion.

In the same way, *Hansenula* and *Pichia* were separated only by the ability or lack of ability to utilize nitrate. This phenotypic characteristic became invalid when Kurtzman (1984) combined these genera on the basis of results of DNA hybridization studies.

Another limitation is the problem of the instability of the physiological characters of yeasts. Scheda and Yarrow (1966) observed enough variation in the fermentation and carbon assimilation patterns of a number of *Saccharomyces* species to cause difficulties in the assignment of yeast strains to different species. Another problem regarding the limitations of the conventional taxonomic system is the relation of the biochemical tests to the metabolism of the organisms. Originally it was not taken into consideration that various carbon sources are not necessarily assimilated independently but may be metabolized by common pathways. This suggests that yeasts that assimilate one carbon compound can also assimilate a structurally related one by the same metabolic pathway (Gorin and Spencer, 1970).

A problem that mainly concerns taxonomists in the wine making and brewing industries is the rapidly changing nomenclature of yeasts (Barnett, 1986). These changes are most inconvenient to these scientists who have to serve an industry where changes are not accepted easily.

An example of such changes is the "lumping" of different wine-making and brewery strains of *Saccharomyces cerevisiae, Sacch. bayanus, Sacch. carlsbergensis, Sacch. uvarum* and *Sacch. logos* to one species, namely *Sacch. cerevisiae*. This "lumping" process has obvious advances for the pure taxonomist, but the wine- and brewing taxonomist are required to distinguish between these yeasts (Hough *et al.*, 1982).

For instance, *Sacch. bayanus* is known for its high alcohol tolerance, making it a most suitable yeast to reinoculate stuck fermentations (Rosini *et al.*, 1982) or secondary fermentations in champagne production.

Although some problems are encountered with the current system of classification, it must be recognized that phenotypic classification does serve its purpose and that not all characters utilized are unstable.

In the search for supplementary taxonomic characteristics, a number of new, more stable criteria have been proposed which include comparison of

ascospore surfaces by scanning electron microscopy (Kurtzman *et al.*, 1972, 1975); serology (Campbell, 1971; Tsuchiya *et al.*, 1974); proton magnetic resonance spectra of cell wall mannans (Gorin and Spencer, 1970); classification of the Coenzyme Q system (Yamada *et al.*, 1973, 1976, 1977) ; DNA hybridization studies (Kurtzman and Smiley, 1979; Kurtzman, 1984); electrophoretic enzyme patterns (Baptist and Kurtzman, 1976) and genome comparisons (Price *et al.*, 1978).

1.5 PURPOSE OF THE RESEARCH

It has been found that chemical compounds, such as DNA, RNA enzyme proteins and mannose containing polysaccharides of yeast cell components, vary from species to species. This has led to a great interest in the chemotaxonomy of yeast cells, using as criteria chemical compounds as well as the physical and immunological properties of macromolecules (Gorin and Spencer, 1970).

1.5.1 The value of long-chain fatty acid composition in the taxonomy of wine- and related yeasts (See Chapter 2)

Lipid analyses are a well established criterion in bacterial taxonomy and have also provided suitable characteristics for the classification and identification of many Coryneform and Actinomycete genera (Collins and Shah, 1984 and Athalye *et al.*, 1985).

Long-chain fatty acids are considered chemically as non-volatile acids ranging from C8 to C30 and can be divided into odd- and even-chain fatty acids. The fatty acids of yeast lipids consist mainly of C16 and C18 acids, although a variety of other acids have been observed. A total of 33 acids, ranging from C8 to C22, including significant amounts of isoprenoid-type acids, have been detected in *Sacch. cerevisiae* (Rattray *et al.*, 1975). Welch and Burlingame (1973), however, found that C20 to C30 acids accounted for only 1 to 2% of the total fatty acid components. A minor polythenoid acid component, as well as C8 to C12 acids were found in baker's yeast (Suomalainen and Keränen, 1968). All these fatty acids were located in membranous structures and intracytoplasmic elements such as nuclei, vacuoles, mitochondria and lipid particles (Rozijn and Tonino, 1964; Matile and Wiemken, 1967; Indge, 1968; Holley and Kidby, 1973; Clausen *et al.*, 1974).

The pathways of fatty acid synthesis in yeasts have been documented (Hunter and Rose, 1971) but the mechanisms of regulation are less

well-defined. The initial step of the *de novo* biosynthesis of fatty acids involving acetyl-coenzyme A (CoA) carboxylase has been suggested as being under negative feedback control by long-chain fatty acyl CoA (Gill and Ratledge, 1973a, 1973b; Sumper, 1974). This is again influenced by the extent of fatty acyl CoA incorporation into membraneous systems (Sumper, 1974). It has been noted that the presence of long-chain fatty acids reduces the cellular content of acetyl CoA carboxylase (Kamiryo and Numa, 1973) and may be significant in the observed inhibition (Mishina *et al.*, 1973) of fatty acid biosynthesis by higher odd-chain fatty acids. The ability of acetyl CoA synthetase to form CoA esters from short-chain acids in *Sacch. cerevisiae* grown aerobically, is inhibited markedly by long-chain fatty acyl CoA (Satyanarayana and Klein, 1973). It was also found by these authors that different proteins were involved in the synthetase activity in aerobic as well as anaerobic cells. It has been shown that *Candida tropicalis*, grown on n-tetradecane, requires four different types of acyl CoA synthetase, each having specific substrate requirements and intracellular location.

Studies on yeasts growing on different n-alkanes showed that two mechanisms occur in fatty acid synthesis (Mishina *et al.*, 1973). Odd-chain fatty acids originated from the elongation of odd-chain fatty acid precursors and even-chain fatty acids by *de novo* synthesis. A similar elongation system was recognized (Orme *et al.*, 1972) in a mutant of *Sacch. cerevisiae* that could synthesize higher acids from C13 to C17 acid supplements and could not perform *de novo* synthesis. Erwin (1973) discussed the formation of unsaturated fatty acids which are influenced especially by the presence or absence of oxygen (Rattray *et al.*, 1975).

The cellular lipid content and -composition is influenced by numerous factors, i.e. the growth cycle (Dawson and Craig, 1966; McMurrough and Rose, 1971); sporulation (Illingworth *et al.*, 1973); nutrients such as nitrogen and phosphorus (Ratledge, 1968; Johnson *et al.*, 1972); growth factors such as inositol (Lewin, 1965; Johnston and Paltauf, 1970; Paltauf and Johnston, 1972), vitamin B6 (Haskell and Snell, 1965) and biotin (Suomalainen and Keränen, 1968): sodium chloride (Combs *et al.*, 1968); choline (Palmer, 1971); benzopyrene (Baraud *et al.*, 1973); propanediol (Suzuki and Hasegawa, 1974): oxygen (Hunter and Rose, 1972; Kovác *et al.*, 1967); temperature (Hunter and Rose, 1972; Kates and Paradis, 1973) and pH (Rattray *et al.*, 1975).

Abel *et al.* (1963) was the first to employ gas-liquid chromatography for the classification of bacteria on the basis of their cellular fatty acid composition. Since then a number of studies on the cellular fatty acid

composition and the taxonomic relationship have been reported (Shaw, 1974; Kaneko *et al.*, 1976; Hossack and Spencer-Martins, 1978; Nishimura *et al.*, 1979; Chen, 1981; Moss *et al.*, 1982; Athalye *et al.*, 1985; Cottrell *et al.*, 1985; Kock *et al.*, 1985; Kock *et al.*, 1986).

It was found that the fatty acid composition of microorganisms varies between species of a genus and also with culture age, medium composition and growth temperature (Deinema, 1961; Merdinger and Devine, 1965; McMurrough and Rose, 1967; Brown and Rose, 1969; Hunter and Rose, 1972; Drucker and Veazey, 1977; Tornabene, 1985 and Viljoen *et al.*, 1986). It is therefore of utmost importance to use standardized conditions for growth in order to obtain reproducible results in a taxonomic study.

In this thesis the long-chain fatty acid compositions of yeasts associated with wine environments were investigated as an aid in identification and classification.

1.5.2 The use of volatile metabolites in the identification of yeasts associated with wine

Many of the volatile constituents associated with the bouquet and flavour of wines are produced by yeasts. The isolation and identification of these yeast metabolites (mainly carbonyl compounds, alcohols and fatty acid esters) have been studied in considerable detail using gaschromatography with subsequent mass-spectrometry (Hardy and Ramshaw, 1970; Killian and Ough, 1979; Schreier *et al.*, 1980).

It was found that the volatile metabolites produced by yeasts vary between different yeast species and strains (Wenzel, 1966; Di Stefano *et al.*, 1981; Soles *et al.*, 1982) and could therefore have taxonomic implications.

A method to determine these volatile metabolites on a qualitative-, as well as quantitative basis will have notable advantages over the organoleptic tests (Lodder, 1970) used in the present conventional classification system.

In this investigation, the use of different volatile metabolites in the identification of some wine yeasts was investigated in a preliminary study.

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CHAPTER 2

THE VALUE OF CELLULAR LONG-CHAIN FATTY ACID COMPOSITION IN THE TAXONOMY OF WINE- AND RELATED YEASTS

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CHAPTER 2

THE VALUE OF CELLULAR LONG-CHAIN FATTY ACID COMPOSITION IN THE TAXONOMY OF WINE- AND RELATED YEASTS

ABSTRACT

The cellular long-chain fatty acid compositions of 103 yeast strains representing 38 species associated with the wine industry were determined by gas-liquid chromatography.

It was possible to differentiate between all the species examined except *Schizosaccharomyces malidevorans* and *S. pombe* which had a similar fatty acid composition, as well as between some strains within *Saccharomyces cerevisiae* and within other species examined. Of importance to the wine industry is the fact that *Sacch. cerevisiae* had an unique fatty acid profile.

This method resulted in identification within three days which compares favourably with the seven to ten days and longer required for conventional methods.

A general correlation was found between the presence of linoleic- and linolenic acid and the complexity of cell differentiation. Two phylogenetic lines were obtained in *Kluyveromyces* by comparing long-chain fatty acid compositon, genetic recombination, pseudomycelium formation and carbon source and ethylamine utilization. These lines correspond with the proposed conventional phylogenetic scheme for *Kluyveromyces*. A correlation was found in this genus between the long-chain fatty acid composition and the ability to form pseudomycelium, to utilize carbon sources as well as ethylamine and the ability to hybridise. In the genus *Saccharomyces*, a similar correlation was found between the presence of linoleic- and linolenic acid and the ability to utilize a large number of carbon sources. A developmental line was found which corresponds with a sequential acquirement of the ability to utilize carbon sources, the ability to form pseudomycelium, loss of resistance to cycloheximide as well as the acquirement of linoleic- and linolenic acid.

2.1 INTRODUCTION

The present yeast classification system aims to assign yeast strains to species and genera on the basis of their morphological characteristics, sexual reproduction and certain physiological and biochemical features (Phaff *et al.*, 1978; Barnett *et al.*, 1983).

Certain difficulties are encountered when the above mentioned criteria are applied. For instance, the genera *Candida* and *Torulopsis* are separated only on the ability of the former to produce pseudohyphae (Lodder *et al.*, 1958). It was, however, observed that these species can produce two or more types of pseudomycelium simultaneously or at different stages of growth (Van Uden and Buckley, 1970). Wickerham and Burton (1954) reported the presence of both spherical and hat-shaped ascospores in strains of *Pichia ohmeri* at a time when it was thought that spore shape was a constant characteristic of a species. Stelling-Dekker (1931) proposed that *Hansenula* and *Pichia* be separated primarily on their ability to assimilate nitrate as a sole source of nitrogen. Since this criterion cannot always be successfully applied, the difference between these two genera could disappear and one generic name will have to be used. Scheda and Yarrow (1966) observed enough variability in the fermentation and carbon assimilation patterns of a number of *Saccharomyces* species to cause difficulties in the assignment of their yeast strains to specific species.

Since some morphological differences are unreliable for taxonomy, and biochemical and physiological criteria are also sometimes variable, new criteria, which are more stable, should be examined. These include a number of macromolecular comparisons such as proton magnetic resonance (Gorin and Spencer, 1970), serology (Campbell, 1971), classification of the isoprenoid quinones in the electron transport system (Yamada *et al.*, 1977), electrophoretic patterns of isozymes and enzymes (Baptist and Kurtzman, 1976), DNA hybridization (Kurtzman and Smiley, 1979; Kurtzman, 1984), genome comparisons (Price *et al.*, 1978) and scanning electron microscopy (Kurtzman *et al.*, 1975). Taxonomic schemes based on Adansonian analyses of the traditional phenotypic characters have also been proposed (Campbell, 1974).

Since the introduction of lipid analyses by gas-liquid chromatography (GLC), various investigations concerning the identification and classification of bacteria and fungi (Shaw, 1974; Miura *et al.*, 1983; Collins and Shah, 1984; Athalye *et al.*, 1985) and yeasts were undertaken (Moss and Dees, 1975; Rattray *et al.*, 1975; Chen, 1981; Moss *et al.*, 1982). As cultivation procedures may influence the cellular fatty acid

composition (Rattray *et al.*, 1975) it is not possible to utilize the results of the above mentioned studies for taxonomic purposes.

Recent work in this field resulted in a reproducible technique for the cultivation of yeasts and analysis of cellular fatty acids (Cottrell *et al.*, 1985; Kock *et al.*, 1985; Kock *et al.*, 1986; Viljoen *et al.*, 1986). In this study, the fatty acid compositions of yeast strains representing 38 species of 21 genera associated with the wine industry were determined by using the method of Kock *et al.* (1985).

The application of long-chain fatty acid compositions in the identification and classification of wine-associated and related yeasts is discussed as follows:

2.1.1 The use of cellular long-chain fatty acid composition in the identification of yeasts associated with the wine industry.

2.1.2 The value of cellular long-chain fatty acid composition in the taxonomy of wine- and related yeasts. This includes:

- 2.1.2.1 The relation between long-chain fatty acid composition and the degree of mycelium formation.
- 2.1.2.2 The value of long-chain fatty acid composition in the phylogeny of the genus *Kluyveromyces*.
- 2.1.2.3 The value of long-chain fatty acid composition in the taxonomy of the genus *Saccharomyces*.

2.2 MATERIALS AND METHODS

Strains: One hundred and three strains comprising 38 species were obtained from the Centraalbureau voor Schimmelcultures, Yeast division, Delft, The Netherlands (CBS); Professor J.P. van der Walt, Council for Scientific and Industrial Research, Pretoria, South Africa (CSIR-Y); the Viticultural and Oenological Research Institute, Stellenbosch, South Africa (N) and the American Type Culture Collection (ATCC) (Table 1).

Cultivation of strains: The inoculum was prepared from stock cultures maintained on YM (Wickerham, 1951) slants. These were then cultured in triplicate for 16 h at 30°C on a rotary shaker at 160 rpm (throw = 50 mm) in 150 mℓ Erlenmeyer flasks. Each flask contained 40 mℓ of medium, consisting of 80 g/ℓ glucose (Merck) and 6.7 g/ℓ yeast nitrogen base (YNB) (Difco).

Ten mℓ quantities of the precultured strains (Klett = 200) were then inoculated into 400 mℓ of glucose YNB liquid medium in 1 ℓ conical flasks and cultured for 2 days under the conditions described. Since a constant and reproducible fatty acid composition was found in the yeast cells during stationary phase by Viljoen *et al.* (1986), the cells were then harvested during this phase by centrifugation at 8000 x *g* for 5 min at 4°C. The sediment was washed three times with cold 0.85% saline solution and lyophilized.

Extraction of the fatty acids and preparation of methyl esters: Fatty acids were extracted from 0.12 g lyophilized yeast cells suspended in 5 mℓ of 15% KOH in 50% methanol. The suspension (in sealed screw-capped test tubes) was heated in a boiling waterbath for 1 h, the saponified material cooled to room temperature and the pH adjusted to 2.0 with 6N HCl. The free fatty acids were then methylated with 3 mℓ of 20% borontrifluoride in methanol (Merck, Darmstadt) in a boiling waterbath for 15 min while shaking. Again the suspension was cooled to room temperature and 0.25 mℓ of a saturated NaCl solution was added. The methyl esters were then extracted by vigorous shaking with three 6 mℓ portions of a 1:4 chloroform-hexane mixture. The chloroform-hexane mixtures were recovered by centrifugation at approximately 500 rpm for 3 minutes. The solvent mixture was evaporated by means of nitrogen gas and the dried methyl ester fraction dissolved in 1.8 mℓ hexane.

Separation of fatty acids by gas-liquid chromatography: The methyl esters of the total fatty acids were analysed by GLC on a Hewlett Packard model 5830A gas chromatograph equipped with dual flame-ionization detectors. Identification of the esters was based on the comparison of retention times with known standards of C14:0 (myristic acid), C14:1 (myristoleic acid), C16:0 (palmitic acid), C16:1 (palmitoleic acid), C18:0 (stearic acid), C18:1 (oleic acid), C18:2 (linoleic acid) and C18:3 (linolenic acid) (Serva, Heidelberg, Germany). All analyses were carried out using glass columns (4 mm I.D. x 1.5 m) packed with 5% diethyleneglycol succinate on Chromosorb W (80-100 mesh). The flow rate of the carrier gas (nitrogen) was 30 cm^3 min^{-1} at a column temperature of 160°C. Relative amounts of given fatty acids were calculated from their respective peak areas.

Pseudomycelium and mycelium formation: This morphological characterstic was determined using the Dalmau plate technique as described by Van der Walt (Lodder, 1970).

2.3 RESULTS AND DISCUSSION

2.3.1 The use of cellular long-chain fatty acid composition in the identification of yeasts associated with the wine industry

It was possible to differentiate between these organisms within 3 days which is a marked improvement on the usual 7 to 10 days and longer required with the conventional methods of Barnett *et al.* (1983). The high resolution, sensitivity and speed of this identification system can also complement the physiological, morphological and serological techniques conventionally used for yeast differentiation.

The results obtained were reproducible when the strains were grown under standard conditions. The standard deviation for triplicates was between 2% and 7%. The 38 species are characterized by the presence (or absence) of varying amounts of myristic acid (C14:0), myristoleic acid (C14:1), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) calculated as relative percentages.

It was possible to distinguish between most of these species as is shown in Table 1. The fatty acids used for differentiation are also highlighted. A detailed discussion concerning the differentiation of these species is now presented.

Division into groups

The strains can be divided into seven major groups according to their fatty acid content (Table 1). Groups I to IV are characterized by the absence of linoleic acid (C18:2) and linolenic acid (C18:3) while strains in group V contain linoleic acid (C18:2) and strains in groups VI and VII contain both linoleic acid (C18:2) and linolenic acid (C18:3).

Group I, characterized by the presence of palmitoleic acid (C16:1) and oleic acid (C18:1) as major fatty acids and the absence of linoleic acid (C18:2) and linolenic acid (C18:3), comprises the 41 strains of *Saccharomyces cerevisiae*.

Group II: The strains representing this group, have fatty acid compositions similar to group I, but are differentiated by a significantly ($P < 0.05$) lower mean percentage palmitoleic acid (C16:1) and a significantly higher mean percentage oleic acid (C18:1) ($P < 0.05$) compared

to groups I, III and IV (student's t-test). This group includes the strains of *Schizosaccharomyes malidevorans, S. octosporus* and *S. pombe*.

Group III includes strains of *Hanseniaspora uvarum, H. valbyensis, Saccharomyces exiguus, Sacch. unisporus* and *Saccharomycodes ludwigii* and they have higher mean percentages of palmitoleic acid (C16:1) and significantly smaller mean percentage of oleic acid (C18:1), compared to groups I, II and IV. These strains contain no linoleic acid (C18:2) or linolenic acid (C18:3).

Group IV comprises only *Wickerhamiella domercqiae* which contains a lower percentage palmitoleic acid (C16:1) compared to groups I and III and a lower percentage oleic acid (C18:1) compared to group II.

Group V is characterized by the presence of linoleic acid (C18:2) and the absence of linolenic acid (C18:3). This group comprises strains of *Endomyces fibuliger, Pichia etchellsii, Torulaspora delbrueckii, Zygosaccharomyces microellipsoides* and *Z. rouxii*.

Group VI: The strains representing this group contain linolenic acid (C18:3) which is not present in the previous groups. This group includes strains of *Candida tenuis, Cryptococcus albidus, Debaryomyces hansenii, Filobasidium capsuligenum, Hansenula anomala, H. canadensis, H. subpelliculosa, Metchnikowia reukauffii, Pichia guilliermondii* and *P. membranaefaciens*.

Group VII includes the strains of *Candida albicans, C. rugosa, C. steatolytica, Debaryomyces hansenii, Hyphopichia burtonii, Issatchenkia terricola, Kluyveromyces marxianus, K. thermotolerans, Lodderomyces elongisporus, Pichia fermentans, Rhodotorula mucilaginosa, Saccharomyces kluyveri, Williopsis saturnus* and *Zygosaccharomyces baillii*. These strains contain lower mean percentages linoleic acic (C18:2) compared to those in group VI and also contain linolenic acid (C18:3).

Subdivision within groups

Group I: This group of 41 *Saccharomyces cerevisiae* strains can be divided into five different subgroups according to their oleic acid (C18:1) content (Table 1).

Subgroup a. In this group, *Saccharomyces cerevisiae* strain N17 contains

a lower mean percentage palmitic acid (C16:0) and a higher mean percentage palmitoleic acid (C16:1) compared to strain N34.

Subgroup b. Strain CSIR-Y2 contains the lowest and strain N18 contains the highest mean percentage palmitic acid (C16:0). Strain N13 contains the highest, while N29 contains the lowest mean percentage palmitoleic acid (C16:1). Differentiation between the remainder of the strains was not attempted.

Subgroup c. The fatty acid compositions of strains CBS 1907, N1, N3, N4, N7, N8, N9, N14, N19, N23, N25, N32 and N41 are similar and they contain a lower mean percentage palmitic acid (C16:0) compared to the other strains in the subgroup. Further subdivision may therefore be possible, but was not attempted.

Subgroup d. In this subgroup, strains N26 and N27 are similar and may be distinghuished from the other strains on the basis of their lower palmitoleic acid (C16:1) content. Strain N5 contains the highest mean percentage palmitic acid (C16:0), while strain N31 contains the highest mean percentage palmitoleic acid (C16:1) in the group.

Group II. In this group, the strains of *Schizosaccharomyces malidevorans* and *S. pombe* contain similar fatty acid compositions. These strains contain lower mean percentages palmitic acid (C16:0) and higher mean percentages palmitoleic acid (C16:1) and oleic acid (C18:1) than strains of *S. octosporus*.

Group III. The *Saccharomyces exiguus* strain contains a lower mean percentage of palmitoleic acid (C16:1) and also a higher mean percentage stearic acid (C18:0) compared to the other strains. The strains of *Hanseniaspora uvarum* and *H. valbyensis* contain the highest mean percentage palmitoleic acid (C16:1) and also the lowest mean percentage oleic acid (C18:1). *H. uvarum* contains a lower mean percentage palmitoleic acid (C16:1) compared to *H. valbyensis*. *Saccharomyces unisporus* contains the lowest mean percentage palmitic acid (C16:0) compared to the strains of *Saccharomycodes ludwigii* which contain the highest mean percentage of this fatty acid. *S. ludwigii* CSIR-Y8 contains a higher mean percentage oleic acid (C18:1) compared to strain CSIR-Y22.

Group V. In this group the strains of *Endomyces fibuliger* contain the highest, while *Torulaspora delbrueckii* N30 and *Zygosaccharomyces rouxii* CSIR-Y364 contain the lowest mean percentage palmitic acid (C16:0). Strain CSIR-Y643 of *E. fibuliger* contains a high mean percentage linoleic acid

(C18:2) compared to strain CSIR-Y269. *T. delbrueckii* CSIR-Y138 contains the lowest mean percentage linoleic acid (C18:2), while *T. delbrueckii* N30 contains the lowest mean percentage oleic acid (C18:1). The strain representing *Z. microellipsoides* CSIR-Y263 contains the highest mean percentage oleic acid (C18:1) while *Z. rouxii* CSIR-Y364 contains the highest mean percentage linoleic acid (C18:2) within this group.

Group VI. *Candida tenuis* strain CSIR-Y604 contains the highest mean percentage of linoleic acid (C18:2) and also the lowest mean percentage oleic acid (C18:1) while strain CSIR-Y565 contains the highest mean percentage stearic acid (C18:0) in the group. *Cryptococcus albidus* contains the highest mean percentage oleic acid (C18:1) in the group while the strains of *Debaryomyces hansenii* contains the lowest mean percentage of linoleic acid (C18:2) in the group.

Filobasidium capsuligenum is characterized by the highest mean percentage of palmitic acid (C16:0) and the lowest mean percentage of palmitoleic acid (C16:1) and linolenic acid (C18:3) in the group.

Hansenula canadensis contains the highest mean percentage linolenic acid (C18:3) in the group.

H. subpelliculosa contains, next to *Metchnikowia reukauffii*, the lowest mean percentage stearic acid (C18:0) in the group and is differentiated from *H. anomala* also by the higher palmitoleic acid (C16:1) content.

M. reukauffii contains the highest mean percentage palmitoleic acid (C16:1) while *Pichia membranaefaciens* contains the lowest mean percentage palmitic acid (C16:0) in the group.

P. guilliermondii is characterized by its linolenic acid (C18:3) content which is lower than *H. canadensis* but higher than the rest of the group.

Group VII. This group is divided into 5 subgroups on the basis of their oleic acid (C18:1) content.

Subgroup a. *Kluyveromyces marxianus* CBS 2745 contains the highest mean percentage oleic acid (C18:1) and the lowest mean percentage palmitoleic acid (C16:1) and stearic acid (C18:0), while *K. thermotolerans* N48 is characterized by the highest mean percentage palmitic acid (C16:0) and palmitoleic acid (C16:1) and the lowest mean percentage linoleic acid (C18:2) and linolenic acid (C18:3). The strain of *Saccharomyces kluyveri* contains the lowest mean palmitic acid (C16:0) and highest mean percentage stearic acid (C18:0) and linolenic acid (C18:3).

Subgroup b. Palmitoleic acid (C16:1) is present in the highest amount in *Kluyveromyces marxianus* CBS 4857 while strain CSIR-Y293 contains the

highest mean percentage palmitic acid (C16:0) and the lowest mean percentage linoleic acid (C18:2) and linolenic acid (C18:3). *Pichia fermentans* contains the lowest mean percentage palmitic acid (C16:0) as well as the highest mean percentage linolenic acid (C18:3) in the subgroup.

Subgroup c. The three strains of *Debaryomyces hansenii* are characterized by the highest mean percentage palmitic acid (C16:0). Strain CSIR-Y959 contains the lowest mean percentage palmitic acid (C16:0) and stearic acid (C18:0) compared to the other two strains of *D. hansenii*. *Hyphopichia burtonii* is characterized by a mean percentage palmitic acid (C16:0) which is lower than in the three strains of *D. hansenii* and higher than in the other species in the subgroup. The strain representing *Issatchenkia terricola* is characterized by a higher mean percentage linoleic acid (C18:2) compared to the three strains of *D. hansenii*, *K. marxianus* and *Williopsis saturnus* and a lower mean percentage linoleic acid (C18:2) compared to *Hyphopichia burtonii*, *Kluyveromyces thermotolerans* and *Zygosaccharomyces baillii*. The lowest mean percentage linoleic acid (C18:2) is found in *K. marxianus* while *K. thermotolerans* contains the highest mean percentage palmitoleic acid (C16:1).

Williopsis saturnus is characterized by the highest mean percentage linolenic acid (C18:3) while *Zygosaccharomyces baillii* contains the lowest mean percentage palmitic acid (C16:0).

Subgroup d: The strain representing *Candida albicans* is characterized by the lowest mean percentage linolenic acid (C18:3) and the lowest mean percentage palmitic acid (C16:0). The strains of *C. rugosa* contain the lowest mean percentage palmitoleic acid (C16:1) while strain CSIR-Y299 contains a higher mean percentage palmitic acid (C16:0) compared to strain CSIR-Y295. Stearic acid (C18:0) is present in the highest mean percentage in *L. elongisporus* while *W. saturnus* contains the highest mean percentage palmitoleic acid (C16:1) and the lowest mean percentage stearic acid (C18:0) and linoleic acid (C18:2).

Subgroup e: *Candida steatolytica* contains the lowest mean percentages of both stearic acid (C18:0) oleic acid (C18:1) and the highest mean percentage linoleic acid (C18:2).

With gas-liquid chromatography of the total cellular fatty acids it was therefore possible to differentiate between all the wine yeast species examined with the exception of *Schizosaccharomyces malidevorans* and *Schizosaccharomyces pombe*. It was also possible to differentiate between some strains within the species *Saccharomyces cerevisiae* and within other species examined.

2.3.2 The value of cellular long-chain fatty acid composition in the taxonomy of wine- and related yeasts

In this section the long-chain fatty acid compositions obtained for the wine yeasts (Table 1) are compared with other phenotypic characters i.e. mycelium formation, carbon compound utilization, ethylamine utilization, etc.

2.3.2.1 The relation between long-chain fatty acid composition and the complexity of mycelium formation (Fig. 1)

On the basis of long-chain fatty acid composition, the wine yeasts analyzed, fall into three distinct groups as shown in Fig. 1.

Group A, differentiated by a less complex fatty acid composition (absence of linoleic- and linolenic acid), comprises:

a) Strains of taxa which produce mainly single cells (*S. malidevorans, S. octosporus, S. pombe, K. phaffii, K. delphensis, K. blattae, Sacch. exiguus, Sacch. unisporus, H. uvarum* and *W. domerqiae*).

b) Strains of taxa which form single cells and rudimentary pseudomycelium (*K. lodderi, K. polysporus, Sacch. cerevisiae, H. valbyensis* and *S. ludwigii*).

This group represents groups I to IV as given in Table I.

Group B, differentiated by the absence of linolenic acid and the presence of linoleic acid, comprises:

a) Strains of taxa which produce mainly single cells (*Z. microellipsoides, Z. rouxii* and *K. africanus*).

b) Strains of taxa producing single cells as well as rudimentary pseudomycelium (*P. etchellsii* and *T. delbrueckii*).

c) Strains of *E. fibuliger* characterized by the ability to form single cells, well-developed pseudomycelium as well as true hyphae.

d) Strains of taxa (*S. japonicus* var. *japonicus* and *S. japonicus* var. *versatilis* which are characterized by the production of single cells, true hyphae and sometimes arthrospores.

This group represents group V of Table 1.

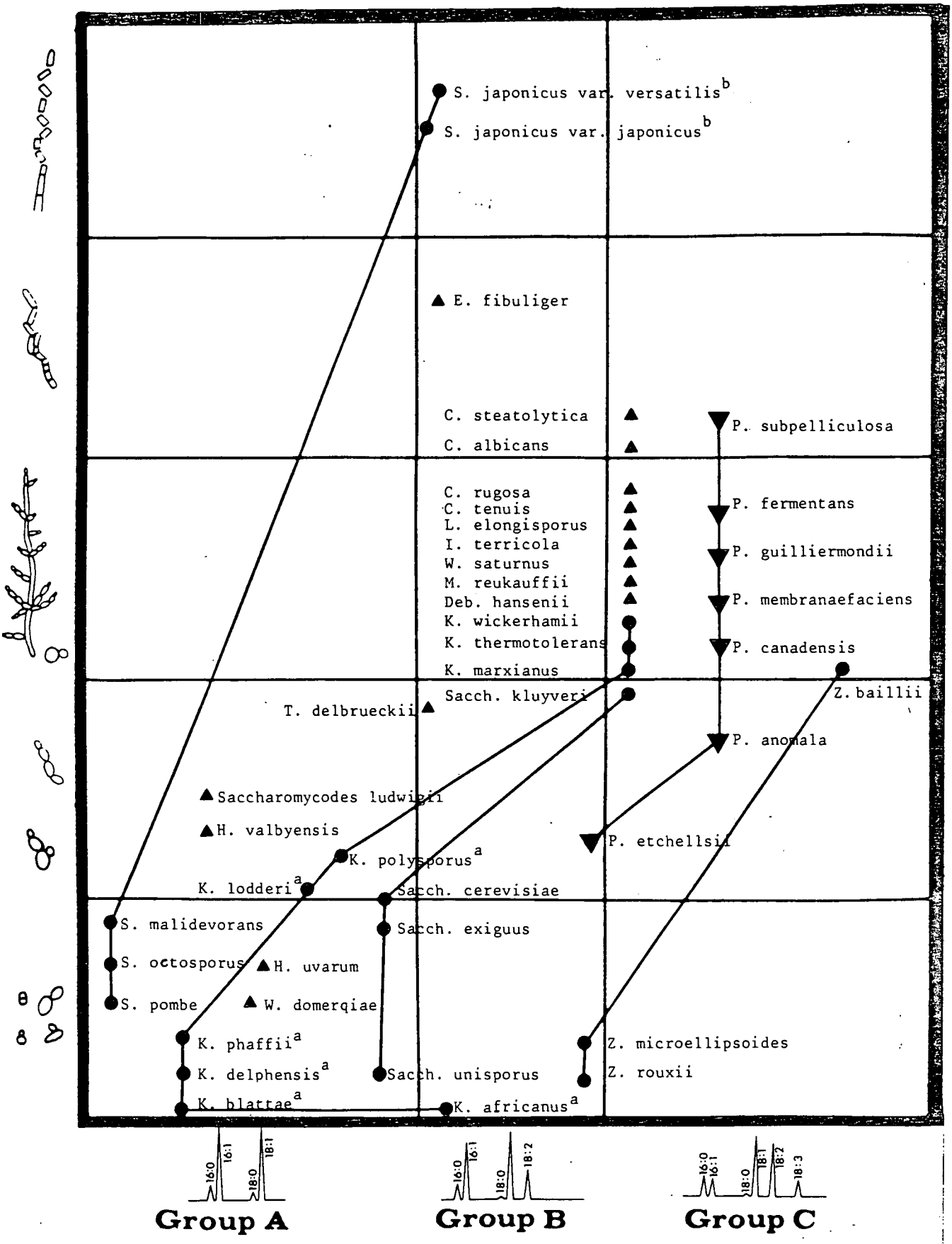
Group C, characterized by a complex fatty acid composition (including linoleic- and linolenic acid), comprises:

Figure 1: Relationship between long-chain fatty acid composition and complexity of cell differentiation in 43 wine- and related yeast species.

a) Indicates fatty acid data of strains derived from Cottrell *et al.*, 1985.

b) Indicates fatty acid data obtained from Van der Walt and Kock, 1986.

FIG. 1


S. japonicus var. versatilis[b]
S. japonicus var. japonicus[b]
E. fibuliger
C. steatolytica
C. albicans
P. subpelliculosa
C. rugosa
C. tenuis
L. elongisporus
I. terricola
W. saturnus
M. reukauffii
Deb. hansenii
K. wickerhamii
K. thermotolerans
K. marxianus
P. fermentans
P. guilliermondii
P. membranaefaciens
P. canadensis
Sacch. kluyveri
T. delbrueckii
Z. baillii
P. anomala
Saccharomycodes ludwigii
H. valbyensis
K. polysporus[a]
P. etchellsii
K. lodderi[a]
Sacch. cerevisiae
S. malidevorans
Sacch. exiguus
S. octosporus
H. uvarum
S. pombe
W. domerqiae
K. phaffii[a]
K. delphensis[a]
K. blattae[a]
Sacch. unisporus
K. africanus[a]
Z. microellipsoides
Z. rouxii
16:0
16:1
18:0
18:1
18:2
18:3
Group A
Group B
Group C

a) Strains of taxa characterized by the presence of single cells as well as rudimentary pseudomycelium (*Sacch. kluyveri* and *P. (Hansenula) anomala*).

b) Strains representing *K. marxianus, K. thermotolerans, K. wickerhamii, P. (Hansenula) canadensis, P. membranaefaciens, P. guilliermondii, P. fermentans, Z. baillii, C. rugosa, C. tenuis, L. elongisporus, I. terricola, W. saturnus, M. reukauffii* and *Deb. hansenii* producing single cells and well-developed pseudomycelium.

c) Strains representing *P. (Hansenula) subpelliculosa, C. albicans* and *C. steatolytica* produced single cells, well developed pseudomycelium as well as true hyphae.

Group C corresponds with groups VI and VII in Table 1.

From these results the following conclusions can be drawn:

1) A general correlation exists between the presence of linoleic acid (C18:2) and linolenic acid (C18:3) and the complexity of cell differentiation i.e. the ability to form pseudomycelium and true hyphae. Strains of taxa which did not contain linoleic- and linolenic acid, were generally found not to produce pseudomycelium while certain strains produced only rudimentary pseudomycelium. Strains of taxa which did produce both linoleic- and linolenic acid, generally formed rudimentary to well-developed pseudomycelium and sometimes true hyphae.

2) Each genus is characterized by its own developmental line. This line is formed by species with a specific relation of cell differentiation to fatty acid composition.

2.3.2.2 The value of long-chain fatty acid composition in the phylogeny of the genus *Kluyveromyces*

The evolutionary development of species representing the yeast genus *Kluyveromyces* from "primitive" ancestors was postulated by several investigators (Lodder, 1970).

On the basis of ascospore shape they arranged the members of this genus into two basic phylogenetic lines radiating from the non-hybridizing "primitive" ancestors, some associated with specific habitats, towards the more evolved taxa, which are less dependent on specific habitats and are capable of hybridizing, utilizing a large number of polyalcohols and di- and trisaccharides.

In the construction of these phylogenetic lines, conventional characteristics were mainly used, while no attempt was made to include other criteria, such as long chain fatty acid composition or pseudomycelium formation.

In this section, the fatty acid results obtained in Table 1 were combined with tjat pf pseudomycelium formation, carbon source- and ethylamine utilization (Lodder, 1970) as well as genetic recombination (Johannsen, 1980). A phylogenetic scheme was then constructed and compared to the present conventional scheme as proposed for *Kluyveromyces*.

The scheme was constructed as follows (Fig. 2):
The upper and lower horizontal axis represent carbon sources arranged in an increasing order of utilization by the species examined. The lower horizontal axis also represents cell differentiation ranging from single cells to the formation of well-developed pseudomycelium. The left vertical axis presents the long-chain fatty acid compositions in ascending order of complexity, as well as the ability of species to hybridize. The right vertical axis presents the ability of species to utilize arbutin as well as ethylamine.

The different species are arranged in this scheme according to the results obtained for the characteristics indicated on the horizontal and vertical axis. Two lines were constructed on the basis of ascospore morphology (line 1 and line 2a,b). Two subsidiary lines (2c and 2d) were drawn from *K. lodderi* representing species producing more than four reniform ascospores.

According to long-chain fatty acid composition, genetic recombination, pseudomycelium formation and carbon source utilization, the *Kluyveromyces* spp. analysed fall into two groups as shown in Fig. 2. Group 1 (*K. wickerhamii, K. thermotolerans* and *K. marxianus*) is differentiated by a more complex fatty acid composition, the ability to hybridize (Johannsen, 1980), the formation of well-developed pseudomycelium and the utilization of a large number of carbon sources as well as ethylamine (Barnett *et al.*, 1983).

Group 2 is characterized by the absence of linolenic acid (C18:3), the inability to hybridize (Johannsen, 1980), the formation of mainly a yeast phase (sometimes rudimentary pseudomycelium) and the utilization of a small number of carbon sources (Barnett *et al.*, 1983) and comprises:

a) Strains of taxa (*K. delphensis, K. phaffii* and *K. lodderi*) which are characterized by the sequential acquirement of the ability to utilize galactose, sucrose and raffinose and also ethylamine, as sole nitrogen source (Fig. 2 - line 2a). Of these species, *K. delphensis* is

Figure 2: Correlation between long-chain fatty acid composition, cell differentiation, carbon source- and ethylamine utilization and genetic relatedness in the genus *Kluyveromyces* indicating two phylogenetic lines.

Indicates positive or variable results.

f Indicates fermentation tests.

a Indicates assimilation tests.

S Slow assimilation.

W Weak assimilation

\+ Positive assimilation.

\- Negative assimilation.

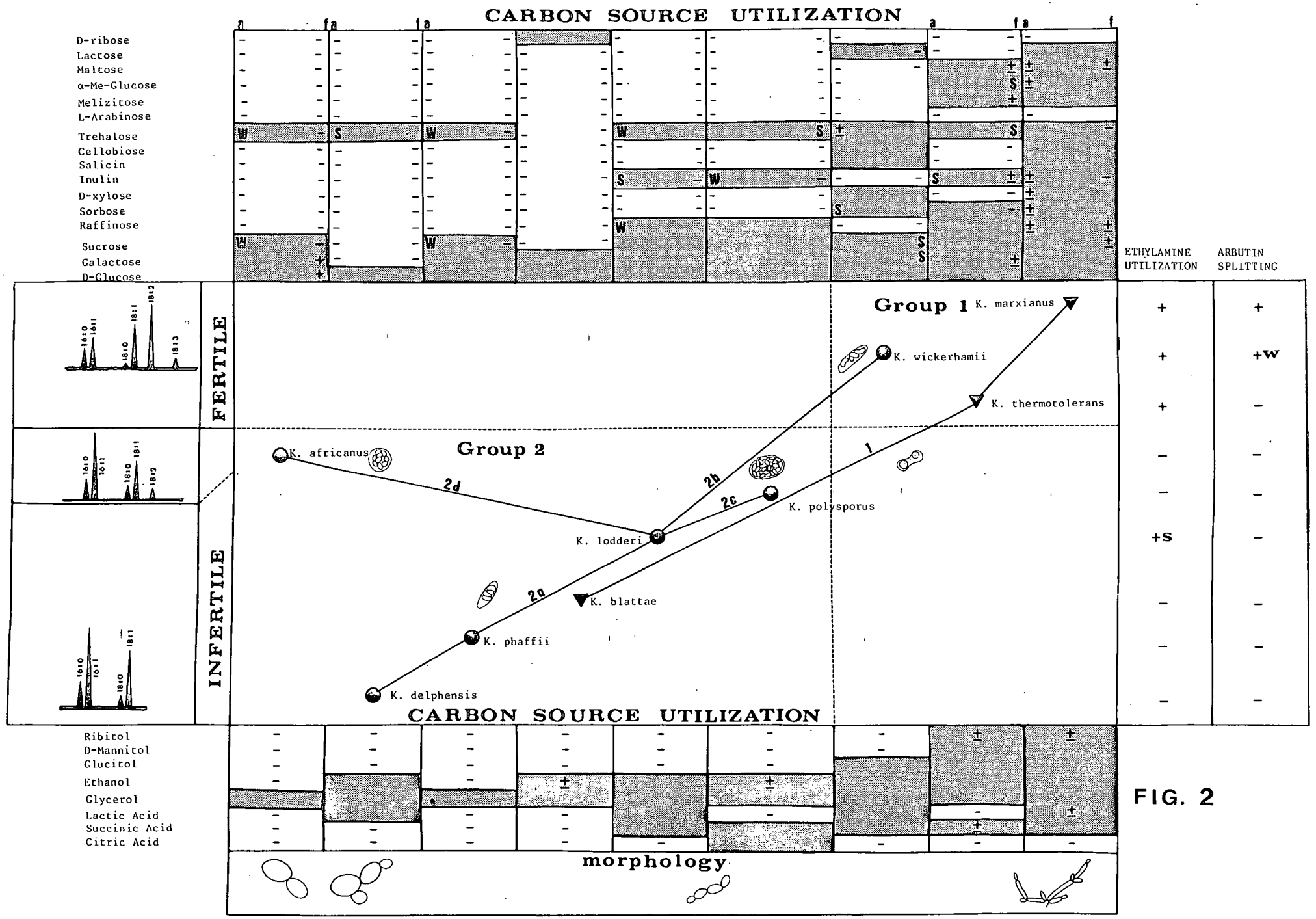

CARBON SOURCE UTILIZATION
D-ribose
Lactose
Maltose
α-Me-Glucose
Melizitose
L-Arabinose
Trehalose
Cellobiose
Salicin
Inulin
D-xylose
Sorbose
Raffinose
Sucrose
Galactose
D-Glucose
ETHYLAMINE UTILIZATION
ARBUTIN SPLITTING
FERTILE
INFERTILE
Group 1
K. marxianus
K. wickerhamii
K. thermotolerans
Group 2
K. africanus
K. lodderi
K. polysporus
K. blattae
K. phaffii
K. delphensis
2d
2b
2c
1
2a
CARBON SOURCE UTILIZATION
Ribitol
D-Mannitol
Glucitol
Ethanol
Glycerol
Lactic Acid
Succinic Acid
Citric Acid
morphology

FIG. 2

associated with a specialized habitat i.e. the sugary efflorescens of dried figs.

b) Strains of *K. polysporus* and *K. africanus* which are characterized by the ability to produce more than the usual one to four reniform ascospores (Figs. 2 lines 2c and d). *K. africanus* is also characterized by the production of linoleic acid (C18:2) which is absent in the other taxa representing group 2.

c) Strains of *K. blattae* which are associated with a specialized habitat i.e. the intestinal tracts of cockroaches (*Blatta orientalis*) and are capable of utilizing only a few carbohydrates (Barnett *et al.*, 1983) (Fig. 2 - line 1).

From our results, the following conclusions are drawn:

1) The phylogenetic lines obtained by comparing long-chain fatty acid composition, genetic recombination, pseudomycelium formation and carbon source- and ethylamine utilization agree with the proposed phylogenetic scheme (Lodder, 1970). According to the results, *K. delphensis, K. phaffii* and *K. lodderi* (Fig. 2 - line 2a) constitute the more primitive group. From this group, two species, *K. africanus* and *K. polysporus* (Fig. 2 lines 2c and 2d) presumably evolved by the acquirement of the ability to produce more than the usual one to four ascospores.

   K. delphensis, K. phaffii, K. lodderi as well as *K. blattae* presumably developed into the more advanced taxa *K. wickerhamii, K. thermotolerans* and *K. marxianus* (Fig. 2 -line 1 and 2b) which are characterized by the acquirement of the ability to utilize more carbon sources.

2) There is a correlation between the long-chain fatty acid composition and the ability to form pseudomycelium, to utilize carbon sources as well as ethylamine and the ability to hybridize.

2.3.2.3 The value of long-chain fatty acid composition in the taxonomy of the genus *Saccharomyces* (Fig. 3)

A similar scheme as for the genus *Kluyveromyces* was constructed from the fatty acid results in Table 1 and the results obtained for pseudomycelium formation, carbon source utilization as well as cycloheximide resistance (Lodder, 1970; Barnett *et al.*, 1983).

On the basis of long-chain fatty acid composition, the *Saccharomyces* species analyzed, fall into two distinct groups. Group 1, represented by

Figure 3: Relationship between long-chain fatty acid composition, carbon source utilization, complexity of cell differentiation and resistance to cycloheximide in the genus *Saccharomyces*.

	Indicates positive or variable results.
f	Indicates fermentation tests.
a	Indicates assimilation tests.
S	Slow assimilation.
W	Weak assimilation
+	Positive assimilation.
-	Negative assimilation.

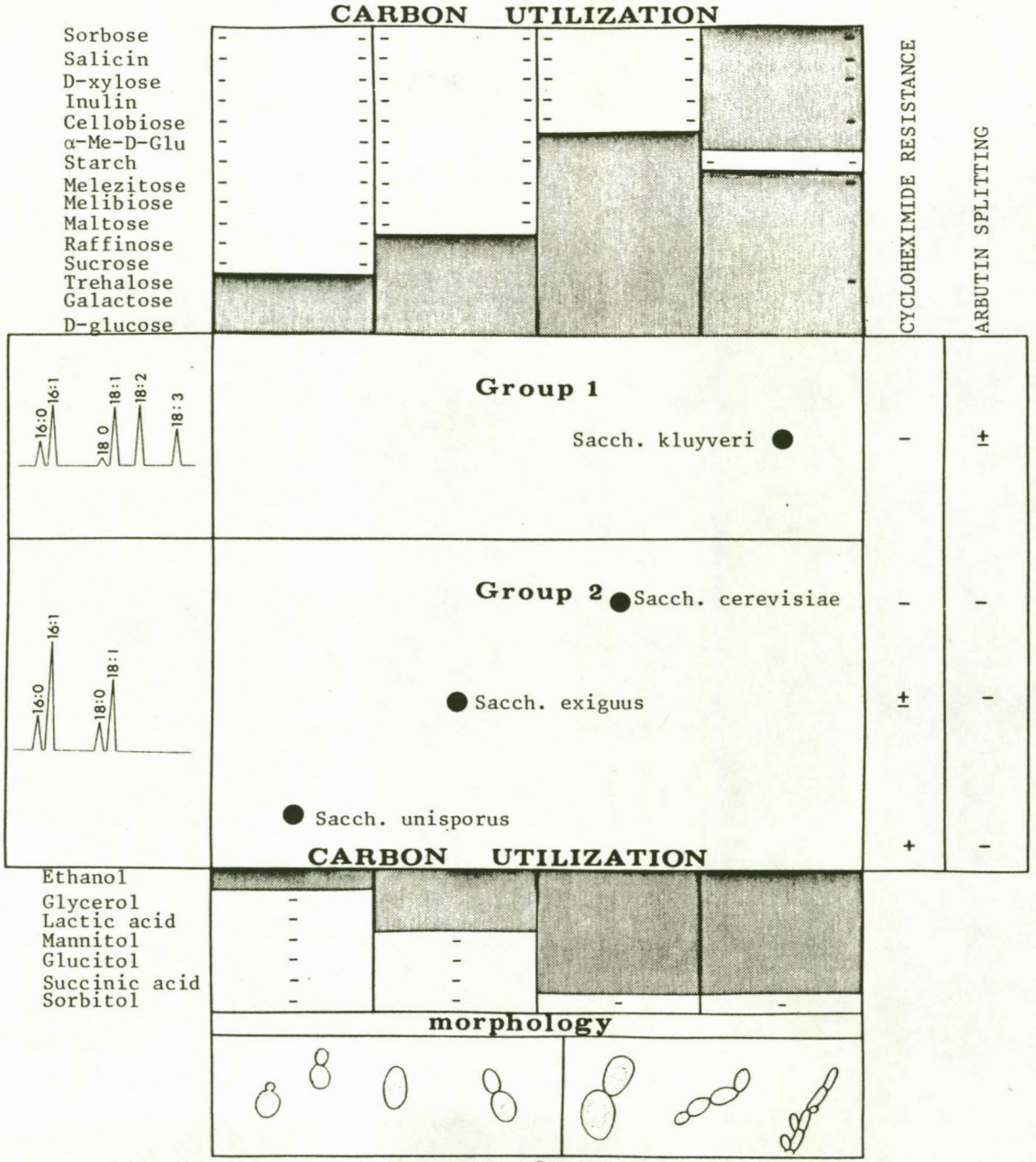

CARBON UTILIZATION
Sorbose
Salicin
D-xylose
Inulin
Cellobiose
α-Me-D-Glu
Starch
Melezitose
Melibiose
Maltose
Raffinose
Sucrose
Trehalose
Galactose
D-glucose
CYCLOHEXIMIDE RESISTANCE
ARBUTIN SPLITTING
16:0
16:1
18 0
18:1
18:2
18:3
Group 1
Sacch. kluyveri
– ±
16:0
16:1
18:0
18:1
Group 2
Sacch. cerevisiae
– –
Sacch. exiguus
± –
Sacch. unisporus
+ –
CARBON UTILIZATION
Ethanol
Glycerol
Lactic acid
Mannitol
Glucitol
Succinic acid
Sorbitol
morphology

FIG. 3

Sacch. kluyveri, is differentiated from group 2 by a more complex fatty acid composition and the utilization of more carbon sources.

From Figure 3 it is apparent that a developmental line exists from *Sacch. unisporus* to *Sacch. kluyveri*. This line includes strains of *Sacch. unisporus*, *Sacch. exiguus*, *Sacch. cerevisiae* and *Sacch. kluyveri*, characterized by a sequential acquirement of the ability to utilize carbon sources, the ability to form pseudomycelium and loss of resistance to cycloheximide.

From our results, the following conclusions are drawn:

1) On the basis of the results obtained with *Kluyveromyces* (section 2.3.2.2) it is proposed that *Sacch. unisporus* and *Sacch. exiguus* represent the more primitive organisms, while *Sacch. kluyveri* represents the more advanced species. *Sacch. cerevisiae* can be considered to be a transitional organism in the group.
2) A correlation exists between long-chain fatty acid composition, the utilization of carbon sources, the ability to form pseudomycelium and resistance to cycloheximide.

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TABLE 1. Grouping of yeast strains associated with wine on the basis of their cellular long-chain fatty acid composition.[a]

GROUP 1:

	Fatty acids[b]							
Strain	14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3
Subgroup a: *Saccharomyces cerevisiae* (C18:1 = 30-35%)								
N17	1.6	0.6	10.7	53.1	2.7	31.3	0	0
N34	1.2	0.2	15.7	49.4	1.3	32.2	0	0
$\bar{X}$	1.4	0.4	13.2	51.2	2.0	31.8	0	0
Subgroup b: *Saccharomyces cerevisiae* (C18:1 = 35 - 40%)								
CSIR-Y2	0.5	0.4	5.6	51.7	1.9	39.6	0	0
N11	1.4	0.5	8.5	51.1	1.8	36.6	0	0
N12	1.1	0.5	8.3	52.5	1.7	35.7	0	0
N13	1.0	0.6	6.1	54.1	1.9	36.2	0	0
N18	1.1	0.4	9.1	50.9	2.1	36.4	0	0
N29	1.2	0.6	7.7	49.4	2.5	38.6	0	0
N16	1.2	0.5	7.7	51.5	1.4	37.5	0	0
N15	0.8	0.2	8.5	50.2	1.7	38.6	0	0
$\bar{X}$	1.0	0.5	7.7	51.4	1.9	37.8	0	0
Subgroup c: *Saccharomyces cerevisiae* (C18:1 = 40 - 45%)								
ATCC 26602	0.5	0.1	9.4	42.4	4.8	42.2	0	0
CBS 1907	0.9	0.5	6.7	45.0	4.1	42.9	0	0
N1	1.0	0.5	6.5	48.1	2.6	41.4	0	0
N3	0.8	0.5	5.0	48.3	2.2	43.1	0	0
N4	1.0	0.6	5.8	48.1	1.8	42.6	0	0
N7	0.5	0.2	4.2	49.6	2.3	42.9	0	0
N8	1.0	0.8	4.5	49.4	2.0	42.2	0	0
N9	0.6	0.2	5.5	48.8	2.0	42.9	0	0

Table 1 (continued)

Strain	Fatty acids[b] 14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3
N10	0.7	0.1	11.0	43.2	1.6	43.4	0	0
N14	0.9	0.5	5.2	52.3	1.6	40.3	0	0
N19	0.8	0.4	6.1	50.1	1.8	40.7	0	0
N20	0.8	0.4	12.7	39.5	2.7	42.5	0	0
N21	1.6	0.8	9.2	45.7	2.5	40.0	0	0
N23	0.9	0.5	5.7	48.1	2.8	42.0	0	0
N25	0.9	0.6	5.4	48.3	2.3	42.6	0	0
N28	0.6	0.0	12.7	43.6	1.8	41.3	0	0
N32	0.9	0.6	6.6	45.8	2.8	43.3	0	0
N35	1.2	0.2	14.9	40.2	2.2	41.1	0	0
N36	1.5	0.3	15.4	38.3	3.2	42.0	0	0
N38	0.4	0.1	13.8	39.4	2.9	43.4	0	0
N39	0.7	0.1	14.6	39.1	2.7	42.6	0	0
N40	0.7	0.1	12.6	43.7	1.6	41.0	0	0
N41	0.7	0.7	3.5	48.8	2.1	44.3	0	0
$\bar{X}$	0.9	0.4	8.6	45.4	2.4	42.2	0	0
Subgroup d: *Saccharomyces cerevisiae* (C18:1 = 45 - 50%)								
ATCC 26603	0.6	0.3	4.1	44.9	1.2	48.4	0	0
N2	0.7	0.3	6.0	44.2	2.3	46.4	0	0
N5	1.0	0.2	10.2	41.6	1.4	45.7	0	0
N6	0.8	0.4	4.7	45.2	2.0	46.8	0	0
N26	0.6	0.2	7.4	38.6	4.2	49.2	0	0
N27	0.6	0.2	7.2	39.8	3.5	48.6	0	0
N31	0.9	0.7	5.7	47.6	2.5	46.6	0	0
$\bar{X}$	0.7	0.3	6.5	43.1	2.4	47.4	0	0
Subgroup e: *Saccharomyces cerevisiae* (C18:1 = 50 - 55%)								
N22	0.4	0.07	8.1	35.8	1.7	53.8	0	0

Table 1 (continued)

Strain		Fatty acids[b] 14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3
GROUP II									
Schizosaccharomyces malidevorans	CSIR-Y933	0	0	7.8	1.1	6.6	84.5	0	0
Schizosaccharomyces octosporus	CBS 371	0	0	16.6	0.4	4.0	79.0	0	0
	CBS 6206	0	0	16.0	0.2	4.1	79.7	0	0
	CBS 6207	0	0	15.0	0.3	6.1	78.6	0	0
	CSIR-Y934	0	0	15.9	0.3	4.8	79.0	0	0
Schizosaccharomyces pombe	CBS 356	0	0	6.3	0.7	8.9	84.1	0	0
	CBS 374	0	0	9.5	1.3	6.2	83.0	0	0
	CBS 5680	0	0	9.6	0.9	5.8	83.7	0	0
	CSIR-Y468	0	0	7.3	0.7	8.3	83.7	0	0
	CSIR-Y830	0	0	8.6	1.0	5.0	85.4	0	0
	N52	0.2	0	7.7	2.5	7.5	81.2	0	0
	N53	0.3	0	9.0	2.2	4.8	83.1	0	0
	$\bar{X}$	0	0	10.8	1.0	6.0	82.1	0	0
GROUP III									
Hanseniaspora uvarum	CSIR-Y898	0.6	0.2	13.9	63.6	0.7	20.9	0	0
Hanseniaspora valbyensis	CSIR-Y895	1.2	0.3	13.0	67.0	0.5	18.0	0	0
Saccharomyces exiguus	CSIR-Y847	2.5	0.4	13.6	42.7	11.5	28.2	0	0
Saccharomyces unisporus	CSIR-Y550	4.7	1.0	12.3	56.9	2.9	21.8	0	0
Saccharomycodes ludwigii	CSIR-Y8	1.1	0.3	15.2	53.0	2.4	28.0	0	0
	CSIR-Y22	1.5	0.6	15.6	58.5	1.5	22.3	0	0
	$\bar{X}$	1.9	0.5	13.9	57.0	3.3	23.2	0	0

Table 1 (continued)

Strain	Fatty acids[b] 14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3
GROUP IV								
Wickerhamiella domercqiae CSIR-Y889	0.5	0	13.8	25.7	2.3	57.8	0	0
GROUP V								
Endomyces fibuliger CSIR-Y269	2.2	0	24.6	5.2	3.2	34.3	30.5	0
CSIR-Y643	0.5	0	17.4	3.8	3.1	34.0	41.2	0
Pichia etchellsii CSIR-Y858	0	0.2	14.1	7.8	2.9	33.0	41.6	0
Torulaspora delbrueckii CSIR-Y138	1.3	0.2	14.9	35.4	4.1	32.8	11.4	0
N30	1.5	0.8	6.0	45.9	1.5	24.5	19.6	0
Zygosaccharomyces microellipsoides CSIR-Y263	0.7	0.2	9.2	32.3	1.6	39.0	16.9	0
Zygosaccharomyces rouxii CSIR-Y364	0.4	0	6.2	11.2	3.3	32.6	46.2	0
$\bar{X}$	0.7	0.2	11.3	22.7	2.7	32.6	29.5	0
GROUP VI								
Candida tenuis CSIR-Y565	0.2	0	14.8	4.6	5.2	27.0	45.4	2.8
CSIR-Y604	0.1	0	12.8	6.7	3.1	20.0	53.9	3.4
Cryptococcus albidus CSIR-Y73	0.9	0	13.2	3.4	1.9	45.3	30.5	4.6
Debaryomyces hansenii CSIR-Y953	0.1	0	17.0	4.9	3.6	36.7	29.8	8.0
N55	0.8	0	20.3	12.3	1.8	27.5	29.8	7.5
Filobasidium capsuligenum CSIR-Y302	1.8	0	29.2	1.8	1.8	27.5	35.8	2.0

Table 1 (continued)

Strain	Fatty acids[b] 14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3
Hansenula anomala								
CSIR-Y207	0.40	0	14.4	3.8	3.0	38.6	34.8	5.0
N54	0.30	0	12.3	6.5	1.5	36.0	34.3	9.0
Hansenula canadensis								
CBS 1992	0.3	0	14.2	6.4	1.7	31.0	32.9	13.6
CBS 2431	0.5	0	15.4	6.2	1.3	28.8	30.5	17.4
Hansenula subpelliculosa								
CBS 5767	0.30	0	14.0	7.5	0.7	38.5	33.8	4.5
Metchnikowia reukauffii								
CSIR-Y13	0.36	0	13.9	14.2	0.2	32.1	34.5	4.6
Pichia guilliermondii								
CBS 2030	0.20	0	12.4	10.5	2.0	34.4	30.4	10.1
Pichia membranaefaciens								
CBS 107	0.3	0	10.6	11.9	1.9	22.2	45.9	7.1
$\bar{X}$	0.5	0	15.3	7.2	2.1	31.8	35.9	7.1
GROUP VII								
Subgroup a (C18:1 = 20 - 30%)								
Kluyveromyces marxianus								
CBS 2745	1.6	0	12.7	21.8	1.6	26.0	24.8	11.5
Kluyveromyces thermo-tolerans N48	1.4	0	15.0	39.8	2.9	20.0	16.5	4.0
Saccharomyces kluyveri								
CSIR-Y273	0.3	0	9.8	24.0	3.9	23.3	24.0	14.5
$\bar{X}$	1.1	0	12.5	28.5	2.8	23.1	21.8	10.0
Subgroup b (C18:1 = 31 - 40%)								
Kluyveromyces marxianus								
CBS 4857	0.5	0	12.0	23.1	1.8	37.4	21.9	2.5
CSIR-Y293	0.7	0	15.6	17.8	2.8	40.9	20.9	2.0

Table 1 (continued)

Strain	Fatty acids[b] 14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3
Pichia fermentans								
CBS 187	0.2	0	9.5	19.4	1.5	40.6	22.4	6.2
$\bar{X}$	0.5	0	12.4	20.1	2.0	39.6	21.6	3.6
Subgroup c (C18:1 = 41 - 50%)								
Debaryomyces hansenii								
CSIR-Y955	0.1	0.1	23.7	4.0	6.9	47.8	17.4	0.2
CSIR-Y956	0.1	0.1	23.0	4.8	6.6	48.0	17.3	0.2
CSIR-Y959	0.2	0	21.9	6.3	3.1	48.1	17.9	2.3
Hyphopichia burtonii								
CSIR-Y608	0.2	0	16.3	4.8	1.8	46.6	28.0	2.3
Issatchenkia terricola								
CSIR-Y644	0.3	0	14.2	8.4	6.7	48.9	19.1	2.3
Kluyveromyces marxianus								
CSIR-Y236	0.4	0	10.7	23.9	5.4	44.9	11.9	2.8
Kluyveromyces thermotolerans CSIR-Y478	0.5	0	12.1	25.7	5.6	45.0	27.8	3.7
Williopsis saturnus								
CSIR-Y17	0.9	0	15.2	15.1	0.3	47.7	15.2	5.4
Zygosaccharomyces bailli								
CSIR-Y126	0.1	0	6.6	10.3	1.1	49.8	28.7	3.3
$\bar{X}$	0.4	0.0	15.7	10.7	3.9	47.2	21.4	2.9
Subgroup d (C18:1 = 51 - 60%)								
Candida albicans								
CSIR-Y240	0.3	0	9.6	15.3	1.3	56.3	15.3	1.8
Candida rugosa CSIR-Y295	0.2	0	14.5	2.6	2.5	60.6	17.4	2.1
CSIR-Y299	0.3	0	19.2	2.9	2.2	55.8	16.8	2.6
Lodderomyces elongisporus								
CSIR-Y162	0.6	0	13.9	8.9	5.1	51.1	18.0	2.4

Table 1 (continued)

Strain	Fatty acids[b] 14:0	14:1	16:0	16:1	18:0	18:1	18:2	18:3
Williopsis saturnus								
CSIR-Y140	0.8	0.1	17.4	19.8	0.3	52.0	7.5	2.2
$\bar{X}$	0.4	0.0	14.9	9.9	2.3	55.1	15.0	2.2
Subgroup e (C18:1 > 61%)								
Candida steatolytica								
CSIR-Y535	0.2	0	13.0	2.9	1.5	67.1	15.2	0.2
Rhodotorula mucilaginosa								
CSIR-Y93	0.6	0	12.5	1.0	5.8	75.3	3.9	1.0
$\bar{X}$	0.4	0	12.7	1.9	3.6	71.2	9.5	0.6

a. Values are the mean of three or more repetitions. The standard deviation of the values were about 5% of the mean (range 2-7%).

b. Fatty acids designated as number of carbon atoms:number of double bonds.

CHAPTER 3

THE USE OF VOLATILE METABOLITES IN THE IDENTIFICATION OF YEASTS ASSOCIATED WITH WINE

3.1 INTRODUCTION

The routine identification of yeast strains traditionally relies on the use of a range of morphological, sexual as well as physiological characters (Barnett *et al.*, 1983). The production of esters has been found to have limited application as a diagnostic criterion, although the genus *Brettanomyces* has long been characterised by the characteristic aroma it produces (Lodder, 1970).

However, in these conventional tests, ester production is usually detected organoleptically in a liquid or on a solid medium (Van der Walt, 1970). No quantitative or qualitative determinations of these esters were performed to be used as taxonomic criteria.

Several workers have investigated the production of esters by yeasts in wine and beer with the aid of gas-liquid chromatography (Nordström, 1963a, 1963b, 1964a, 1964b; Webb and Muller, 1972; Daudt and Ough, 1973; Nykänen and Nykänen, 1977; Killian and Ough, 1979; Suomalainen and Lehtonen, 1979; Di Stefano *et al.*, 1981; Soles *et al.*, 1982; Brock *et al.*, 1984; Akhtur *et al.*, 1985; Nykänen, 1986).

It has been shown by Sponholz and Dittrich (1974) that *Pichia (Hansenula) anomala* and *Candida krusei* produced more ethyl acetate than *Saccharomyces cerevisiae*, *Schizosaccharomyces pombe* and *Pichia membranaefaciens*. On the other hand, *P.(H.) anomala* and *C. krusei* form the lowest amount of ethyl esters of octanoic, decanoic and lauric acids. Nykänen and Nykänen (1977) found that strains of *Sacch. cerevisiae* produced more isopentyl acetate, ethyl hexanoate, ethyl octanoate, ethyl decanoate and phenylethyl acetate than *Sacch. uvarum* yeasts.

Nordström (1964b) found that ester production depended on environmental factors which may influence the formation and consumption of acyl-CoA and the ester forming reactions. Ester production was also found to be genetically controlled. Different strains of brewer's yeasts formed different amounts of volatile esters which stresses the genetic character of this metabolic function. Differences were also observed between top yeasts and bottom yeasts, but this is ascribed rather to the fermentation

procedure than to the yeast itself.

In this chapter, the production of volatile metabolites, including esters, by some yeasts associated with the wine industry, was investigated in a study to determine its value to differentiate between yeast species and strains.

3.2 MATERIALS AND METHODS

Strains: The yeast strains analysed, were obtained from the culture collection of the Viticultural and Oenological Research Institute, Stellenbosch, Republic of South Africa and included one strain of *Schizosaccharomyces pombe* (N53) and nine strains of *Saccharomyces cerevisiae* (N6, N59, N66, N76, N81, N87, N88, N91 and N92).

Cultivation of strains: The same cultivation procedure as described in Section 2.2 was followed. In this case, the supernatant was frozen directly after centrifugation and stored at -12°C until analysed.

Extraction apparatus: The methods of Marais and Pool (1979) and Marais (1986) were used in the extraction and analysis of the volatile metabolites. Samples of the defrosted supernatant were extracted by Freon 11 in a continuous extractor (Marais, 1986). The extraction was also performed on the YNBG medium as a control.

Extraction technique:

1) Defrosted supernatant (250 mℓ) was cooled to 0°C prior to extraction to lessen the degree of emulsification at the Freon/sample interface.
2) Internal standards was added to the sample in a 250 mℓ measuring flask:
   0,5 mℓ of a 80 µg/ℓ 2-ethyl hexanol solution
   0,5 mℓ of a 80 µg/ℓ tetradecanol solution
3) Twenty millilitres of Freon 11 was poured into the extraction apparatus and a tuft of sylilated glass wool was placed on the Freon surface. The sample containing the internal standards was then carefully poured into the extraction apparatus.
4) The extraction apparatus was then installed with its bottom immersed 50 mm in ice. The collecting funnel and a condenser, through which water at about -5°C was circulated, were fitted to the extraction unit.
5) A 25 mℓ pear-shaped collecting flask, containing 20 mℓ Freon 11, was fitted to the side arm of the extraction unit and immersed in a waterbath at 35°C.
6) Extraction was carried out for 20 hours at a controlled room temperature of 19°C $\pm$ 1°C.

Concentration of the Freon extract:

1) After 20 hours of extraction, the pear-shaped flask was removed from the extraction apparatus and a Vigreux column (270 x 20 mm) and air condenser (550 x 13 mm) fitted on top to facilitate reflux. The flask was clamped immersed in a waterbath at 35°C and room temperature controlled at 19°C ± 1°C. The Freon was evaporated under partial reflux to a concentrate of approximately 2 mℓ.
2) After concentration, the flask was placed in solid CO_2 to freeze out possible traces of water from the extract. The dry extract was then transferred to a 3 mℓ tapered-tip pear-shaped flask by means of a cooled Pasteur pipette. A small air condenser (220 mm x 8 mm) with a Teflon spiral of 1 x 0,5 mm was fitted onto the flask and concentration was done under partial reflux to approximately 0,1 mℓ.
3) Extracts were stored at -12°C prior to analysis.

Gas chromatographic conditions:

Gas chromatograph	:	Hewlett Packard 5880 with automatic dual integrators
Column	:	50 m x 0,31 mm (i.d.), Carbowax 20M fused silica capillary (Hewlett Packard)
Injection temp.	:	200°C
Detector	:	Flame ionization
Detector temp.	:	250°C
Temperature program:		60°C for 10 min 60°C to 190°C at 1°C/min 190°C for 30 min
Carrier gas	:	Helium
Column flow rate	:	1,5 mℓ/min
Split flow rate	:	120 mℓ/min
Split ratio	:	90:1
Septum purge	:	6 mℓ/min
H_2 flow rate	:	30 mℓ/min
Air flow rate	:	300 mℓ/min
Injection volume	:	1 µℓ
Analysis time	:	170 min

Confirmation of the identity of volatile metabolites by mass-spectrometry:

Identities of volatile substances were indicated in a screening study. The mass spectra and retention times were compared with a library of known standards.

GC-MS conditions were as follows:

GC-MS	:	Finnigan 4600
GC-MS column	:	50 m x 0,32 mm (i.d.), CARBOWAX column
Injection temp.	:	220°C
Interface temp.	:	220°C
Manifold temp.	:	90°C
Temperature program:		60°C for 10 min
		60°C to 180°C at 1°C/min
		180°C for 40 min
Carrier gas	:	Helium
Column flow rate	:	2,2 mℓ/min
Split ratio	:	10:1
Electron energy	:	70 eV
Electron multiplier voltage	:	1200 volts
Scanning rate	:	From 35 to 350 Amu each second with a 0,05 second delay between scans

Calibration of the volatile compounds:

The response factor for the internal standard (fa = 1) was also used for the other volatile metabolites and their concentrations were consequently calculated as relative concentrations. The internal standard calibration method entails the following:

$$\text{Conc. (b)} = \frac{\text{area (b) x factor (b) x concentration (a)}}{\text{area (a) x factor (a)}}$$

where a = internal standard

b = unknown compound

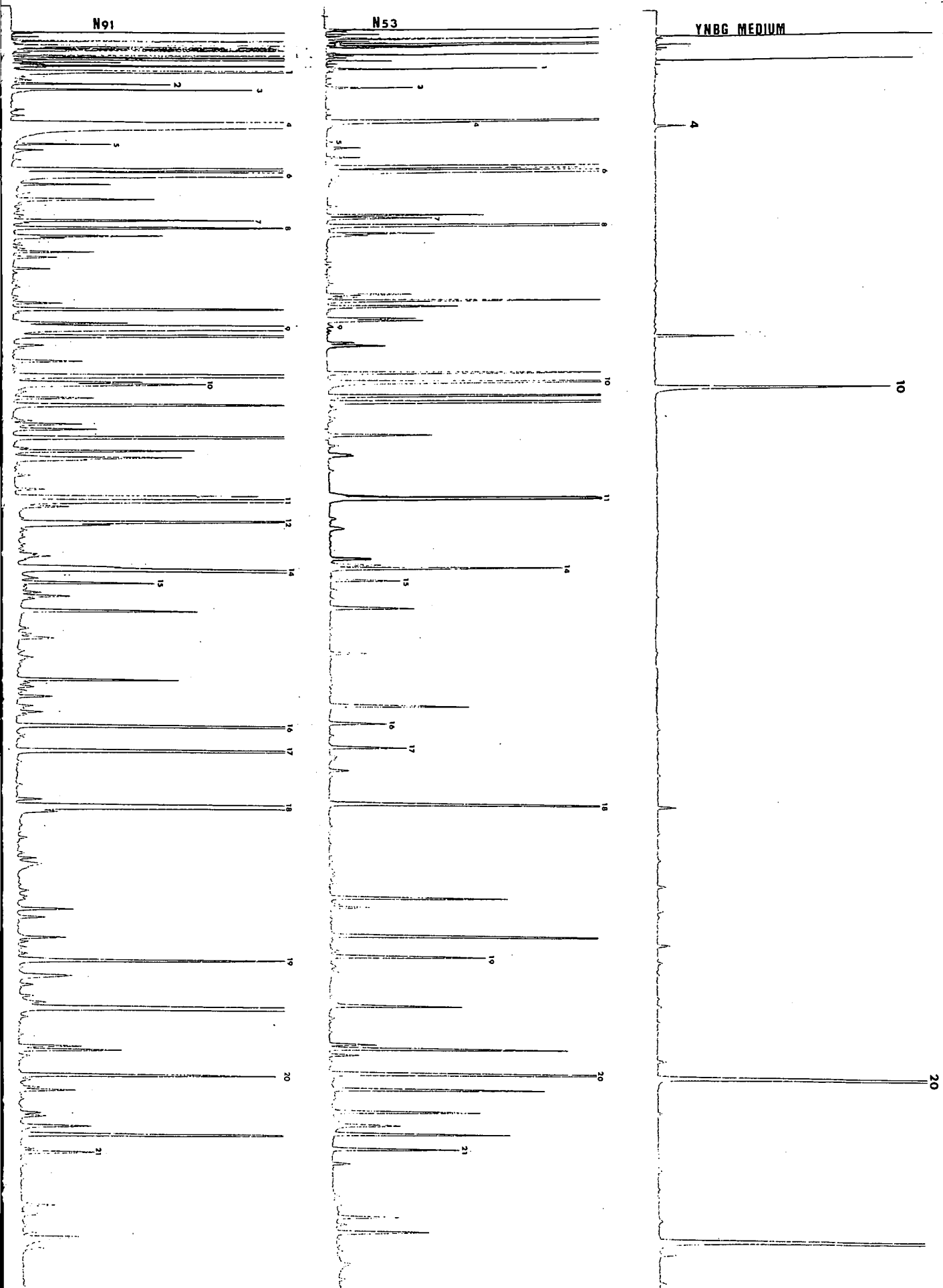
3.3 RESULTS AND DISCUSSION

The results obtained were reproducible (deviation of the mean value less than 10%) and indicate quantitative - as well as qualitative differences between the yeast strains studied (Table 1). The components present in the YNBG medium did not interfere with the identified components, except for a small (± 5 µg/ℓ) amount of isoamyl alcohol that was present (Fig. 1).

Approximately 80 individual peaks were obtained for each of the different *Sacch. cerevisiae* strains analyzed by GC. Only eighteen of these compounds were identified by GC-mass spectrometry (Fig. 1).

Figure 1: Chromatograms of volatile metabolites produced by *Schizosaccharomyces pombe* (N53) and *Saccharomyces cerevisiae* (N91) compared to the control (YNBG medium).

FIG. 1



Schizosaccharomyces pombe N53 is differentiated from the *Sacch. cerevisiae* strains on the basis of the inability to produce isoamyl acetate and n-butyric acid and the production of isobutanol, isoamyl alcohol, ethyl hexanoate, ethyl oxtanoate, γ-butyrolactone, isovaleric acid, 2 phenylethyl acetate, hexanoic acid, 2-phenylethanol and octanoic acid only in small quantities.

Sacch. cerevisiae strain N59 produced the lowest amount of ethyl lactate and the highest amount of octanoic- and decanoic acid. *Sacch. cerevisiae* N6 produced the highest amount of hexanol, while strain N66 formed the lowest quantity of *n*-butanol, hexyl acetate and octanoic acid. *Sacch. cerevisiae* N76 and N81 produced the highest amounts of isoamyl acetate, γ-butyrolactone, isovaleric acid, diethyl succinate and 2-phenylethanol respectively.

Sacch. cerevisiae strain N87 formed the lowest amount of isoamyl acetate while strain N88 produced the highest amount of hexanol and lowest amount of decanoic acid. Strain N91 formed the highest concentrations of isobutanol, n-butanol, isoamyl alcohol, hexyl acetate, ethyl lactate, ethyl octonoate, n-butyric acid, 2-phenylethyl acetate, hexanoic acid and the lowest amount of hexanol. It is interesting to note that this strain could not produce γ-butyrolactone. Finally, *Sacch. cerevisiae* N92 produced the lowest concentration of diethyl succinate.

With the aid of this method, it was possible to differentiate between the 10 strains representing *Sacch. cerevisiae* and *Schizosaccharomyces pombe*.

On the basis of the present findings, differentiation of *Sacch. cerevisiae* strains appears to be possible by analyzing volatile metabolites present in the supernatant of the culture medium. The high sensitivity and speed (less than 5 days) of this technique may certainly complement the long-chain fatty acid method as well as physiological, morphological and serological techniques used in yeast differentiation.

This procedure may prove to be of great value in the differentiation of *Sacch. cerevisiae* strains. It should be noted that this is only a preliminary study. Further work is needed on more wine yeast isolates and more volatile metabolites must be identified to construct an identification system.

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TABLE 1: Some volatile components and concentrations (mg/ℓ) produced by ten wine yeast strains under controlled conditions

Compound	Nr	N53	N59	N6	N66	N76	N81	N87	N88	N91	N92
isobutanol	1	10,0	226,6	313,4	231,1	659,3	91,7	57,9	182	697	39,4
isoamyl acetate	2	-	22,2	59,1	32,7	89,6	11,2	1,5	5,5	19,8	4,3
n-butanol	3	3,6	12,1	7,2	2,6	36,6	9,9	6,1	11,1	40,7	4,1
isoamyl alcohol	4	17,1	2436,4	4234,3	3325,1	5701,9	1965,2	777,8	1892,6	7362,2	745,8
ethyl hexanoate	5	0,5	7,3	15,4	1,4	18,2	12,3	2,3	5,5	17,0	3,2
Hexyl acetate	6	1063,2	1601,0	1326,4	519,1	3785,2	3436,7	887,8	2765,3	7308,3	1753,2
ethyl lactate	7	14,2	6,5	7,5	6,8	12,6	22,3	13,8	16,6	58,4	24,0
hexanol	8	234,1	151,2	606,4	53,8	226,5	227,7	401,7	595,9	109,2	264,5
ethyl octanoate	9	0,3	49,9	55,7	15,9	261,3	240,9	4,7	21,6	844,4	21,7
γ-butyrolactone	11	86,9	258,7	374,5	147,8	904,0	895,7	180,0	410,5	-	370,0
n-butyric acid	12	-	24,8	31,7	13,6	162,5	44,4	4,2	14,8	871,4	4,4
isovaleric acid	14	40,2	130,8	200,1	78,2	536,9	512,2	100,2	216,4	522,1	206,2
diethyl succinate	15	9,7	8,6	15,8	39,8	67,0	43,7	5,8	17,7	38,4	0,9
2-phenylethyl acetate	16	10,1	37,0	63,8	23,4	214,8	177,3	37,5	68,4	237,1	72,6
hexanoic acid	17	11,7	107,6	147,0	21,5	211,2	170,4	63,8	88,1	228,2	62,2
2-phenylethanol	18	63,6	1201,4	2017,0	1977,0	2345,1	3306,2	617,7	983,8	2750,4	1264,2
octanoic acid	19	25,4	478,1	335,4	27,9	76,7	186,8	275,1	68,2	118,1	173,1
decanoic acid	21	11,4	50,6	57,2	13,4	15,7	46,5	35,8	7,8	29,5	26,9

CHAPTER 4

GENERAL DISCUSSIONS AND CONCLUSIONS

The traditional use of morphological, physiological and biochemical features in yeast taxonomy have been the basis with which especially the Delft School of taxonomists classified yeasts into groups of natural taxa.

The weaknesses of this sytem already became apparent by 1954 when Wickerham and Burton (1954) discovered variation in the shape of ascospores within the genus *Pichia* while Scheda and Yarrow (1966) found it difficult to differentiate between species of *Saccharomyces* because of unstable physiological characteristics.

Owing to this apparent instability, new and more stable criteria must be developed in order to differentiate between yeasts. Consequently, the use of long-chain fatty acid composition and volatile metabolite production in the differentiation of some yeasts associated with wine were investigated.

4.1 THE VALUE OF CELLULAR LONG-CHAIN FATTY ACID COMPOSITION IN THE TAXONOMY OF WINE- AND RELATED YEASTS

In this study the use of long-chain fatty compositions in the identification and taxonomy of wine- and related yeasts are discussed.

4.1.1 The use of cellular long-chain fatty acid composition in the identification of yeasts associated with the wine industry

This study deals with the use of fatty acid analyses as a differentiating technique between cultured *Sacch. cerevisiae* and so-called "wild yeasts" in the wine industry. It was possible to differentiate *Sacch. cerevisiae* strains from other yeast species by its unique fatty acid fingerprint (Fig. 1). This result has considerable significance for the wine industry because it allows differentiation from wild yeasts by fatty acid analyses. It was also possible to group the 103 yeast strains representing 38 species into seven distinct groups on the basis of their long-chain fatty acid composition.

With this method it was also possible to differentiate between all the species examined with the exception of *Schizosaccharomyces pombe* and *S. malidevorans*. Differentiation was possible between some strains of the species examined. In relating these data in terms of the practical

Figure 1: Representative chromatograms of the long-chain fatty acid compositions of seven groups of winery-related yeasts.

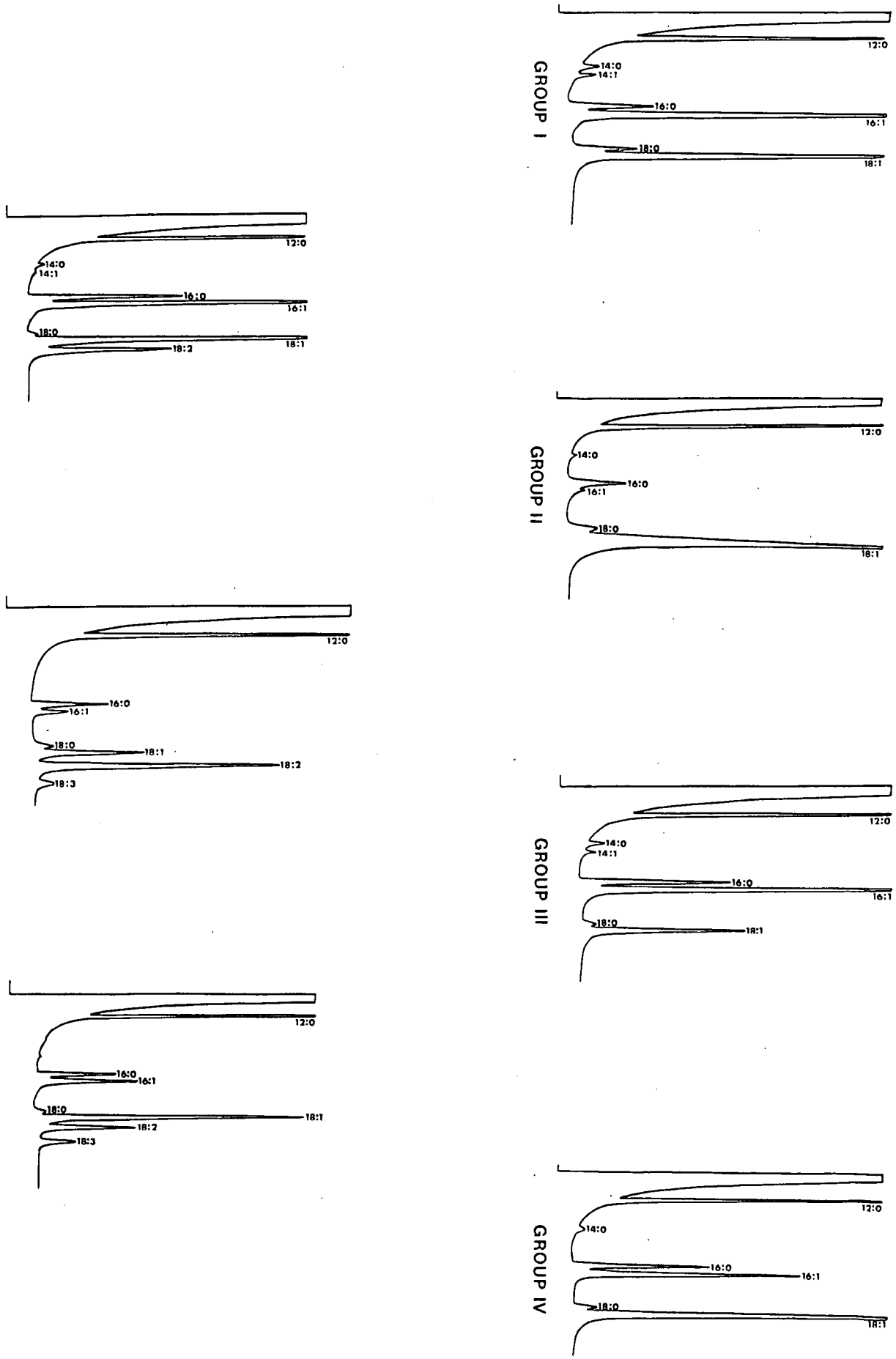
GROUP I
12:0
14:0
14:1
16:0
16:1
18:0
18:1
GROUP II
12:0
14:0
16:0
16:1
18:0
18:1
GROUP III
12:0
14:0
14:1
16:0
16:1
18:0
18:1
GROUP IV
12:0
14:0
16:0
16:1
18:0
18:1
GROUP V
12:0
14:0
14:1
16:0
16:1
18:0
18:1
18:2
GROUP VI
12:0
16:0
16:1
18:0
18:1
18:2
18:3
GROUP VII
12:0
16:0
16:1
18:0
18:1
18:2
18:3

FIG. 1

identification of the yeasts associated with the wine industry, it becomes apparent that long-chain fatty acid profiles may be used for differentiation purposes. While these analyses admittedly underline differences between yeasts, it may also complement the existing taxonomic system. The technique, however, cannot be used to delimit species within a genus, but may be useful as a supporting chemotaxonomical tool.

4.1.2 The value of cellular long-chain fatty acid composition in the taxonomy and phylogeny of some wine- and related yeasts

The long-chain fatty acid compositions obtained for these yeasts (Table 1 - Section 2.3.1) were compared with the organism's ability to form pseudomycelium, carbon compound- and ethylamine utilization and cycloheximide resistance. A general trend was found between the presence of linoleic acid (C18:2) and linolenic acid (C18:3) and the complexity of cell differentiation i.e. the ability to form pseudomycelium and true hyphae.

The different genera studied, were characterized by their own developmental lines with its own characteristic relation of cell differentiation to fatty acid composition.

The value of long-chain fatty acid composition in the phylogeny of *Kluyveromyces* was also investigated. The phylogenetic scheme obtained by comparing long-chain fatty acid composition, genetic recombination, pseudomycelium formation and carbon source- and ethylamine utilization agrees with the proposed phylogenetic scheme (Lodder, 1970). A relation was found between the long-chain fatty acid composition and these phenotypic and genetic characteristics. (Martini *et al.* 1972)

Likewise- in the genus *Saccharomyces*, a correlation was found between long-chain fatty acid composition, cell differentiation, utilization of carbon sources and the resistance to cycloheximide.

It is interesting to note that a relation also exists between DNA relatedness and the similarity in long-chain fatty acid compositions (Table 1).

According to the results presented in Table 1, the following conclusions are drawn:

a) Yeasts with different fatty acid compositions (presence and absence of linoleic and linolenic acid) appear to show low DNA homologies.

b) Similarities in long-chain fatty acid compositions do not necessarily imply a high percentage DNA reassociation (*K. marxianus* var. *lactis* x *K. marxianus* var. *dobzhanskii*).

c) Long-chain fatty acid composition may therefore be used to scan yeasts prior to DNA reassociation studies in order to select possible related yeasts.

4.2 THE USE OF VOLATILE METABOLITES IN THE IDENTIFICATION OF YEASTS ASSOCIATED WITH WINE

In this preliminary study, the feasibility of using the production of volatile metabolites i.e. esters, alcohols and acids as a phenotypic criterion in the identification of *Schizosaccharomyces pombe* and several *Saccharomyces cerevisiae* strains was investigated. The same culture conditions and culture age was used in the fatty acid identification procedure, with the difference that the supernatant was analyzed for volatile metabolites.

With the aid of this method it was possible to differentiate between the strains of *Sacch. cerevisiae* and *S. pombe*. In order to make an overall taxonomic evaluation, it is necessary to analyse more strains and species and identify more volatile metabolites. However, the possibility of including volatile metabolites for taxonomic purposes, should be investigated.

4.3 FUTURE RESEARCH

In conclusion, it is recommended that future research should include the following:

a) The continuation of fatty acid- and volatile metabolite analyses of more strains of wine-associated yeasts and the eventual construction of a data bank for identification purposes.

b) The use of capillary columns and mass spectrometry in order to obtain a more accurate separation and identification of long-chain fatty acids and volatile metabolites.

c) The development of new techniques in order to differentiate between different yeast strains, especially *Sacch. cerevisiae* used in wine fermentations. These techniques may include electrophoresis (including electrophoretic karyotyping), and other chemotaxonomic techniques.

d) The possible application of volatile metabolites to yeast taxonomy and phylogeny in the same manner as is evident with long-chain fatty acid composition.

TABLE 1 Relation between long-chain fatty acid compositions and DNA relatedness

Strains	% DNA reassociation	Representative groups according to long-chain fatty acid composition (Section 2.3.1)
Sacch. cerevisiae x Sacch. kluyveri	- 3% (Martini and Kurtzman, 1985)	Group 1 x Group VII
Sacch. cerevisiae x K. marxianus	0 - 20% (Bicknell and Douglas, 1970; Ouchi et al., 1970)	Group 1 x Group VII
Sacch. cerevisiae x K. wickerhamii	0% (Bicknell and Douglas, 1970)	Group 1 x Presence of C18:2; C18:3 (Cottrell et al., 1985)
Sacch. cerevisiae x Z. rouxii	0 - 11% (Bicknell and Douglas, 1970; Ouchi et al., 1970)	Group 1 x Group V
Sacch. cerevisiae x P. membranae-faciens	12 - 18% (Ouchi et al., 1970)	Group 1 x Group VI
Sacch. cerevisiae x P. anomala	11 - 14% (Ouchi et al., 1970)	Group 1 x Group VI
Sacch. cerevisiae x S. octosporus	11 - 13% (Ouchi et al., 1970)	Group 1 x Group II
K. marxianus var. bulgaricus x K. marxianus var. cicerisporus	117% (Martini and Phaff, 1973)	Both contain C18:2 and C18:3 (Cottrell et al., 1985)
K. marxianus var. fragilis x K. marxianus var. marxianus	93% (Martini and Phaff, 1973)	Both contain C18:2 and C18:3 (Cottrell et al., 1985)

Table 1 (continued)

Strains	% DNA reassociation	Representative groups according to long-chain fatty acid composition (Section 2.3.1)
K. marxianus var. cicerisporus x K. marxianus var. wikenii	103% (Martini and Phaff, 1973)	Both contain C18:2 and C18:3 (Cottrell et al., 1985)
K. marxianus var. drosophilarum x K. marxianus var. phaseolosporus	70% (Martini and Phaff, 1973)	Both contain C18:2 and C18:3 (Cottrell et al., 1985)
K. marxianus var. lactis x K. marxianus var. vanudenii	97% (Martini and Phaff, 1973)	Both contain C18:2 and C18:3 (Cottrell et al., 1985)
K. marxianus var. lactis x K. marxianus var. dobzhanskii	4% (Martini and Phaff, 1973)	Both contain C18:2 and C18:3 (Cottrell et al., 1985)
Torulaspora delbrueckii x Z. microellipsoides	10,7% (Price et al., 1978)	Group II x Group II

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SUMMARY

A) In Chapter 1 the need for a yeast identification system in the wine industry is highlighted. The definition, as well as taxonomic development of the ascomycetous yeasts are discussed as well as the problems encountered.

B) In Chapter 2 the cellular long-chain fatty acid compositions of 103 yeast strains representing 38 species related to the wine industry were determined gaschromatographically. It was possible to differentiate between most species examined as well as between some strains within species. A correlation was observed between long-chain fatty acid composition and complexity of cell differentiation, genetic recombination, carbon source- and ethylamine utilization and resistance to cycloheximide. A phylogenetic scheme for the genus *Kluyveromyces* was constructed on the basis of the abovementioned features.

C) Chapter 3 includes the use of volatile metabolites in the identification of winery-associated yeasts. According to the results it was possible to differentiate between the *Sacch. cerevisiae* and *S. pombe* strains.

D) A Discussion and Conclusions is presented in Chapter 4. This includes a discussion on the identification of wine yeasts and the relation between long-chain fatty acid composition, pseudomycelium formation, genetic recombination, carbon source- and ethylamine utilization and resistance to cycloheximide. A possible relation between the similarity in long-chain fatty acid compositions and DNA homology between yeasts strains is indicated. The use of volatile metabolites in the identification of wine yeasts is also discussed.