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Identification of yeast species by fatty acid profiling as measured by gas-liquid chromatography

Ibrahim El Menyawi, Markus Wögerbauer, Helga Sigmund, Heinz Burgmann*, Wolfgang Graninger

Division of Infectious Diseases, Department of Internal Medicine I, University Hospital of Vienna, Währinger Gürtel 18-20, 1090 Vienna, Austria

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Abstract

An improved rapid method for the identification of yeasts and yeast-like fungi from clinical sources which is based on fatty acid profiles obtained by gas-liquid chromatography (GLC) is described. The fatty acid profile database is based upon internal standardisation and using the relative retention times and the retention index of the analysed fatty acids. Differentiation between yeast species was achieved by the quantitative and qualitative comparison of measured fatty acid profiles with those in the database. A total of 1024 clinical isolates were analysed by GLC to test the validity of the database. 96.2% of all tested samples were identified correctly to the species level by the improved GLC method.

Keywords: Yeast; Fatty acids

1. Introduction

The rising number of severe fungal infections is a major public health concern. The increase is due to the number of immunocompromised patients e.g. AIDS victims, transplantion, cancer patients and patients receiving immunosuppressive therapy. Extensive use of broadspectrum antibiotics, intravenous catheters and drug abuse contributes to the predisposition for yeast infections. *Candida albicans* is the most prevalent yeast species of the normal endogenous microflora and is readily isolated from the skin, oropharynx and gastrointestinal tract of healthy humans [1]. The same fungus detected in human blood samples should be considered significant [2]. Present-day diagnosis is either time consuming and labour intensive or inaccurate [3]. A delayed diagnosis may lead to a considerable increase in financial expenditure for an effective therapy and an increased number of fatalities. Rapid identification of the invading pathogen is of profound clinical relevance, due to the number of rapidly emerging fungal pathogens resistant to antimycotic agents, because it could be decisive for the effectiveness and outcome of the treatment [4–8]. Identification of yeast strains from clinical specimens is routinely performed by time-consuming commercially available systems

*Corresponding author. Tel.: +43-1-40400-4440; fax: +43-1-40400-4418.

E-mail address: heinz.burgmann@akh-wien.ac.at (H. Burgmann)

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such as API ID 32 C (bioMerieux), Vitek Biochemical Card (bioMerieux), BBL Chromagar (Becton Dickinson) or combinations of these. Conventional identification methods such as Gram staining and morphological studies, germ tube tests, enzymatic, fluorogenic as well as carbohydrate assimilation tests [9] are also used. More recently, molecular biological techniques like PCR have attracted attention as diagnostic tools [10]. An ideal identification strategy would combine rapid characterization, accuracy and low work expenditure.

The first step towards this goal was the introduction of the characterization of cellular fatty acids by gas-liquid chromatography for taxonomic purposes. This approach has proved to be successful for the species-specific identification of gram-negative nonfermentative bacteria, anaerobic bacilli and mycobacteria from environmental and clinical sources [11–16]. In comparison to bacteria, yeasts show a relatively limited range of cellular fatty acids. While some authors think that this low variability in the cellular fatty acid pool of fungi provides an insufficient base for chemotaxonomy [17], other groups have shown that species-specific fatty acid profiling of yeasts and yeast-like fungi is useful [18]. Prerequisites for the effective species-specific characterization of clinical yeast samples are: (i) the strict management and standardisation of growth conditions (e.g. growth temperature, media, incubation) since the composition of the cellular fatty acid pool can vary considerably with changes in sample cultivation [19]; (ii) a fatty acid extraction procedure capable of also retaining easily volatile fatty acids; (iii) the construction of a large database containing species-specific fatty acid profiles from clinical samples and (iv) the internal standardisation of fatty acid profiles measured by GLC by including the relative retention time (RRT) and the retention index (RI).

The aim of our study was to improve the species-specific identification of yeasts from clinical samples based upon characterization of cellular fatty acids by gas-liquid chromatography. The establishment of a large fatty acid profile database and the internal standardization of the GLC measurements overcome the limitations of other commercially available GLC-systems.

2. Materials and methods

2.1. Strains

Fatty acids extracted from 1024 clinical samples (University Hospital of Vienna, Austria) and 23 ATCC-strains (American Type of Culture Collection) were analysed by GLC. The samples had been previously identified by conventional cultural and biochemical tests [API (bioMerieux, Marcy l'Etoile, France), BBL CHROMagar (Becton Dickinson, Basel, Switzerland)]. The following ATCC-strains were used:

C. albicans (ATCC 90028, 90029), *C. famata* (ATCC 26418), *C. glabrata* (ATCC 90030, 34138), *C. guilliermondii* (ATCC 20474), *C. kefyr* (ATCC 28838, 34137), *C. krusei* (ATCC 20405, 32545), *C. lambica* (ATCC 28789), *C. lusitaniae* (ATCC 64125), *C. parapsilosis* (ATCC 90018, 28476), *C. tropicalis* (ATCC 20401, 24887), *C. spherica* (ATCC 8632), *C. utilis* (ATCC 28955), *C. valida* (ATCC 36355), *Cryptococcus neoformans* (ATCC 90113), and *Saccharomyces cerevisiae* (ATCC 42130, 42243).

For source of clinical samples (isolated once per proband from the location cited) see Table 1.

2.2. Media and growth conditions

All samples were incubated on Sabouraud dextrose agar (Difco Laboratories, Detroit MI, USA) at 30°C for 24 h and single colonies were then isolated. The isolates were frozen and stored as described [20] until further tests were performed. In order to isolate the cellular fatty acids, the strains were incubated on Sabouraud dextrose agar plates at 30°C, examined visually for growth after 24 h and then identified by their growth rate, colony morphology and biochemical properties (see below).

2.3. Incubation period and growth temperature

For some strains, the 24 h standard incubation period was extended to 48 h to evaluate variations in the cellular fatty acid pool due to the prolonged growth period. A temperature of 30°C was chosen as

Table 1
Strains and samples tested

Species	No. of strains	Source of isolate
C. albicans	525	bronchoalveolar lavage (BAL), throat swab, bronchial and cervical secretion, vaginal swab
C. glabrata	107	stool, bronchial and cervical secretion
C. guilliermondii	9	urine, stool, wound swab
C. pseudotropicalis (kefyr)	28	throat swab, stool
C. krusei	95	stool, sputum, bronchial secretion
C. lusitaniae	24	wound swab, stool, urine
C. parapsilosis	80	stool, bronchial secretion
C. tropicalis	111	throat swab, stool, bronchial secretion
Cryptococcus neoformans	17	kidney transplantation (2 blood and 15 tissues)
Saccharomyces cerevisiae	28	stool, sputum, bronchial secretion

the standard incubation temperature to match the conditions required by API ID 32 C.

2.4. Biochemical identification

The yeast identification system API ID 32 C (bioMerieux, Marcy l'Etoile, France) consists of 32 cupules, each containing a dehydrated carbohydrate substrate for standard assimilation tests. Inoculum suspensions were obtained from mature cultures grown on Sabouraud dextrose agar at 30°C for up to 48 h, according to the manufacturer's instructions [21]. BBL CHROMagar Candida is a differential culture medium for the isolation and identification of clinically important yeast species. Colour changes caused by the formation of chelate complexes of heavy metal ions with some fermentation products are specific for particular yeast species. BBL CHROMagar Candida medium was supplied as ready-for-use dishes by Becton Dickinson (Basel, Switzerland). All strains were cultivated on these plates at 30°C for 24 and 48 h.

2.5. Reagents and reference compounds

Sodium hydroxide, methanol, ether, hydrochloric acid, *n*-hexane and water were HPLC grade (Merck, Darmstadt, Germany). The following fatty acid methyl esters were used as reference substances (all supplied by Sigma Inc., St. Louis, MO, USA): Decanoic ($C_{10:0}$), undecanoic ($C_{11:0}$), 2-hydroxydecanoic (2-OH-$C_{10:0}$), dodecanoic ($C_{12:0}$), tridecanoic ($C_{13:0}$), 2-hydroxydodecanoic (2-OH $C_{12:0}$), 3-hydroxydodecanoic (3-OH-$C_{12:0}$), cis-9-tetradecenoic ($C_{14:1}9$), tetradecanoic ($C_{14:0}$), 13-methyltetradecanoic (iso-$C_{15:0}$), 12-methyltetradecanoic (antiso-$C_{15:0}$), 2-hydroxytetradecanoic (2-OH-$C_{14:0}$), 3-hydroxytetradecanoic (3-OH-$C_{14:0}$), pentadecanoic ($C_{15:0}$), 14-methylpentadecanoic (iso-$C_{16:0}$), cis-9-hexadecenoic ($C_{16:1}9$), hexadecanoic ($C_{16:0}$), 15-methylhexadecanoic (iso-$C_{17:0}$), cis-9,10-methylenehexadecanoic (cis-$C_{17:0}$), heptadecanoic ($C_{17:0}$), 2-hydroxyhexadecanoic (2-OH-$C_{16:0}$), cis-9,12-octadecadienoic ($C_{18:2}9,12$), cis-9-octadecenoic (cis-$C_{18:1}9$), trans-9-octadecenoic (trans-$C_{18:1}9$), cis-11-octadecenoic (cis-$C_{18:1}11$), octadecanoic ($C_{18:0}$), cis-9,10-methyleneoctadecanoic (cis-$C_{19:0}$), nonadecanoic ($C_{19:0}$) and eicosanoic ($C_{20:0}$) acid. 2-hydroxy eicosanoic (2-OH-$C_{20:0}$), 2-hydroxy docosanoic (2-OH-$C_{22:0}$), 2-hydroxy trieicosanoic (2-OH-$C_{23:0}$) and trieicosanoic ($C_{23:0}$) acid.

2.6. Sample preparation

Two 4 mm loops (approx. 80 mg) of yeast colonies were collected from Sabouraud dextrose agar plates and transferred to a glass tube (13×100 mm, fitted with a Teflon lined cap) containing 1 ml of 15% (wt./vol.) NaOH in 50% aqueous solution of methanol and 1 mg trieicosanoic acid ($C_{23:0}$) (internal standard). The suspension was boiled for 30 min at 100°C in a water bath to extract the fatty acids from the cellular lipids and to saponify them to sodium salts. After cooling to room temperature, 2 ml of 6 N hydrochloric acid in methanol was added. The samples were incubated at 80°C for another 10

min. The methylated fatty acids were extracted with ether and hexane. The organic phase was washed in 1 ml of 5% (wt.) NaOH solution to remove any non derivatised fatty acids. After phase separation, the organic layer was transferred to a sealed 1 ml vial and stored at room temperature until gas chromatographic analysis.

2.7. Gas chromatography and standard conditions for the analysis of fatty acids

Cellular fatty acids were analysed as methyl esters using a HP-5890A series II gas chromatograph equipped with a hydrogen flame ionisation detector. The analytical column was prepared with fused capillary silica (HP-Ultra 2, 25 m by 0.22 mm I.D. and 0.33 μm film thickness) containing methylphenyl silicone as the stationary phase. Operating parameters: injector temperature 250°C; detector temperature 300°C; oven temperature rising from 170 to 300°C at 5°C per min and held at 300°C for 5 min before recycling to the initial temperature.

2.8. Qualitative and quantitative analysis of fatty acids

Each fatty acid was identified by the relative retention time (RRT) and the retention index (RI). RRT is the ratio of the retention time of a substance relative to the retention time of a standard. RI characterises the retention behaviour of a substance under standardised GLC conditions. To obtain a linear fit of a homologous series of fatty acids (e.g. saturated fatty acids) the logarithm of the RRT is plotted against the carbon number (CN) of the substance under investigation. The RRTs of 36 reference fatty acid methyl esters from three different homologous series (saturated, unsaturated and hydroxylated fatty acids) were calculated using trieicosanoic acid methyl ester ($C_{23:0}$) as the reference compound. The RI of the fatty acid methyl esters were calculated as described [22]. Using the standardised chromatographic conditions described above, the RRT of unknown fatty acid methyl esters from ATCC-yeast strains were compared to RRT and RI of known fatty acid methyl ester reference substances to calculate the RI of each unknown fatty acid. The identities of the remaining fatty acids were established by gas chromatographic–mass spectrometry (GC–MS). The mean area under the peak and the standard deviation for each fatty acid were calculated for each species. These values were obtained as percentages of the total peak area relative to the peak area of the internal standard ($C_{23:0}$) to eliminate inoculum size variation. This combination of qualitative (RRT, RI) and quantitative information (relative amount of each fatty acid detected) forms the basis of our method.

2.9. Reproducibility of fatty acid analysis

The reproducibility of the chromatographic technique was demonstrated by at least three analyses of each isolate using the standardised growth conditions (see above). The samples were plated on Sabouraud agar dishes and isolated for each new test run. The coefficient of variation calculated as (standard deviation/mean)×100 was determined for each peak in the chromatogram.

2.10. Recovery of fatty acids after methylation

The recovery of the volatile $C_{10:0}$ fatty acid from a lipid matrix that did not contain $C_{10:0}$ acid was determined: 1 mg of $C_{10:0}$ was added to 10 mg lipid. Recovery of $C_{10:0}$ after methylation was calculated relative to the initial concentration of $C_{10:0}$ methyl ester used.

2.11. Correlation and statistical analysis

Fatty acid profiles of reference strains and reliably characterised strains were used to set up the reference database for the automatic identification of yeasts and yeast-like fungi. Identification is based upon the calculation of similarity coefficients of different yeast strains [23]. This is accomplished by comparing the fatty acid profile of the unknown strain to the profiles in the database. A selection of individual strains was analysed by correlation and subsequently by cluster analysis [23–25] to evaluate the ability of this method to group the strains into clear species-related clusters. Several methods have been published for calculating the similarity coefficient of GLC-data [24,26–32]. In this study, multivariant projection components (PC) analysis was

used [33] to detect sample differences. One of these projections was sample-related, and the other was variable-related. The sample-related projection correlated to similarities among the samples, the variable-related projection was used to detect similarities among the variables. The latter also indicated which of the variables contributed to the sample-related projection.

3. Results

Five fatty acids (set A) proved to be sufficient for the successful identification of the following yeast species: *C. guilliermondii, C. krusei, C. lusitaniae, C. parapsilosis, C. tropicalis, C. utilis* and *C. valida* (see Fig. 1a, b). Set A consisted of hexadecanoic acid ($C_{16:0}$), hexadecenoic acid ($C_{16:1}9$), octadecanoic acid ($C_{18:0}$), octadecenoic acid ($C_{18:1}9$) and octadecadienoic acid ($C_{18:2}9,12$). *C. albicans, C. kefyr, C. glabrata,* and *Saccharomyces cerevisiae* could not be discriminated, however, using this set. For their effective differentiation, the spectrum of fatty acids had to be expanded to include set B1 ($C_{10:0}$, $C_{12:0}$) or set B2 ($C_{12:0}$ and $C_{14:0}$). Generally, the fatty acids in sets B1 and B2 tend to only appear in small quantities, however in *S. cerevisiae*, the relative amounts of $C_{10:0}$ and $C_{12:0}$ were 2.5% and 6%, respectively. The relative amounts of $C_{12:0}$ and $C_{14:0}$ in *C. kefyr* were 2% and 5%. The inclusion of set B1 or set B2 for taxonomic purposes proved to be successful and enabled the differentiation of *C. albicans* and *C. kefyr*, and *C. glabrata* and *S. cerevisiae* (see Figs. 2 and 3).

The fatty acid profiles used for setting up the database were obtained from strains that were reliably identified by API ID 32 C (id%>99.9 and $T>=0.75$, according to manufacturer's instructions) [34]. Any strains under investigation that were not identified by API ID 32 C were identified using BBL CHROMagar, Vitek or subsequent GLC-testing. The effectiveness of the GLC method was determined by direct comparison with the results obtained by API ID 32 C (Table 2). All strains correctly identified by API ID 32 C and/or other methods (Gram staining, germ tube test, urease test, colorimetric tests for the enzymes L-proline aminopeptidase and β-galactosaminidase, nitrate assimilation and phenol oxidase test) – taken arbitrarily to be 100% identification – were again analysed using the GLC method (Fig. 4). When set A only was used for characterisation, 98.7% of all *C. albicans* samples were correctly identified. Applying set A and set B1 for identification, 99.4% of the 525 clinical samples of *C. albicans* samples were correctly identified (Table 2). The accuracy of identification could be significantly increased by the inclusion of set B2 for the analysis of *C. glabrata* (set A alone: 87.9% vs. set A+set B2: 96.3%), *C. guilliermondii* (77.8% vs. 100%) and *Saccharomyces cerevisiae* (82.1% vs. 96.4%).

The GLC and API ID 32 C methods gave almost identical results for *C. albicans* (GLC: 99.4% vs. API: 99.6%). GLC analysis proved to be superior to API ID 32 C for the identification of *C. glabrata* (96.3% vs. 90.7%), *C. guilliermondii* (100% vs. 88.9%), *C. kefyr* (96.4% vs. 89.3%), *C.* krusei (94.7% vs. 92.6%), *C. lusitaniae* (100% vs. 91.7%), *C. parapsilosis* (98.8% vs. 95%), *C. tropicalis* (96.4% vs. 81.1%), *Cryptococcus neoformans* (100% vs. 76.5%) and *Saccharomyces cerevisiae* (96.4% vs. 82.1%) (see Table 2).

The amount of $C_{16:0}$ detected is almost constant for all species tested (mean value: 14.45% (wt.)), with the exception of *C. glabrata* and *S. cerevisiae*, which showed amounts varying from 7.4 to 10.6% and 8.8 to 14.1%, respectively (see Table 3). The concentration of $C_{16:1}$ differed in all species, the highest amounts were found in *C. glabrata* and *S. cerevisiae* (45–62%), moderate in *C. albicans*; *C. kefyr*; *C. tropicalis* (7.7–15.7%) and low amounts in *C. krusei* and *C. parapsilosis* (2.8–5.6%). $C_{18:1}$ proved to be a predominant component of the cellular fatty acid pool in the species tested so far and tended to be higher in *C. krusei* (51.3–57.6%) and lower in *S. cerevisiae*. The average amount of $C_{18:0}$ was between 1 and 5.9%. $C_{18:2}$ was common in all species tested and varied from 15.4 to 47.9%. The absence of $C_{18:0}$ and $C_{18:2}$ is characteristic for both *C. glabrata* and *S. cerevisiae*. Tetradecenoic acid ($C_{14:1}$), pentadecenoic acid ($C_{15:1}$), heptadecanoic acid ($C_{17:0}$), 2-hydroxy-hexa-decanoic acid (2-OH-$C_{16:0}$) and 2-hydroxy-octadecanoic acid (2-OH-$C_{18:0}$) were not detectable in all species and, generally, tend to appear only in trace amounts ([19], and Table 3). The detection limit of absolute amounts of individual fatty acids were in the range of 10^{-9} g.

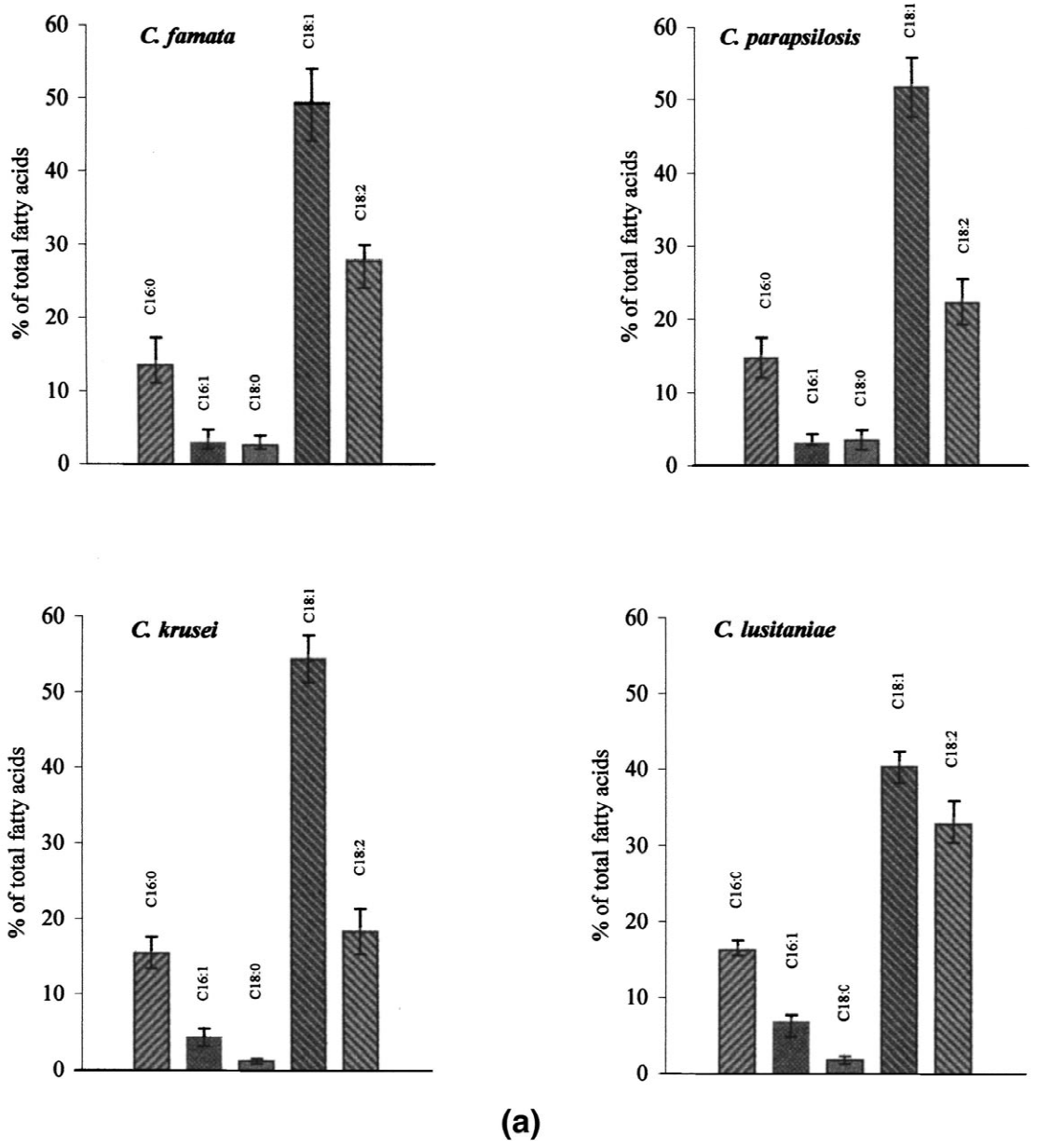


Fig. 1. (a) and (b) Relative amounts of fatty acids (set A) within analysed species.

BBLChromagar showed a good rate of identification for clinical samples of *C. albicans*, *C. glabrata*, *C. krusei* and *C. tropicalis* within 48 h of incubation. For all other isolates, the colour reactions were too ambiguous to be reliable (data not shown).

4. Discussion

Conventional or commercial systems for the identification of yeasts require 24–72 h before the results are known. A yeast identification system with

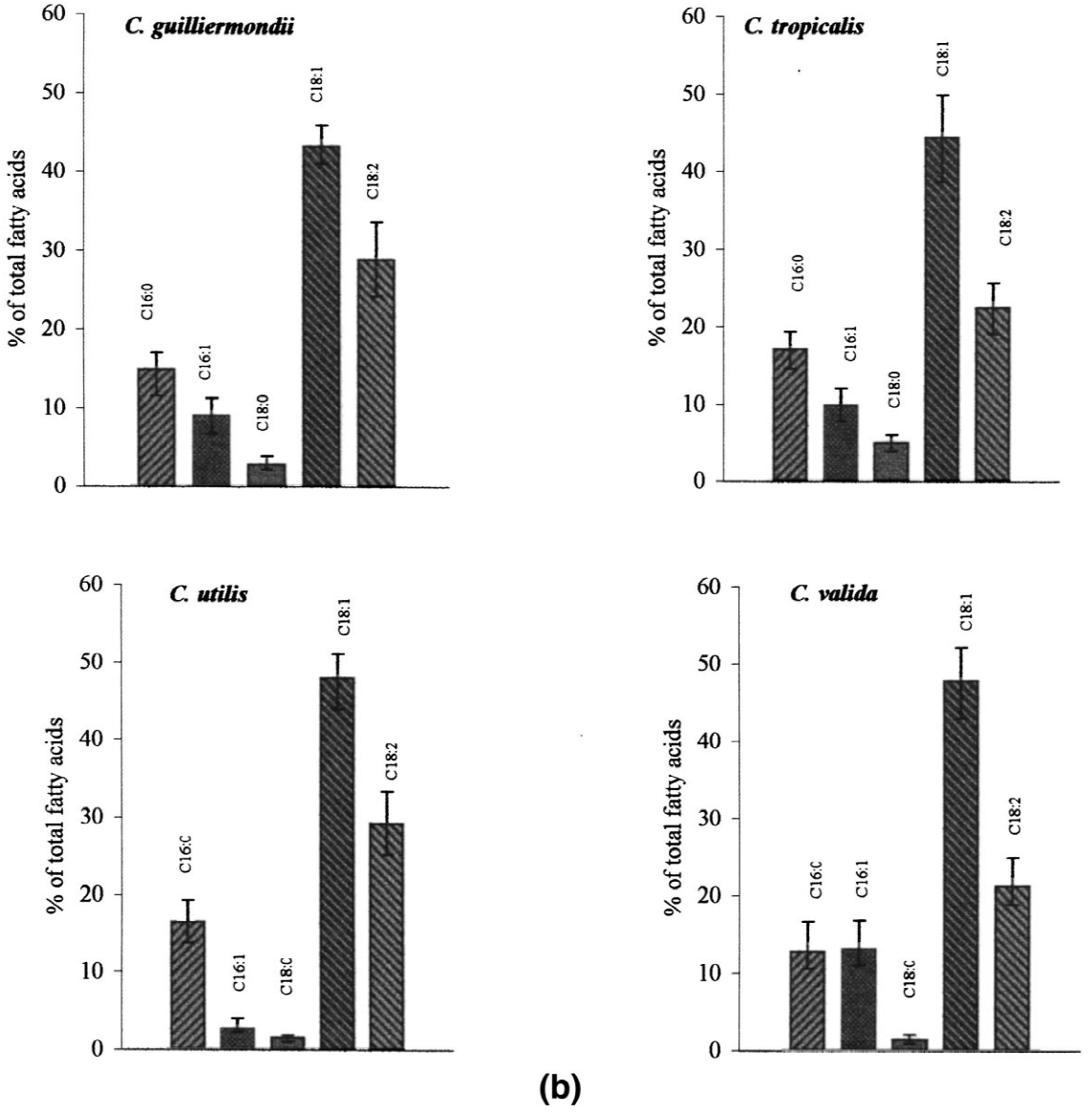
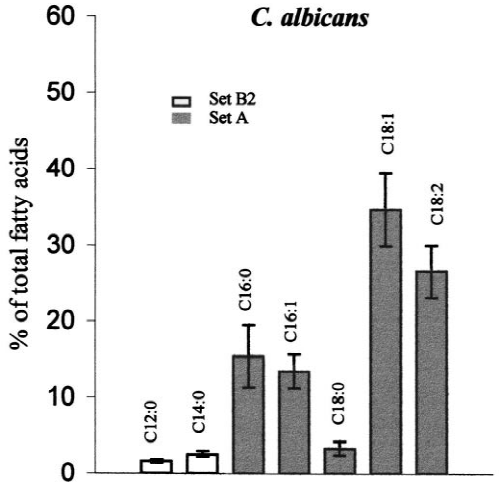


Fig. 1. (*continued*)

the most benefit to the patient would be one that yields results with high selectivity and specificity on the same day that the sample is taken. Analysis of cellular fatty acids has been used extensively over recent years for microbial identification, and now appears to be very promising for the routine identification of clinically significant yeasts and yeast-like fungi, since it combines rapid identification with acceptable accuracy. A commercially available GLC system can identify yeasts within hours of harvest from Sabouraud dextrose agar plates but the results often lack accuracy [3]. This technique for the species-specific identification of clinical yeast samples can be improved by: (i) including the RRT and RI into the analysis of GLC derived fatty acid profiles; (ii) establishment of a large database with



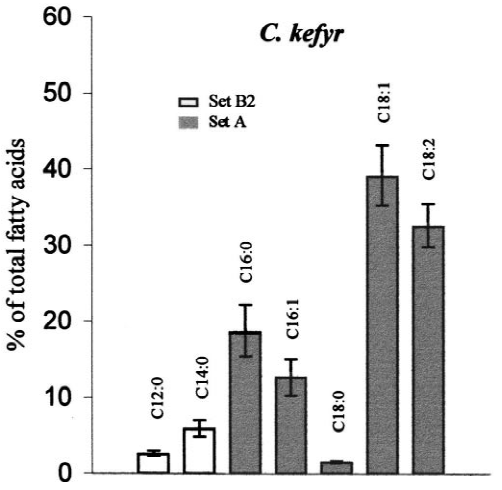
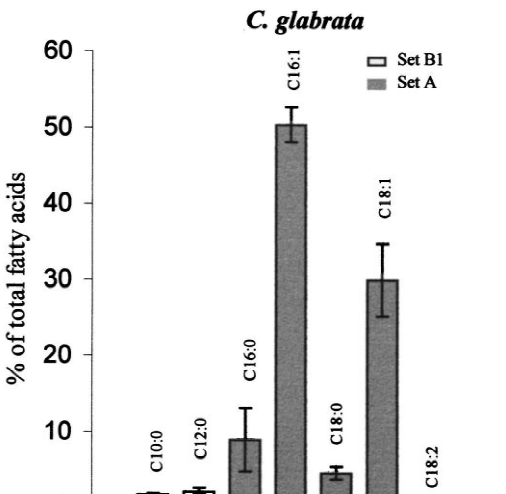


Fig. 2. Differentiation between *C. albicans* and *C. kefyr* by their fatty acid profiles, including set A and set B2 (C12:0, C14:0).



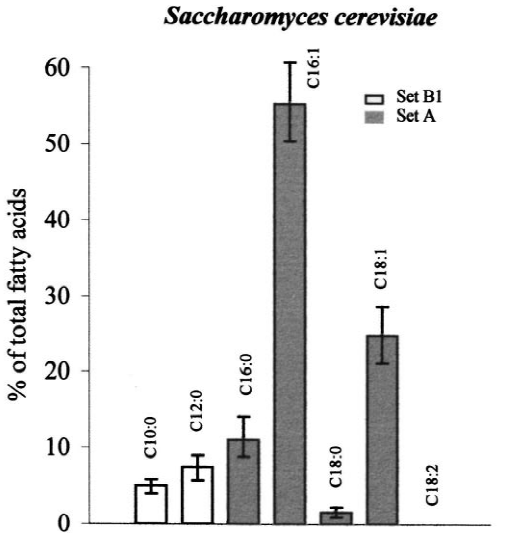


Fig. 3. Differentiation between *C. glabrata* and *S. cerevisiae* by their fatty acid profiles, including set A and set B1 (C10:0, C12:0).

species-specific fatty acid profiles from clinical isolates; and (iii) the exact identification and determination of relative concentrations of each fatty acid measured under standardised GLC conditions. The incorporation of these analysis tools yields an overall species-specific identification rate of 96.2% in the 1024 clinical yeast samples under investigation. Statistical analysis (χ^2-test) showed that the GLC method can perform with excellent accuracy with respect to certain fungal species.

Exact calibration and standardisation of growth conditions are crucial features, since the lipid content can vary significantly due to variation of environmental parameters [19]. Our database contains fatty acid profiles from 461 clinical yeast isolates and 22 ATCC strains grown on Sabouraud dextrose agar for 24 h at 30°C and then identified by API ID 32 C. Although there are databases available which are

Table 2
Accuracy of identification with API ID 32 C and GLC

Species	Tested	Database	No. of strains identified correctly			No. of strains not identified correctly		
			by API 32C (%)	by GLC with set A[a] (%)	set A+B1[b]+B2[c] (%)	by API 32C (%)	by GLC with set A (%)	set A+B1+B2 (%)
C. albicans	525	284	523 (99,6)	518 (98,7)	522 (99,4)	2 (0,4)	7 (1,3)	3 (0,6)
C. glabrata	107	35	97 (90,7)	94 (87,9)	103 (96,3)	10 (9,3)	13 (12,1)	4 (3,7)
C. guilliermondii	9	2	8 (88,9)	7 (77,8)	9 (100)	1 (11,1)	2 (22,2)	0 (0,0)
C. kefyr[d]	28	10	25 (89,3)	26 (92,9)	27 (96,4)	3 (10,7)	2 (7,4)	1 (3,6)
C. krusei	95	30	88 (92,6)	90 (94,7)	90 (94,7)	7 (7,4)	5 (5,3)	5 (5,3)
C. lusitaniae	24	11	22 (91,7)	24 (100)	24 (100)	2 (8,3)	0 (0,0)	0 (0,0)
C. parapsilosis	80	29	76 (95,0)	79 (98,8)	79 (98,8)	4 (5,0)	1 (1,2)	1 (1,2)
C. tropicalis	111	38	90 (81,1)	107 (96,4)	107 (96,4)	21 (18,9)	4 (3,6)	4 (3,6)
Cryptococcus neof.	17	10	13 (76,5)	17 (100)	17 (100)	4 (23,5)	0 (0,0)	0 (0,0)
S. cerevisiae	28	12	23 (82,1)	23 (82,1)	27 (96,4)	5 (17,9)	5 (17,9)	1 (3,6)
Total	1024	461	965	985	1005	59	39	19

[a] Set A contains: C16:0, C16:1, C18:0, C18:1 and C18:2 fatty acids.
[b] Set B1 contains: C10:0, C12:0 fatty acids.
[c] Set B2 contains: C12:0, C14:0 fatty acids.
[d] *C. kefyr = C. pseudotropicalis.*

based on yeasts grown at 28°C or 35°C, we decided to incubate the isolates at 30°C like others [19], because this temperature is also required by API ID 32 C for the identification of the yeast species. This temperature permitted adequate growth for all species tested and sufficient fatty acid recoveries (data not shown).

The identity and relative amount of each fatty acid was established by RRT and RI of 36 saturated, unsaturated and hydroxylated fatty acids. The relative retention time was introduced into the calculation because variations in the retention time of a compound can be significantly influenced by chromatographic conditions. This can easily lead to a wrong identification of some fatty acids when several compounds have very similar retention times. Setting up the database is the most time-consuming step of the whole procedure but has to be performed only once. Afterwards, the procedure can be easily automated. Currently 30 samples can be identified by one GLC system within a day. Prolonging the growth period from 24 h to 48 h did not reduce the accuracy of the species-specific identification. The total lipid amount was not significantly lowered and the ratio of the major fatty acids used for identification remained essentially unchanged.

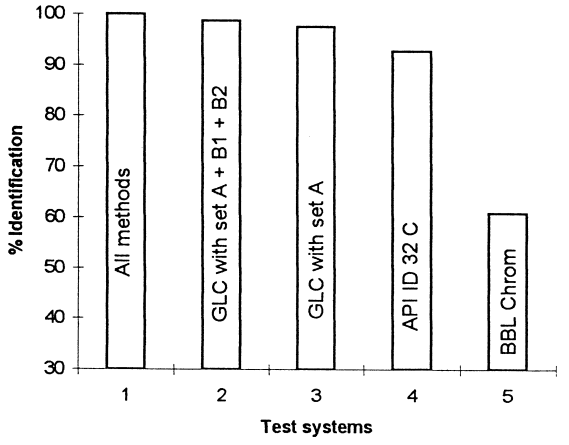


Fig. 4. Comparison between different test systems.

In order to assess the efficiency of identification of yeasts and yeast-like fungi, the recovery of the fatty acids and reproducibility of the procedure must be taken into account. Recovery studies of volatile fatty acids ($C_{10:0}$ and $C_{12:0}$) showed a pronounced reduction in concentration after methanolysis. Methylation, which has been used extensively over recent years for isolation of fatty acids from whole microbial cells, has serious drawbacks with respect to the

Table 3
Distribution of major fatty acids, mean and (range)

Species	$C_{16:0}$	$C_{16:1}$	$C_{18:1}$	$C_{18:2}$	$C_{18:0}$	Others[a]
C. albicans	15.37 (11.26–19.48)	13.43 (11.20–15.66)	34.74 (29.95–39.53)	26.58 (23.13–30.03)	3.31 (2.45–4.17)	3.01
C. famata	13.52 (11.10–17.22)	2.87 (1.94–4.72)	49.31 (44.21–54.11)	27.89 (24.15–29.95)	2.60 (2.00–3.90)	2.40
C. glabrata	8.99 (7.41–10.57)	50.45 (45.58–55.33)	30.01 (27.05–32.97)	ND[2]	4.69 (3.74–5.98)	2.58
C. guilliermondii	14.82 (11.38–16.97)	8.90 (6.52–11.24)	43.09 (40.94–45.89)	28.77 (24.10–33.67)	2.65 (1.94–3.87)	2.83
C. pseudotropicalis	17.49 (14.36–20.62)	12.77 (10.60–14.94)	34.67 (30.69–38.65)	28.83 (24.70–32.96)	1.94 (1.60–2.28)	8.29
C. krusei	15.52 (13.40–17.67)	4.29 (3.14–5.55)	54.46 (51.34–57.58)	18.39 (15.40–21.38)	1.26 (0.88–1.64)	3.97
C. lambica	12.85 (9.95–15.95)	18.43 (15.18–22.63)	37.15 (33.94–40.45)	26.08 (23.20–29.72)	0.95 (0.65–1.40)	4.66
C. lusitaniae	16.31 (15.58–17.61)	6.87 (4.88–7.73)	40.45 (38.35–42.41)	32.95 (30.41–35.91)	1.87 (1.24–2.35)	1.92
C. parapsilosis	14.78 (12.03–17.53)	3.06 (2.84–4.28)	51.84 (47.81–55.87)	22.45 (19.34–25.56)	3.52 (2.15–4.89)	2.63
C. tropicalis	16.95 (14.45–19.39)	9.80 (7.67–11.93)	44.22 (38.59–49.85)	22.32 (18.95–25.69)	4.86 (3.74–5.98)	2.92
C. sphaerica	13.75 (10.95–16.90)	18.50 (14.55–23.62)	29.95 (27.06–33.07)	31.53 (27.40–34.83)	1.45 (1.10–1.90)	3.80
C. utilis	16.40 (13.73–19.36)	2.65 (2.15–4.07)	47.85 (43.83–51.16)	29.08 (25.10–33.70)	1.46 (0.90–1.80)	1.88
C. valida	12.70 (10.35–16.64)	13.01 (10.76–16.79)	47.70 (42.78–52.23)	21.18 (18.82–25.05)	1.29 (0.75–1.90)	3.30
Cryptococcus neoformans	15.27 (11.35–19.02)	ND[b]	37.50 (34.35–40.03)	41.80 (36.60–47.90)	4.50 (2.55–6.28)	1.90
Saccharomyces cerevisiae	11.07 (8.86–14.16)	55.31 (50.46–62.01)	24.92 (21.16–28.68)	ND[b]	1.58 (0.95–2.23)	6.48
Trichosporon spp.	15.42 (12.07–18.91)	5.18 (3.49–6.98)	34.37 (31.17–38.57)	36.75 (31.85–41.73)	2.10 (1.56–3.00)	3.41
Aspergillus fumigatus	13.58 (12.35–14.82)	0.54 (0.39–0.62)	31.89 (29.74–34.44)	44.18 (41.13–47.36)	2.70 (2.15–3.37)	6.69

[a] Most analyses show presence of C10:0, C12:0, C14:0, C14:1, C17:0, C17:1 fatty acids.
[b] ND: Not detected.

analysis of volatile esters of $C_{10:0}$, $C_{12:0}$ or shorter fatty acids, because these substances can be easily lost during reaction. These volatile fatty acids are reported to appear only occasionally and in trace amounts [19] but there are also reports indicating a more frequent occurrence [18]. We believe that these inconsistencies are mainly due to technical inadequacies when performing methanolysis, because we were able to extract these volatile fatty acids reproducibly in sufficient quantities for analysis. In contrast to other reports [18] we found that $C_{10:0}$ and $C_{12:0}$ play a decisive role in discriminating between *S. cerevisiae* and *C. glabrata*. $C_{12:0}$ and $C_{14:0}$ are crucial for the differentiation between *C. albicans* and *C. kefyr*. It has been recommended that volatile fatty acids should not be transferred by methanolysis, but should be analysed separately after extraction into hexane [35,36]. A direct comparison of recovery rates after alcoholysis in methanol, ethanol, propanol and butanol showed, in agreement with other reports [18], that 15–20% of $C_{12:0}$ disappears during evaporation of methanolic HCl and concentration of acid. Under the extreme conditions for esterification (e.g. boiling for 1 h or more) 40–50% of $C_{10:0}$ is lost. Esters of $C_{14:0}$–$C_{24:0}$ acids are easily recovered and analysed efficiently by gas chromatography. Our GLC measurements of overall fatty acid recoveries gave results comparable to those of other studies [18].

A major drawback of the commercial GLC system (Microbial Identification System, Newark, DE, USA) is its inability to discriminate between *S. cerevisiae* and *C. glabrata*, or between *C. tropicalis* and *C. lusitaniae* [3]. We believe this to be due to an intrinsic limitation in its capabilities to discriminate correctly between similar fatty acids. This system also shows limitations in identifying $C_{10:0}$ and $C_{12:0}$ because it can only detect trace amounts of these compounds, which is useless for fatty acid profile analysis [18]. In addition, internal standardisation is not used, which may be the major reason for inconsistencies in the identification of similar compounds [3]. We have no problems in discriminating between *C. tropicalis* and *C. lusitaniae*, because we analyse the fatty acid pool much more accurately due to the incorporation of RRT and RI. Also, we are able to differentiate between *S. cerevisiae* and *C. glabrata* by including volatile fatty acids in the analysis. We have found that when some additional fatty acids ($C_{10:0}$, $C_{12:0}$ and $C_{14:0}$) are correctly detected and included in the analysis, the fatty acid profiles of the four species mentioned above become more distinct and enable adequate identification as shown in Fig. 2 and 3.

We found that for detection of a yeast pathogen – especially with blood, liquor or bronchoalveolar specimens – only one sample was indicative. Repeated testing of different specimens from the same patient collected at different time intervals (after 1 day, 7 days and 2 weeks) only confirmed the initial diagnosis. Species-specific characterisation of the infectious agent was established with the first analysed sample from the patient or inconclusive throughout the test period. Species-specific identification was not improved by repeated testing or by testing specimens obtained from different locations or taken at different time intervals from the same location (data not shown). Thus, species-specific identification and diagnosis of the infection may be facilitated by a single positive sample by the improved GLC-based method. However we recommend to perform an equal number of GLC analyses usually applied in diagnostic laboratories with conventional methods for confirming a fungal infection.

Although the specimens collected from the patients also have to be plated onto Sabouraud dextrose agar plates and incubated for 24 h – as it is required by conventional fungal detection methods – our method still leads to a considerable time saving. Whereas API ID 32 C and BBLChromagar consume an additional 24–48 h after plating for species-specific identification of yeast samples our GLC method yields results on the same day. Therefore the minimal time consumptions for each method from collection of the specimen to species-specific identification are: API ID 32 C: 48 h; BBLChromagar: >48 h; improved GLC-method: 28 h. We save approximately 20 h at least compared with the methods mentioned above. Time consumption of the GLC method is comparable with PCR approaches, which rely on agar plating for sample purification. We expect PCRs applied directly onto crude specimens to suffer from false positive results or inhibition, but we actually did not perform comparisons with PCR in our laboratory.

We have shown that our GLC-based method is an improvement in accuracy compared with the API ID 32 C system. Interpretation of the assimilation tests of API ID 32 C is based upon a numerical identification system, which may lead to identification of the sample at the genus level with high accuracy, but discrimination at the species level is only poorly performed or fails completely with some strains [37]. In these cases, GLC-based analysis of fatty acid profiles significantly increases the confidence-level for species-specific identification.

Our study evaluated the effectiveness of gas chromatography for the routine identification of clinically relevant yeast species and as an improvement on standard methods currently in use in clinical mycology laboratories. We have demonstrated that our strategy – after the establishment of a large database containing fatty acid profiles from yeasts – is fast and accurate. The method overcomes the time-consuming steps necessary for API ID 32 C, appears to be superior in species-specific identification and can be easily automated.

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