constant weight.

RESULTS AND DISCUSSION

The oil values determined by the oil signal and the solid-liquid signal methods, moisture content, and F-factor for each sun-dried sample of mustard, sunflower, and soybean have been given in Table I. The mean F-factors of these crops for their sun-dried seeds are 3.22, 2.87 and 2.98, respectively. The correlation in oil values determined by the two methods is 98.8% for mustard, 94.5% for sunflower, and 93.1% for soybean. It might be possible to improve the correlations for sunflower and soybean which have much larger seeds than mustard by analyzing larger samples of ca. 5 g in weight which would provide a more representative sample. This could be achieved by using an NMR probe head with a larger volume over which the RF and the magnetic fields are constant.

In the present experiment, the requirement of the representative sample has been fully met for mustard crop by using ca. 2.5 g seeds. The agreement between the oil values obtained by the two methods is very good for mustard, which means that the solid phase T_2, moisture content, and proton density difference between the two phases of the seeds do not change significantly from sample to sample. The good agreement between the methods shows that it is possible to determine oil in seeds by pulsed NMR without weighing sun-dried seeds in plant breeding.

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Preparation of Heptadecenoic Acid from *Candida tropicallis* Yeast

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ABSTRACT

In this paper a method is described for preparing 10 g or more of heptadecenoic acid (C17:1ω8) of 99 p.100 purity from *Candida tripicallis* yeast. Three cycles of treatment, based on crystallization techniques, were used successively: (1) Crystallization of fatty acids (in free form) from acetone at -25 C induced the elimination of most of the saturated fatty acids, and at -60 C, of all of the polyunsaturated acids. (2) Inclusion formation of fatty acids (as methyl esters) with urea at hC induced the removal of all of the remaining saturated methyl esters and most of methyl oleate. (3) Crystallization of fatty acid methyl esters from acetone at -60 C removed almost all the remaining monounsaturated methyl esters (methyl palmitoleate and methyl oleate). Total efficiency of the preparation was about 17 p.100.

INTRODUCTION

Thorough studies of lipid metabolism involve qualitative and quantitative analyses of lipid classes and their fatty acids in biological samples. Among the different dosage methods, the addition of internal standards (free or esterified fatty acids) in the samples has the following advantages: sensitivity, specificity, and efficiency (simultaneous quantification of several kinds of lipids).

Among all the natural fatty acids readily available, heptadecenoic acid (C17:1ω8) is the one that best possesses all the features which an internal standard should have: scarcity in most animal tissues, length of the carbon chain close to that of the main fatty acids in the biological samples, and easy usage in organic synthesis of esters (triglycerides, phospholipids, and cholesterol esters). Grown on hydrocarbons, *Candida tropicallis* yeast are rich in this acid (27.9 p.100 of total fatty acids). They are therefore an excellent biological source for preparing highly pure heptadecenoic acid.

Fractional distillation, often used in the past, could alter the structure of the fatty acid. Zhukov and Vereshchagin (1) avoided this problem and used preparative gas chromatography. Nevertheless, the authors worked out only some hundred milligrams of pure heptadecenoic acid, while a specialized, expensive apparatus would have been required for the preparation of several grams of this acid needed for our studies.

It is possible to separate great amounts of fatty acids into classes in a single step according to the degree of unsaturation with a simple liquid chromatography device. Florisil columns or ion exchange resins columns impregnated with silver nitrate have been used respectively by Anderson and Hollenbach (2) and Willner (3), and Wuster et al. (4) and Emken et al. (5). This technique, whose various uses and limits have been described by Morris (6), has been applied particularly to purify cis-olefins from trans-isomers by De Vries (7) and Emken et al. (5). It is, however, expensive, and its use is especially valuable for the purification of the polyunsaturated fatty acids.

Fractional crystallization techniques, based on the differences in physical properties of fatty acids, allow a greater field of purification possibilities. They usually do not cause any damage to fatty acids, are cheap and allow great quantities of pure fatty acids to be extracted. Brown (8) and Brown and Kolb (9), on the one hand, described the potentially numerous applications of low temperature fractional crystallization from solvents, and Schlenk (10) and Iverson and Weik (11), on the other hand, showed the advantages of the formation of inclusion complexes with urea. In both cases, the purification of a given fatty acid can be obtained only after several successive steps in the same treatment. Similarly, several authors have worked out methods of preparing highly pure fatty acids (99 p.100 pure or more) based on the use of either one of these two techniques or a combination of both. The raw sources used have been from either vegetable or animal origin: purification of oleic acid from olive oil by Swern and Parker (12), Keppler et al. (13), Rubin and Paisley (14), Fremont and Gozzelino (15), purification of linoleic acid from safflower oil by Brown (8) and Keppler et al. (13), or from corn oil by Schlenk and Holman

(16); purification of arachidonic acid from pork liver by Privett et al. (17), purification of penta and hexaenoic acids from tuna oil by a combination of fractional crystallization and preparative gas chromatography by Stoffel and Ahrens (18), or from menhaden oil by a combination of methods of fractionation and liquid-liquid partition chromatography by Privett and Nickell (19).

The aim of the present work is to describe a method of preparing large quantities (17 g) of heptadecenoic acid (C17:1ω8) from the total lipids of *Candida tropicallis* yeast grown on hydrocarbons. This method is based on a combined use of low temperature fractional crystallization from acetone, on the one hand (free fatty acids or methyl esters), and fractional inclusion complex formation with urea, on the other hand (fatty acids as methyl esters).

MATERIALS AND METHODS

Preparation of Fatty Acids and Methyl Esters

Total lipids were extracted as described by Folch et al. (20), from four samples of 1 kg of *Candida tropicallis* yeast, collected after growth on a mixture of C13 to C18 hydrocarbons. They were saponified by a 10 p.100 solution of alcoholic KOH overnight at 20 C. After extracting unsaponifiable materials with hexane, fatty acids were released by the addition of HCl 6N and were extracted with hexane. They were washed with distilled water until neutral and dried over pure anhydrous sodium sulfate. Fatty acids were methylated by boiling and refluxing the solvent for 30 min with 3 p.100 methanol HCl.

Fatty Acids Crystallization

*From acetone.*The following concentrations were used: 5 p.100 for fatty acids in free form and 10 p. 100 for fatty acids as methyl esters. The solutions were kept at -25 C or -60 C in an ultra Kryostat (UK 80 DW Lauda) in a methanolic bath, for 6 hr, so crystallization could take place. Crystals (C) were separated from the filtrate (F) by filtration under vacuum with a sintered glass funnel n° 0 previously chilled at -25 C. Crystals gathered on the funnel were dissolved in chloroform. Fatty acids or their methyl esters were then recovered from the crystals or the filtrate after the solvent had been withdrawn by evaporation under vacuum.

With urea and methanol. The crystallization of fatty acids (as methyl esters) with urea was generally carried out under the following conditions: (a) 1 part (g) of methyl esters, (b) 10 volumes (ml) of methanol, and (c) 1 part (g) of urea.

Nevertheless, when small amounts of saturated fatty acids were present in the mixture, the quantity of urea was increased two- or threefold. Methyl esters were rendered soluble in the methanol in a glass Pyrex flask, and urea was then added to the solution. Its complete dissolution was obtained by heating the solution with the help of an electric hot plate. Afterwards, the solution was allowed to cool at room temperature and then transferred to a cold room at 4 C overnight. It was filtered under mild vacuum through a sintered glass funnel n° 0 kept at 4 C.

Methyl esters were systematically reextracted from filtrates and crystals. First of all, methanol was removed by vacuum evaporation, and the urea of the remainder was solubilized in a boiling 1 p.100 HCl solution. Methyl esters were extracted with hexane and weighed, and their composition was studied by gas chromatography.

Analytical Techniques

The C17:1ω8 content of every successive fraction was determined by gas chromatography of their methyl esters on an open tubular column of stainless steel impregnated with Carbowax 20M-terephtalic acid (100 m long and 0.5 mm in diameter). The device consisted of a Packard Type 427 chromatograph fitted with a FID detector and connected to an electronic integrator LTT Icap 5 and a Houston Omniscribe potentiometric recorder. The following procedure was adopted: oven temperature, 190 C; detector and injector temperature, 240 C. The carrier gas was nitrogen "u" filtered on an O_2 and H_2 absorber Oxysorb (Airgaz). Its flow rate was 0.35 ml/mn corresponding to, in our operative conditions, a pressure of 0.4 bar. Fatty acid identifications were obtained by calculating their corresponding equivalent chain length (ECL) values from retention times supplied by the electronic integrator and then, by comparing these values either to those of ECL tables from Flanzy et al. (21) or to those worked out in our laboratory from standard commercial fatty acids (Nu Chek Prep) under similar operative conditions. The structure of the last purified fraction of methyl heptadecenoic (fraction C_7*****) was also checked by the analysis of its infrared spectrum, supplied with an infrared Beckman model 420 spectrophotometer with a solvent-free compound in a 0.1 mm cell. The purity control was determined by recording the nuclear magnetic resonance (NMR) spectrum with a deuterochloroform solution on a Varian T60 spectrometer operating at 60 MHZ.

RESULTS AND DISCUSSION

The total lipid composition (13.8 p.100 of dry matter) of *Candida tropicallis* yeast is indicated in Table I. Their phospholipid content was 70 p.100, which explained their low fatty acid content (8.9 p.100). The fatty acids composition of both classes of lipid yeast (phospholipids and triglycerides, Table I) was determined. Since they were very similar, there was no need to separate the two classes. Extraction yielded 346 g fatty acids from 4 kg of yeast. According to its composition, the initial mixture included 96.5 g heptadecenoic acid (C17:1ω8). Apart from this acid (27.9 p.100), total lipids included 45.7 p.100

TABLE I

Extraction of Total Fatty Acids of *Candida tropicallis* Yeast: Some Characteristics of the Fractions at the Main Steps of Preparation

Fractions	Centesimal composition	Weight (g)
Yeast	—	4000
Yeast (dry matter, p.100 of fresh weight)	96.9	
Total lipids (p.100 of dry matter)	13.8	
phospholipids (p.100 of total lipids)	70	
triglycerides (p.100 of total lipids)	30	
Fatty acids (p.100 of dry matter)	8.9	346

saturated fatty acids, 11.2 p.100 polyunsaturated fatty acids (C17:2ω5, C18:2ω6, C18:3ω3), and 15.2 p.100 monounsaturated fatty acids of which 8.3 p.100 were hexadecenoic acid (C16:1ω7) and 6.6 p.100 were oleic acid (C18:1ω9).

Purification required 21 successive steps in three cycles. (The detailed fatty acids composition of every successive crystallization fraction is available upon request.)

The first cycle allowed saturated and polyunsaturated fatty acids to be removed by fractional crystallization from acetone as free fatty acids (Fig. 1). Most of the saturated fatty acids (84 p.100) were removed in the crystals in a single step at -25 C. Elimination of the remaining saturated fatty acids could not be obtained by a second and similar treatment. Assays thus showed that their low content in the mixture F_1 (9.4 p.100) seemed to modify their solubility features in acetone at -25 C, which eliminated any possibility of their selective crystallization. This could be obtained by the formation of urea inclusion complexes, but the methylation of fatty acids would be required. For efficiency, we chose to delay this treatment stage and to maintain the same operating conditions as before until the technique proved unnecessary; thus, most of polyunsaturated fatty acids (97 p.100) were then removed in the filtrate in four successive steps at the temperature of -60 C (Fig. 1). This treatment induced the loss of 46 p.100 of C17:1ω8 but also of 86 and 51 p.100 of C16:1ω7 and C18:1ω9, respectively (Fig. 4). At this stage of purification (fraction C_2***), the composition of the purified mixture (84 g) was 64.5 p.100 of C17:1ω8 (54.2 g), 5.1 p.100 of C16:1ω7, and 13.8 p.100 of C18:1ω9.

The second treatment cycle was aimed at removing all remaining saturated fatty acids as well as most of the C18:1ω9. It was obtained by the formation of urea inclusion complexes of fatty acids (as methyl esters) at 4 C (Fig. 2). First of all, operative conditions were established in order to extract from the crystals 20 and then 40 p.100 of the fatty acid methyl esters. This induced the elimination of saturated fatty acids but also the loss of too much C17:1ω8 (21.8 g). So secondly, fatty acid methyl esters from the crystals (fractions C_3 and C_4) were rehandled under more intense conditions (inclusion of 85 p.100 of fatty acid methyl esters in the crystals) in order to keep a large part of C17:1ω8 (11 g) in the filtrate. At this stage of purification (fraction F_4), the purified mixture (53.3 g) was still loaded with 9.5 p.100 of C18:1ω9. It was thus necessary to continue extracting this acid in the crystals, which was carried out in a four-step procedure under intense conditions (extraction of 70 to 85 p.100 of fatty acid methyl esters in the crystals). When this second cycle of treatment was over, the purified mixture of fatty acid methyl esters (fraction F_5) contained 88.3 p.100 of C17:1ω8 (31.3 g), 7.2 p.100 of C16:1ω7, and 3.0 p.100 of C18:1ω9 (Fig. 4).

The third cycle of treatment was aimed at removing almost all remaining monounsaturated acids (C16:1ω7 and C18:1ω9) as fatty acid methyl esters by fractional crystallization from acetone at -60 C (Fig. 3). The operative conditions were first calculated to crystallize about 70 p. 100 of all fatty acid methyl esters (fraction C_6) which led to a decrease down to 4.1 and 2.1 p. 100, respectively in the C16:1ω7 and C18:1ω9 content, but also a loss of 7g of C17:1ω8 m in the filtrate (fraction F_6). Therefore, the filtrate was rehandled under the same conditions which allowed 4.3 g of C17:1ω8 to be extracted in the crystals. In order to complete the purification of C17:1ω8, crystals were grouped together (fraction C_6) and treated under conditions (10 p.100 solution of fatty acid methyl esters in acetone) which led to the crystallization of 90 p.100 of fatty acid

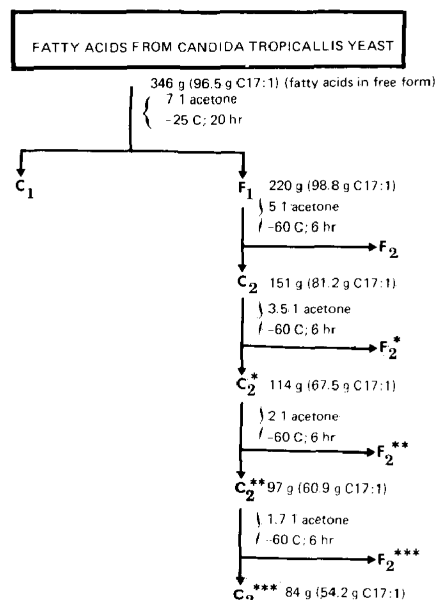

FIG. 1. Preparation of heptadecenoic acid (fatty acids in free form). Elimination of the main part of the saturated fatty acids by crystallization from acetone at -25 C. Elimination of the totality of the polyunsaturated fatty acids from acetone at -60 C. C = crystals; F = filtrate.

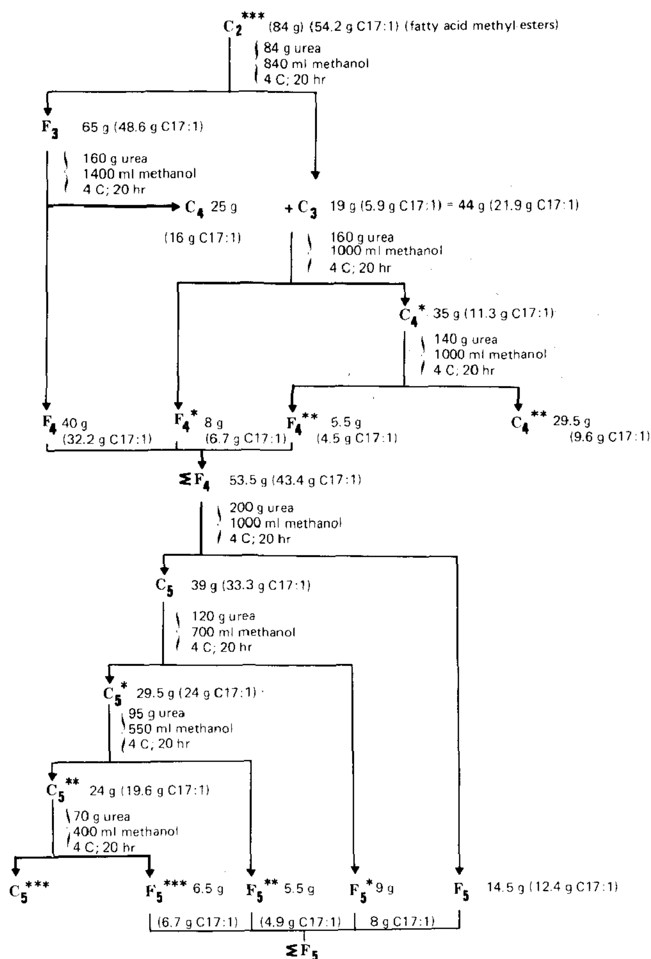

FIG. 2. Preparation of heptadecenoic acid (fatty acids as methyl esters). Elimination of the totality of saturated fatty acids and the main part of oleic acid by crystallization from urea and methanol at 4 C.

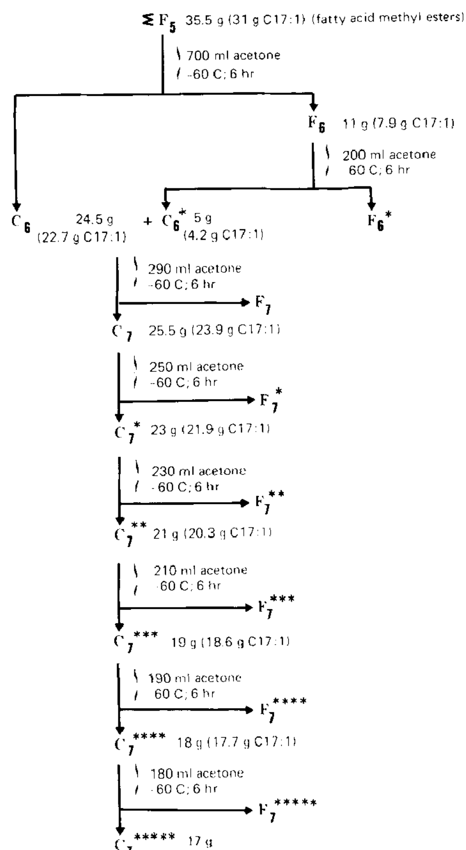

FIG. 3. **Preparation of heptadecenoic acid (fatty acids as methyl esters). Elimination of most of the monounsaturated fatty acids (palmitoleic and oleic acids) from acetone at -60 C.**

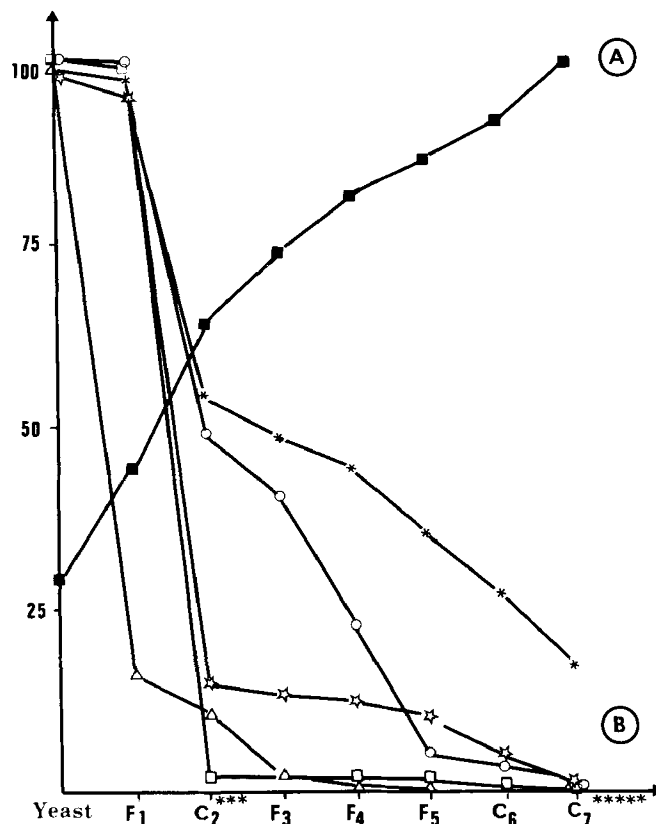

FIG. 4. **(A) Heptadecenoic acid enrichment in the purified samples (p.100, ■); (B) fractional recovery (p.100 from original weight) of main fatty acids at different crystallization phases. * = heptadecenoic acid (C17:1ω8); △ = saturated fatty acids; □ = polyunsaturated fatty acids; ○ = oleic acid; ☆ = palmitoleic acid.**

methyl esters. These conditions restricted the relative crystallization of C16:1ω7 and C18:1ω9 as much as possible, but six successive steps of the same treatment were necessary (Fig. 4). The C17:1ω8 purification ended at stage C_7***** (17 g) with a purity of 99 p.100. The total efficiency of the preparation was about 17 p.100.

Molecular structure of pure methyl heptadecenoate (fraction C_7*****) was then studied by gas liquid chromatography. By comparing the ECL values (16.28 for C16:1 ω7, 17.24 for C17:1, and 18.20 for C18:1ω9), the double bond was very likely located on the ω8 carbon (C17:1ω8) which agrees with previous data (1,21). In addition, the GLC analysis indicated that the double bond has very likely a cis-configuration, again in complete agreement with the results obtained by Zhukov and Vereshchagin (1). IR and RMN spectra analysis confirmed the structure of methyl heptadecenoate and thus the molecule's high degree of purity.

The main difficulty in purifying the C17:1ω8 was the presence of two monounsaturated fatty acids (C16:1ω7 and C18:1ω9) in the original mixture whose physical properties differed very little and moreover in opposite ways from those of C17:1ω8. Fractional crystallization from acetone at -60 C proved suitable for removing C16:1 ω7 (and polyunsaturated fatty acids and urea complexes formation at 4 C for removing C18:1ω9 (and saturated fatty acids). So, preparation of a large amount of very pure (99 p.100) heptadecenoic acid (more than 17 g) from *Candida tropicallis* yeast can be carried out by fractional crystallization with a conventional laboratory device, this present procedure being a compromise between preparation time and purification yield.

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