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Simultaneous determination by GC of free and combined fatty acids and sterols in grape musts and yeasts as silanized compounds

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A rapid and simplified technique was studied for simultaneous profiling of free and bound fatty acid and sterol contents of must and yeast by capillary gas chromatography. The protocol was designed to efficiently separate, identify, and accurately quantify the above compounds. Extracted from must with chloroform, free fatty acids and sterols were both analyzed as trimethylsilyl derivatives using a non-polar polydimethylsiloxane DB1 fused-silica capillary column. Combined fatty acids and sterols in must and yeast were saponified in aqueous ethanolic potassium hydroxide solution before chloroform extraction and silanization for GC analysis. Total run time was 77 min during which the two classes (fatty acids with chain lengths of 7–23 carbons and 10 sterols) were separated with good resolution and identified by relative retention times compared to those of the standards. This method was applied to both a grape must and yeast. Quantitative analysis was carried out with the use of three different internal standards. The simultaneous analysis of fatty acids and sterols (combined and free together) should also be applicable to other natural products and foods.

INTRODUCTION

Fatty acid and sterol analyses are very important in many areas of natural food products and biological chemistry.

Analysis of fatty acids and sterols from plant sources is difficult owing to the chemical nature of these substances, to the small amount present in biological samples and to the structural similarity of the compounds. When they are extracted, these compounds are normally subjected to one or more time-consuming pre-fractionation steps in order to concentrate them.

High-performance liquid chromatography (HPLC) is a technique often used for the determination of fatty acids, mainly using reversed-phase HPLC (RP-HPLC) (Durst *et al.*, 1975; Pei *et al.*, 1975; Scholfield, 1975; Özcimder & Hammers, 1980; Aveldano *et al.*, 1983; Grogan, 1984; Rezanka & Podojil, 1985) with non-aqueous solvents as the mobile phase. However, the lack of chromophoric groups in the fatty acid molecules makes the detection and estimation of the compounds difficult. In fact, with UV detection, only short wavelengths (190–210 nm) are useful and this severely limits the choice of solvents that can be used as mobile phase constituents. The universal refractive index detector exhibits poor sensitivity towards these compounds and cannot be used in conjunction with gradient elution.

Therefore, it is of interest to prepare fatty-acid derivatives containing chromophoric groups, which can be detected in sensitive spectral ultraviolet ranges in which the common HPLC detectors work. In fact, various authors have studied different derivatization reagents such as phenacyl (Borch, 1975; Durst *et al.*, 1975; Grushka *et al.*, 1975; Engelhardt & Elgass, 1978; Wood & Lee, 1983) *p*-bromophenacyl (Pei *et al.*, 1976; Weatherson *et al.*, 1978; Halgunset *et al.*, 1982), *p*-nitrobenzyl (Grushka *et al.*, 1975), *p*-methylthiobenzyl (Vioque *et al.*, 1985) and 1-chlorophenacyl (Gübitz, 1980) esters. An alternative HPLC approach is based on the preparation of fluorescent derivatives such as the 2-naphthacyl (Distler, 1980), 9-diazomethylanthracene (Barker *et al.*, 1980) and 4-bromoethyl-6,7 dimethoxycoumarin (Farinoti *et al.*, 1983) esters.

For determining fatty acids a good alternative option appeared to be gas chromatography preceded by the formation of volatile methyl ester derivative compounds (Metcalfe & Schmitz, 1961; Morrison & Smith, 1964; Glass, 1971; Bertrand & Miele, 1984; Brown *et al.*, 1990). Recently, the preparation of derivatives was

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eliminated by use of specific columns to separate free fatty acids (such as FFAP) (Taylor & Kirsop, 1977; Herraiz *et al.*, 1980; Rozes & Lonvaud-Funel, 1981).

As regards sterols, LC has been routinely employed in separation of free sterols in an apolar chromatographic system and successive confirmation by GLC analysis of the collected effluent (Grunwald, 1969; Hunter *et al.*, 1978). Some improvement was achieved by the introduction of argentated preparative thin-layer chromatography (Ag PLC) as a preliminary step to GLC analysis, since mixtures of sterol acetates could be separated into various zones according to their complexing properties with silver ion (Idler & Safe, 1972).

High-performance liquid chromatography (HPLC) can resolve some mixtures of sterols, provided they are in the form of acetates or benzoates (Rees *et al.*, 1976). The HPLC of free sterols, which is more convenient as well as preferable for quantitative analyses and mass spectrometry, has so far been only partially successful.

Mass spectrometry has often been employed to identify components which have been separated on preparative GLC columns. Various derivatives have been used to improve the volatility, GLC resolution, or mass-spectrometric response of the free sterols. In particular acetates (Nordby & Nagy, 1973), methyl ethers (Idler *et al.*, 1970) and trimethylsilyl ethers (Chambaz & Horning, 1969) have been examined and their GLC and mass-spectrometric performance tabulated (Knights, 1967; Brooks *et al.*, 1968; Brooks *et al.*, 1973).

Coupled gas–liquid chromatography–mass spectrometry (GC–MS) has been employed for the investigations of sterols (Knights, 1967; Brooks & Steel, 1973; Smith *et al.*, 1973; Novotny *et al.*, 1976; Edmonds *et al.*, 1977; Lisboa & Halket, 1978) and has considerable potential for the rapid identification of complex mixtures.

Therefore, the time-consuming steps in most methods of fatty-acid and sterol analysis are due not only to a quantitative and distinct extraction and/or separation of these two classes of lipid component, but also to a complex and difficult procedure of preparation of derivatives for chromatography.

In an attempt to develop a more rapid method of free and combined fatty acid and sterol analysis, we have studied a new method for their simultaneous extraction and separation from grape must and yeast and for their simultaneous derivatization as silanized compounds. This also allows the determination of both acids and sterols by a single GC-FID injection.

MATERIALS AND METHODS

Reagents and chemicals

Two standard solutions were prepared as follows:

(a) Fatty acid standard solution. l-Heptanol ($C_7H_{16}O$ Merck, Darmstadt, F.R.G.) 316 mg/liter, heptanoic acid ($C_7H_{14}O_2$ Merck) 208 mg/liter, ethyl caprylate ($C_{10}H_{20}O_2$ Fluka, Buchs, Switzerland) 200 mg/liter, caprylic acid ($C_8H_{16}O_2$ Fluka) 234 mg/liter, nonanoic acid ($C_9H_{18}O_2$ Merck) 200 mg/liter, ethyl decanoate ($C_{12}H_{24}O_2$ Fluka) 220 mg/liter, capric acid ($C_{10}H_{20}O_2$ Fluka) 200 mg/liter, undecanoic acid ($C_{11}H_{22}O_2$ Sigma, St. Louis, MO, USA) 210 mg/liter, ethyl laurate ($C_{14}H_{28}O_2$ Fluka) 214 mg/liter, lauric acid ($C_{12}H_{24}O_2$ Fluka) 190 mg/liter, tridecanoic acid ($C_{13}H_{26}O_2$ Fluka) 218 mg/liter, tetradecanoic acid ($C_{14}H_{28}O_2$ Sigma) 200 mg/liter, palmitic acid ($C_{16}H_{32}O_2$ Sigma) 200 mg/liter, heptadecanoic acid ($C_{17}H_{34}O_2$ Fluka) 200 mg/liter, linolenic acid ($C_{18}H_{30}O_2$ Sigma) 198·5 mg/liter, linoleic acid ($C_{18}H_{32}O_2$ Sigma) 200 mg/liter, oleic acid ($C_{18}H_{34}O_2$ Sigma) 201·5 mg/liter, stearic acid ($C_{18}H_{36}O_2$ Sigma) 220 mg/liter, nonadecanoic acid ($C_{19}H_{38}O_2$ Fluka) 200 mg/liter, heneicosanoic acid ($C_{21}H_{42}O_2$ Fluka) 300 mg/liter and tricosanoic acid ($C_{23}H_{46}O_2$ Fluka) 300 mg/liter were dissolved in chloroform (Merck) in a volumetric flask.

(b) Sterol standard solution. 5-α-Cholestane ($C_{27}H_{48}$) 210 mg/liter, cholesterol ($C_{27}H_{46}O$) 225 mg/liter, desmosterol ($C_{27}H_{44}O$) 100 mg/liter, ergosterol ($C_{28}H_{44}O$) 600 mg/liter, campesterol ($C_{28}H_{48}O$) 100 mg/liter, stigmasterol ($C_{29}H_{48}O$) 230 mg/liter, lanosterol ($C_{30}H_{50}O$) 100 mg/liter, β-sitosterol ($C_{29}H_{50}O$) 100 mg/liter, stigmastanol ($C_{29}H_{52}O$) 225 mg/liter, and uvaol ($C_{30}H_{50}O_2$) 200 mg/liter, all obtained from Sigma, were dissolved in chloroform (Merck) in a volumetric flask.

Preparation of the must fraction

Laboratory tests were carried out on natural must obtained from sound Chardonnay grapes as follows: the must running spontaneously from grapes crushed in a roller stemmer machine (Zambelli, PD Italy) was put in a laboratory idropneumatic press (Enoagricola Rossi, PG Italy) in order to separate the first 65% fraction (free run juice). After an addition of 75 mg/liter SO_2 as $K_2S_2O_5$ (150 mg/liter), the must was left at room temperature for 4 h. Centrifugation at 3000 × *g* for 10 min was applied to separate solid substances from the must. The limpid supernatant was then frozen at −20°C.

A sample of 0·5 liter of the limpid supernatant was filtered on diatomaceous earth to determine density, total extract, reducing sugars, pH, total acidity, sulfur dioxide and ammonia (see Table 1).

Methods

The fatty acid and sterol standard solutions were mixed in order to optimize the procedure of GC analysis preceded by silanization. Their times were then used as references to identify peaks in grape must samples.

Apparatus and chromatographic conditions

The gas chromatographic analysis was performed with a Varian 3600 gas chromatograph equipped with a

Table 1. Physicochemical characteristics of the must

Density	1.940
Total extract	245 g/liter
Reducing sugars	227 g/liter
pH	3.28
Total acidity	105 meq/liter
Sulfur dioxide	32 mg/liter
Ammonia	0.121 g/liter

capillary column of fused silica, phase DB1 (J. & W. Scientific, Folson, CA, USA), of length 30 m and 0·25 mm i.d., film thickness 0·25 μm, temperature conditions: initially 40°C, rising to 200°C at 6°C/min held for 15 min, then rising at a rate of 6°C/min to 260°C and from 260 to 290°C at 2°C/min, detector flame ionization detector 300°C, injector 280°C, range 12, attenuation 4, carrier gas helium, linear flow rate 1·5 ml/min, pressure 15·7 psig, injection volume 1–2 μl.

The chromatographic data were analyzed on a Varian STAR 2000 GC Star Workstation Software.

Extraction of free fatty acids and sterols

The extraction process consisted in transferring 50 ml of the must sample to a separatory funnel and in adding 1 ml of a 1 : 1 ethanol : water solution of 0·310 mg/ml C_9, 1 mg/ml C_{19} in ethanol and 1 mg/ml cholesterol in ethanol as internal standards (I.S.). These three different internal standards were chosen for their suitable retention times and their absence in vegetable matrices and in yeasts (Ratledge & Evans, 1989). Free fatty acids and sterols were extracted by shaking, with three successive 25 ml portions of $CHCl_3$, in a separatory funnel. In order to break down the water—chloroform emulsion when it occurred, the mixtures were centrifuged for 5 min at 5000 rpm to completely separate the chloroformic and aqueous phases. The organic phase was transferred to a rotary evaporator flask at 30°C for concentrating to about 1 ml and, after the addition of an opportune amount of anhydrous Na_2SO_4, it was transferred into a screw-capped tube washing with approximately 1 ml of $CHCl_3$; 200 μl were then put into a 1 ml vial, dried under a 300 ml/min nitrogen flux and silanized with 100 μl pyridine, 100 μl bis(trimethylsilyl) trifluoracetamide, 10 μl trimethylchlorosilane and incubated 1 hour at 80°C.

Extraction of combined fatty acids and sterols in must and yeast

(a) Must

To 25 ml absolute ethanol was added the remaining aqueous phase from which free fatty acids and sterols were previously extracted in order to precipitate colloidal substances together with suspended solids and eventual yeasts. The precipitate was centrifuged for 15 min at 5000 rpm and the supernatant (ethanol—water solution) was eliminated. In fact, the supernatant did not contain fatty acids and sterols, as was experimentally verified by using a XAD-2 resin extraction followed by GC analysis. The centrifuged pellet was resuspended in approximately 10 ml of distilled water and transferred to a spherical flask.

(b) Yeast

In contrast to the must, for yeast cells, in which almost all fatty acids and sterols are present in combined form in lipid molecules, the procedure used begins with saponification of cells, as a slurry or in the form of dry yeast, rather than extraction of free lipids followed by saponification of the combined ones as in must. Yeast cells (0·02—1·0 g wet weight) were suspended in 10 ml of distilled water and transferred to a spherical flask.

At this point, either (a) or (b) were saponified, extracted and concentrated by the following procedure: 1 ml of a 1 : 1 ethanol : water solution of 0·310 mg/ml C_9, 1 mg/ml C_{19} in ethanol, and 1 mg/ml cholesterol in ethanol were added as internal standards (I.S.); then the whole mixture was saponified by addition of 10 ml of 2 N KOH in ethanol and by refluxing under a Liebig condenser on a heating mantle for 1·5 h. After cooling the saponified material at room temperature the con-

Table 2. Retention times and relative response factors to the three specific internal standards

Peak table	Retention time (min)	Relative response factor to the specific Internal Standard		
		C9	C19	Cholesterol
l-Heptanol	11·26	0·637		
Heptanoic acid	13·13	0·895		
Ethyl caprylate	13·58	1·323		
Caprylic acid	15·46	0·867		
Nonanoic acid	17·70	1·000		
Ethyl decanoate	18·25	1·350		
Capric acid	19·84	0·977		
Undecanoic acid	21·88	1·001		
Ethyl laurate	22·47	1·308		
Lauric acid	23·83	0·970		
Tridecanoic acid	25·69	1·118		
Tetradecanoic acid	27·52	1·179		
Palmitic acid	32·46		0·793	
Heptadecanoic acid	36·16		0·905	
Linolenic acid	38·97		0·779	
Linoleic acid	39·18		0·978	
Oleic acid	39·44		0·753	
Stearic acid	41·20		0·881	
Nonadecanoic acid	45·75		1·000	
Heneicosanoic acid	51·03		1·075	
Tricosanoic acid	55·02		1·213	
5-α-Cholestane	57·00			0·850
Cholesterol	64·04			1·000
Desmosterol	64·83			1·329
Ergosterol	65·96			7·190
Campesterol	66·56			0·879
Stigmasterol	67·31			1·128
Lanosterol	68·55			1·298
β-Sitosterol	68·86			0·813
Stigmastanol	69·20			1·207
Uvaol	75·26			1·165

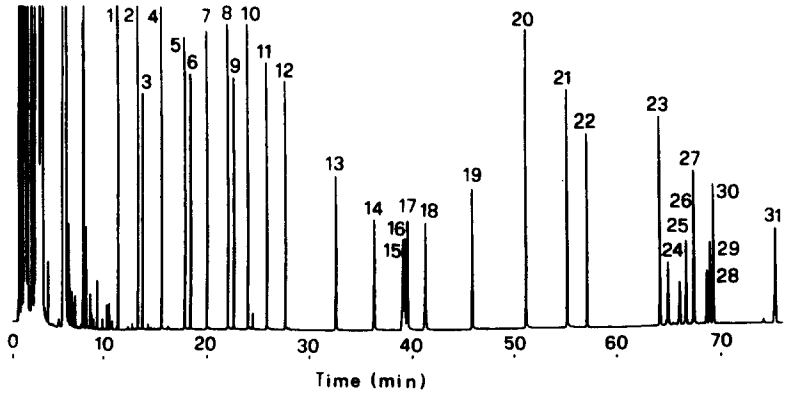

Fig. 1. Optimal separation of the components of the fatty acid and sterol standard mixture. Peaks: 1 = 1-heptanol; 2 = heptanoic acid; 3 = ethyl caprylate; 4 = caprylic acid; 5 = nonanoic acid; 6 = ethyl decanoate; 7 = capric acid; 8 = undecanoic acid; 9 = ethyl laurate; 10 = lauric acid; 11 = tridecanoic acid; 12 = tetradecanoic acid; 13 = palmitic acid; 14 = heptadecanoic acid; 15 = linolenic acid; 16 = linoleic acid; 17 = oleic acid; 18 = stearic acid; 19 = nonadecanoic acid; 20 = heneicosanoic acid; 21 = tricosanoic acid; 22 = 5-α-cholestane; 23 = cholesterol; 24 = desmosterol; 25 = ergosterol; 26 = campesterol; 27 = stigmasterol; 28 = lanosterol; 29 = β-sitosterol; 30 = stigmastanol; 31 = uvaol.

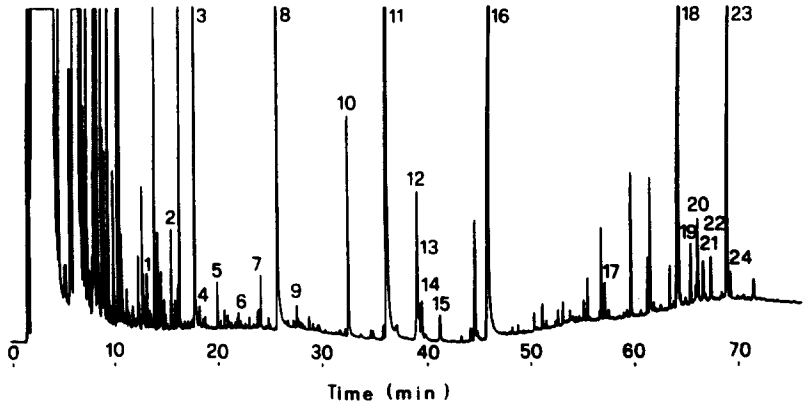

Fig. 2. Example of a chromatogram of the free fatty acids and sterols in a sample must. Peaks: 1 = ethyl caprylate; 2 = caprylic acid; 3 = nonanoic acid; 4 = ethyl decanoate; 5 = capric acid; 6 = undecanoic acid; 7 = lauric acid; 8 = tridecanoic acid; 9 = tetradecanoic acid; 10 = palmitic acid; 11 = heptadecanoic acid; 12 = linolenic acid; 13 = linoleic acid; 14 = oleic acid; 15 = stearic acid; 16 = nonadecanoic acid; 17 = 5-α-cholestane; 18 = cholesterol; 19 = desmosterol; 20 = ergosterol; 21 = campesterol; 22 = stigmasterol; 23 = β-sitosterol; 24 = stigmastanol.

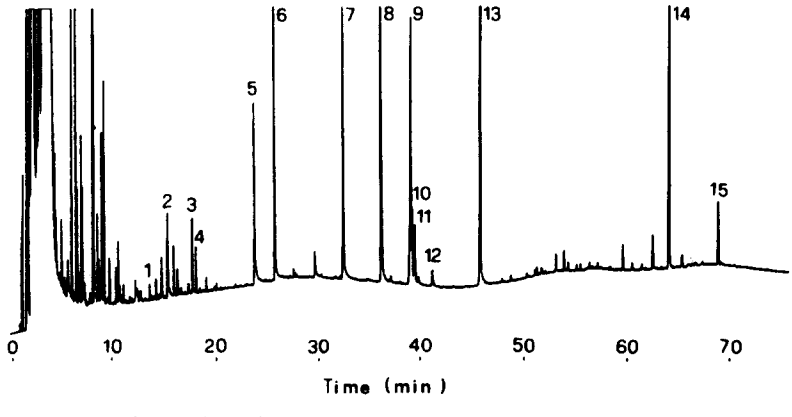

Fig. 3. Example of a chromatogram of combined fatty acids and sterols in the same must as Fig. 2. Peaks: 1 = ethyl caprylate; 2 = caprylic acid; 3 = nonanoic acid; 4 = ethyl decanoate; 5 = lauric acid; 6 = tridecanoic acid; 7 = palmitic acid; 8 = heptadecanoic acid; 9 = linolenic acid; 10 = linoleic acid; 11 = oleic acid; 12 = stearic acid; 13 = nonadecanoic acid; 14 = cholesterol; 15 = β-sitosterol.

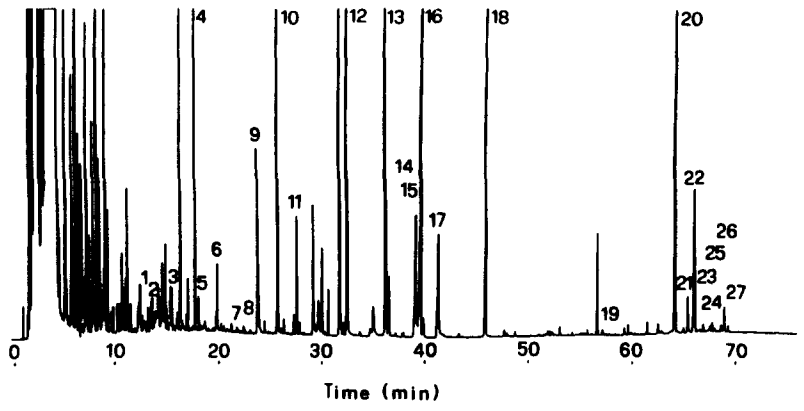

Fig. 4. Additional example of combined fatty acid and sterol chromatographic analysis in (S22b) yeast cells. Peaks: 1 = heptanoic acid; 2 = ethyl caprylate; 3 = caprylic acid; 4 = nonanoic acid; 5 = ethyl decanoate; 6 = capric acid; 7 = undecanoic acid; 8 = ethyl laurate; 9 = lauric acid; 10 = tridecanoic acid; 11 = tetradecanoic acid; 12 = palmitic acid; 13 = heptadecanoic acid; 14 = linolenic acid; 15 = linoleic acid; 16 = oleic acid; 17 = stearic acid; 18 = nonadecanoic acid; 19 = 5-α-cholestane; 20 = cholesterol; 21 = desmosterol; 22 = ergosterol; 23 = campesterol; 24 = stigmasterol; 25 = lanosterol; 26 = β-sitosterol; 27 = stigmastanol.

denser was washed with 10 ml of distilled water and the alcoholic saponified solution was transferred into a separatory funnel, washing the spherical flask with the first 15 ml extraction portion of $CHCl_3$ (see below). Sterols and other non-saponifiable lipids were extracted with three successive 15 ml portions of $CHCl_3$, shaking vigorously for 2 min. The organic phase was concentrated to about 2 ml as previously described. The saponified lipids were acidified with a sufficient amount of 10 N H_2SO_4 to change the litmus paper reaction and then extracted for 2 min with 3 × 15 ml $CHCl_3$. After repeated concentration of the chloroformic phase to about 2 ml, the two organic phases (the first from the non-saponifiable matrix with sterol solution, the second from the matrices saponifiable with fatty acids) were mixed in a screw-capped tube. The resulting trimethylsilyl derivatives of 200 μl of this solution were then analyzed as described above.

Estimation of acid and sterol concentration

The concentration was estimated by converting the areas of the peaks of each acid and sterol into units of concentration (mg of acid or sterol per ml of sample), using the relative response factors with respect to the specific internal standards (see Table 2).

The response factor (RF) for each acid and sterol was calculated from the equation:

$$RF = \frac{(C_s) \cdot (A_{IS})}{(A_s) \cdot (C_{IS})}$$

in which C_s and C_{IS}, the acid or sterol and internal standard concentrations (mg/ml) in the sample respectively and A_s and A_{IS} are the response area of the acid or sterol and the area of the internal standard, respectively.

The concentration was then calculated using the following formula:

$$C_s = \frac{(A_s) \cdot (C_{IS}) \cdot (RF)}{(A_{IS})}$$

Three different internal standards (C_9, C_{19} and cholesterol) were used, each having retention times that were differently distributed on the chromatogram, in such a way that each of them can be located relatively close to the peaks of the compounds to be evaluated and should not be greatly different chemically from the compounds themselves.

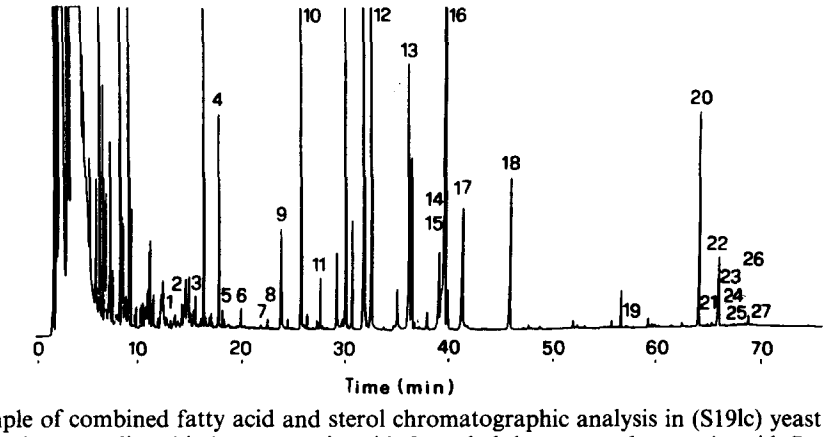

Fig. 5. Additional example of combined fatty acid and sterol chromatographic analysis in (S19lc) yeast cells. Peaks: 1 = heptanoic acid; 2 = ethyl caprylate; 3 = caprylic acid; 4 = nonanoic acid; 5 = ethyl decanoate; 6 = capric acid; 7 = undecanoic acid; 8 = ethyl laurate; 9 = lauric acid; 10 = tridecanoic acid; 11 = tetradecanoic acid; 12 = palmitic acid; 13 = heptadecanoic acid; 14 = linolenic acid; 15 = linoleic acid; 16 = oleic acid; 17 = stearic acid; 18 = nonadecanoic acid; 19 = 5-α-cholestane; 20 = cholesterol; 21 = desmosterol; 22 = ergosterol; 23 = campesterol; 24 = stigmasterol; 25 = lanosterol; 26 = β—sitosterol; 27 = stigmastanol.

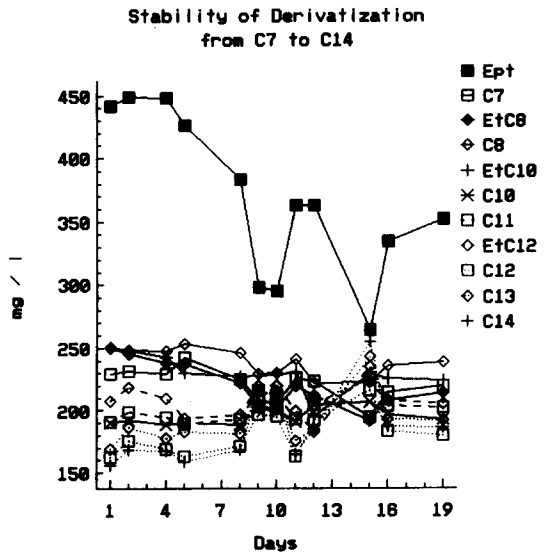

Fig. 6. Stability of derivatization of fatty acid from C_7 to C_{14}. Ept = 1-heptanol; C7 = heptanoic acid; EtC8 = ethyl caprylate; C8 = caprylic acid; EtC10 = ethyl decanoate; C10= capric acid; C11 = undecanoic acid; EtC12 = ethyl laurate; C12 = lauric acid; C13 = tridecanoic acid; C14 = tetradecanoic acid.

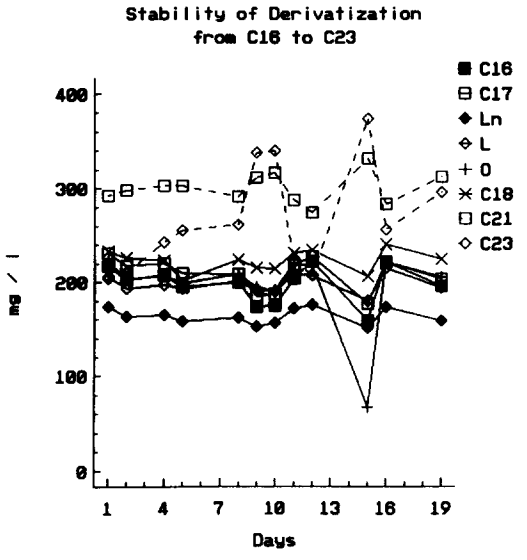

Fig. 7. Stability of derivatization of fatty acid from C_{16} to C_{23}. C16 = palmitic acid; C17 = heptadecanoic acid; Ln = linolenic acid; L = linoleic acid; O = oleic acid; C18 = stearic acid; C21 = heneicosanoic acid; C23 = tricosanoic acid.

Determination of the recovery of fatty acids and sterols in must

In order to determine the repeatability, linearity and the percentage of recovery for each acid and sterol, 1, 2, 3 and 4 ml of the acid and sterol standard solutions were added to four different fractions of the same must (previous verification of its low fatty acid and sterol contents) against a control sample prepared with only the addition of the three internal standards (1 ml of a 1 : 1 ethanol : water solution of 0·310 mg/ml C_9, 1 mg/ml C_{19} in ethanol and 1 mg/ml cholesterol in ethanol). All the samples were then extracted and analyzed by the methods described above.

In order to ascertain the quantitative recovery of the silanization procedure, two aliquots of each organic

Table 3. Repeatibility, linearity and recovery of the method

Peak table	Gradient	Intercept	SD of gradient	SD of intercept	Correlation coefficient	Standard error of estimates
1-Heptanol	286·55	15·16	20·31	49·74	0·993	64·21
Heptanoic acid	179·83	0·41	3·28	8·02	0·999	10·36
Ethyl caprylate	326·82	12·15	15·66	38·36	0·997	49·52
Caprylic acid	224·12	12·16	2·82	6·90	0·999	8·91
Ethyl decanoate	273·84	6·91	4·18	10·23	0·999	13·20
Capric acid	195·04	2·76	1·73	4·23	0·999	5·46
Undecanoic acid	235·99	−5·84	4·72	11·56	0·999	14·92
Ethyl laurate	220·39	−7·77	5·01	12·26	0·999	15·83
Lauric acid	190·41	−10·55	5·26	12·88	0·999	16·63
Tridecanoic acid	185·57	−16·59	6·52	15·97	0·998	20·62
Tetradecanoic acid	181·66	−16·24	7·36	18·04	0·997	23·29
Palmitic acid	199·90	53·32	6·97	17·06	0·998	22·03
Heptadecanoic acid	202·21	10·50	6·41	15·69	0·998	20·26
Linolenic acid	226·30	18·10	4·81	11·79	0·999	15·22
Linoleic acid	220·69	−1·27	7·61	18·64	0·998	24·07
Oleic acid	226·78	9·89	1·38	3·39	0·999	4·38
Stearic acid	222·81	0·84	2·88	7·06	0·999	9·11
Heneicosanoic acid	287·44	13·15	6·44	15·78	0·999	20·37
Tricosanoic acid	302·39	20·25	14·03	34·37	0·997	44·38
5-α-Cholestane	191·50	4·38	3·12	7·65	0·999	9·88
Desmosterol	91·26	11·31	5·04	12·35	0·995	15·94
Ergosterol	343·28	188·06	17·45	42·74	0·996	55·17
Campesterol	127·85	10·67	1·73	4·25	0·999	5·49
Stigmasterol	206·36	15·99	1·09	2·68	0·999	3·45
Lanosterol	86·63	−3·95	1·19	2·91	0·999	3·76
β-Sitosterol	170·75	367·36	3·91	9·58	0·999	12·38
Stigmastanol	192·39	9·97	0·91	2·24	0·999	2·89
Uvaol	207·84	4·09	1·47	3·60	0·999	4·64

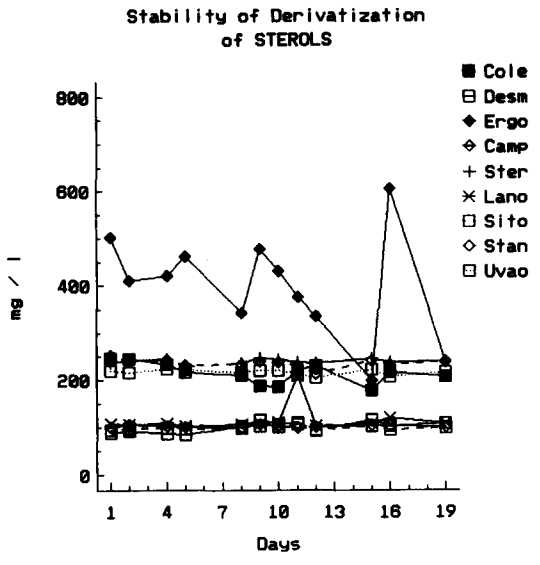

Fig. 8. Stability of sterol trimethylsilyl derivatives. Cole = 5-α-cholestane; Desm = desmosterol; Ergo = ergosterol; Camp = campesterol; Ster = stigmasterol; Lano = lanosterol; Sito = β-sitosterol; Stan = stigmastanol; Uvao = uvaol.

portion were silanized and analyzed. Regression analysis was performed with the software STSC-Statgraphics version 5·1.

The reproducibility of the gas-chromatographic method was verified by repeating the analysis of a 1 : 1 mixture of the standard fatty acid and sterol solutions seven times.

The stability of trimethylsilyl derivatives was tested by repeating the gas-chromatographic analysis of the same standard sample over a period of time.

RESULTS AND DISCUSSION

After several injections, the optimal temperature program was chosen as described in Materials and Methods. All the tested compounds were resolved completely on the chromatogram as can be seen in Fig. 1. Gas-chromatographic separation of different must samples was carried out with the same eluting program used for the standard simulated sample and the data showed them to be relatively free from interfering peaks, as is shown in typical chromatograms relative to extracts of both free (Fig. 2) and combined (Fig. 3) acids and sterols in must and combined acids and sterols in yeasts (Figs 4 and 5).

The linearity and recovery for each acid and sterol exhibit excellent results because the correlation coefficients were over 99% in the case of compounds with relatively high volatility (such as heptanol, EtC8, etc.) and up to 99·9% for the lower volatile compounds (Table 3). Concerning the recovery efficiency, the method generally gives good results: the highest standard errors of estimates were those of heptanol and ergosterol, but their values are always acceptable (Table 3).

Results obtained for reproducibility of the gas-chromatographic method are shown in Table 4. This method yields an average coefficient of variation of 5·63%, since coefficients of variation are less than 15%.

As regards the stability of derivatization, Figs. 6–8 show that, until the fifth day, trimethylsilyl derivatives are relatively stable, while after this point they begin to deteriorate, influencing quantitative analysis.

Table 4. Reproducibility of gas-chromatographic method

Peak table	Mean (mg/liter)	Range of 7 estimations	Standard deviation	Standard error	Coefficient of variation
l-Heptanol	425	372–458	34·2	12·9	8·03
Heptanoic acid	234	225–251	9·06	3·42	3·87
Ethyl caprylate	240	226–250	9·15	3·46	3·81
Caprylic acid	250	242–259	5·55	2·10	2·23
Ethyl decanoate	241	228–250	8·71	3·29	3·67
Capric acid	192	183–201	5·72	2·16	2·99
Undecanoic acid	194	180–206	8·69	3·29	4·48
Ethyl laurate	207	187–222	12·9	4·88	6·22
Lauric acid	169	152–184	113	4·26	6·66
Tridecanoic acid	179	157–197	13·27	5·02	7·44
Tetradecanoic acid	167	144–193	16·6	6·26	9·90
Palmitic acid	206	186–236	16·6	6·26	8·03
Heptadecanoic acid	216	195–237	15·42	5·83	7·15
Linolenic acid	166	159–182	8·84	3·34	5·32
Linoleic acid	200	193–213	7·36	2·78	3·69
Oleic acid	209	199–229	11·83	4·47	5·66
Stearic acid	222	202–234	9·90	3·74	4·46
Heneicosanoic acid	298	273–322	15·1	5·70	5·07
Tricosanoic acid	252	205–305	36·2	13·7	14·4
5-α-Cholestane	229	202–247	18·4	6·95	8·05
Desmosterol	91·5	84·2–101	6·44	2·43	7·04
Ergosterol	472	409–520	44·1	16·7	9·34
Campesterol	102	97·9–106	2·79	1·06	2·75
Stigmasterol	241	229–246	5·56	2·10	2·31
Lanosterol	106	102–110	2·42	0·916	2·28
β-Sitosterol	96·4	88·7–101	4·54	1·72	4·71
Stigmastanol	241	219–254	12·5	4·72	5·17
Uvaol	222	214–232	6·66	2·52	3·00

CONCLUSIONS

The described method appears rapid and accurate for the simultaneous determination of fatty acids and sterols.

This simultaneous chromatographic determination of fatty acids and sterols could be applied to foods or natural plant products in the environment or in yeast matrices.

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