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Identification and determination of individual sophorolipids in fermentation products by gradient elution high-performance liquid chromatography with evaporative light-scattering detection

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ABSTRACT

High-performance liquid chromatography (HPLC) was used for the characterization of sophorolipids, one of the most important types of glycolipid biosurfactants. By using gradient elution with a water–acetonitrile mixture on a reversed-phase (C_{18}) column and evaporative light-scattering detection, resolution of all the important individual sophorolipids present in fermentation products was achieved. In addition to HPLC, a combination of techniques involving selective production by fermentation of sophorolipids, chemical conversions of the products, separation methods and, for identification of lipidic chains of sophorolipids, gas chromatography and mass spectrometry was used. This led to the identification of almost all significant compounds observed in HPLC, including several previously unreported sophorolipids. As a result, a rapid method is now available for investigations of the influence of fermentation conditions on the nature and quantitative distribution of the sophorolipid products obtained.

INTRODUCTION

Among glycolipids, one of the main classes of biosurfactants, sophorose lipids produced from glucose and a lipidic carbon source by yeasts such as *Candida bombicola* have long been the subject of much interest [1,2]. Although the structure of sophorolipids has been studied in detail [1,3–5], much less information is available concerning their production [2], the main point investigated being the relationship between the nature of the lipid carbon source (vegetable oils, fatty acid esters, *n*-alkanes) and the structure of the products formed [6,7]. The production conditions, however, appear to be important both for the yield, which can be fairly high [8], and for composition of the products [6–8].

Analytical difficulties probably account to a large extent for this relative paucity of information, as the sophorolipid mixture produced by fermentation is complex. The purpose of this work was to devise an analytical tool allowing the rapid determination of all quantitatively significant sophorolipid products, in order to be able to follow the progress of fermentation under various conditions. High-performance liquid chromatography (HPLC) with gradient elution was selected for this purpose, detection being achieved with an evaporative light-scattering detector [9–12]. This detector is compatible with gradient elution as its principle makes it independent of the nature of the mobile phase. Further, as its response does not depend on the presence of specific chemical groups, it has been

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shown to be appropriate for the determination of compounds such as lipids [12–18] and surfactants [19]. In the work reported here, HPLC with gradient elution was found in the case of sophorolipids to be a key component in a strategy involving a combination of techniques resulting in identification of individual sophorolipids in a complex mixture.

EXPERIMENTAL

Sophorolipid production

Sophorolipids were produced by *Candida bombicola* CBS 6009 at 25°C in a 4-l laboratory fermenter under conditions of aeration (0.5 v v^{-1} min^{-1}) and of vigorous agitation (800 rpm) produced by a centrifuge propeller. The initial culture medium (2.2 l) had the following mineral composition per litre: 4 g of $(NH_4)_2SO_4$, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$ and 5 g of dried cornsteep liquor (Roquette, Lille, France). During the cultures (185 h), the pH of this medium was maintained constant at 3.5 by automatic addition of a 4 *M* NaOH solution. Ethyl esters of rapeseed oil and glucose were the carbon sources. Ethyl esters were fed continuously at a constant rate of 1.75 ml h^{-1} per litre of the initial culture medium. Glucose was added according to two different procedures. In the first, it was supplied in excess from the beginning to the end of the culture by daily additions of 50 g l^{-1} (referred to the initial fermentation volume). Decantation of sophorolipids was triggered by heating the broth for 10 min at 100°C. The lyophilized sophorolipid layer is referred to as sample A. Sample B was prepared according to the second procedure. Glucose (50 g l^{-1}) was added only to the initial medium without any further addition. The broth was centrifuged and the supernatant cleared of cells was then lyophilized. Owing to the different fermentation procedures, widely different sophorolipid compositions were observed in samples A and B.

Chemical conversions of sophorolipids

Catalytic hydrogenation. A 40-mg amount of freeze dried sophorolipids was dissolved in 3 ml of anhydrous methanol. The solution was placed in a 20-ml seal-stoppered tube with 40 mg of a palladium catalyst (5% on $CaCO_3$). The air volume was replaced with hydrogen under 1 bar relative pressure and the sample was incubated with shaking at 60°C for 4 h. At the end of the reaction, the remaining hydrogen was flushed off with nitrogen and the methanol phase was collected and evaporated at room temperature under a stream of nitrogen.

Methylation. Methyl esters of acidic sophorolipids were obtained by incubating at 60°C with shaking for 4 h 40–60 mg of dried sophorolipids and 100 mg of acidic resin (Amberlyst 15; Rohm and Haas, Philadelphia, PA, USA) in 3 ml of methanol. Methanol was then evaporated under a stream of nitrogen. Methanolysis of hydroxy fatty acid moieties (see below) did not take place under these conditions, but with lactonic forms limited ring opening (10–15%) took place. The procedure was used in order to improve the chromatographic resolution of acidic sophorolipids in samples containing small amounts of lactonic forms.

Alkaline hydrolysis. Alkaline hydrolysis of sophorolipids led to the deacetylated acidic structures of the glycolipids. Sophorolipids (100 mg) were dissolved in 10 ml of a mixture of 5 ml of methanol, 5 ml of water and 1.5 g of NaOH and placed in a tube sealed with a PTFE-lined screw-cap. The tube was heated for 30 min in a boiling water-bath. After cooling, the reaction medium was carefully acidified to pH 3.0 by dropwise addition of concentrated H_2SO_4. A 20-ml volume of methanol was added and the mixture was filtered. The clarified filtrate was evaporated under a stream of nitrogen.

Methanolysis. For the determination of the hydroxy fatty acid moieties, sophorolipids (acids and lactones) were cleaved and transesterified in the presence of methanol and H_2SO_4 to yield the methyl esters of hydroxy acids. Amounts of 20–50 mg of sophorolipids were dissolved in a mixture of 2 ml of 1% H_2SO_4 in methanol and 1 ml of toluene containing 2 g l^{-1} of arachidic acid as internal standard. This mixture was poured into a tube that was tightly sealed with a Viton cap (DuPont, Wilmington, DE, USA) and heated for 1 h at 100°C. The resulting hydroxy fatty acid methyl esters were extracted twice with 5 ml of cyclohexane in the presence of 5 ml of 50

g l^{-1} NaCl solution. The cyclohexane phase was cleared by centrifugation and dried under a stream of nitrogen. These conditions gave satisfactory recoveries of hydroxy fatty acid esters for quantitative determination.

Good reproducibility in gas chromatographic (GC) analysis was achieved by silylation of the hydroxy group. Dried methyl ester samples were treated with 0.25 ml of Sylon TP (Supelco, Bellefonte, PA, USA) and 1.75 ml of pyridine at 60°C for 30 min. The silylated hydroxy fatty acid methyl esters were extracted with 10 ml of heptane in the presence of 2 ml of distilled water and the organic layer was used for GC analysis.

Chromatographic procedures

Thin-layer chromatography. Thin-layer chromatography (TLC) was performed on sophorolipids using 20 cm × 20 cm × 1 mm Kiesegel 60 F_{254} plates (Merck, Darmstadt, Germany). The developing solvent was chloroform–methanol–water (65:15:2). Two detection techniques were used: exposure to iodine vapour and vaporization of a solution of α-naphthol in a mixture of sulphuric acid, ethanol and water.

For recovery of the separated products, the spots were scraped off after detection with iodine vapour. The recovered material was placed in a tube and determination was effected using the anthrone reagent [20]. Sophorolipids could also be eluted with the developing solvent for further chromatographic analysis.

Gas chromatography. Methylated and silylated hydroxy fatty acids proceeding from sophorolipid conversion were analysed using a 30 m × 0.32 mm I.D. DB-5 column (J&W Scientific, Folsom, CA, USA) with temperature programming (from 150 to 290°C at 4°C min^{-1}). Identification of these compounds was performed by GC–MS using a 25 m × 0.32 I.D. CP-Sil 8 CB column (Chrompack, Middelburg, Netherlands) under the same chromatographic conditions and an MS 80 mass spectrometer (Kratos, Manchester, UK).

High-performance liquid chromatography. Isocratic HPLC separations of sophorolipids were performed using a two-column system. The first column was an Ultrabase C_{18}, 5 μm (300 mm × 4.6 mm I.D.), from SFCC-Shandon (Eragny, France) and the second was a Lichrosorb 10 RP-8, 5 μm (250 mm × 4.6 mm I.D.), from Chrompack. Elution was performed with acetonitrile–water (70:30, v/v) at a rate of 0.7 ml min^{-1}. HPLC-grade acetonitrile from SDS (Pepin, France) and triply distilled water were used. The instrumentation involved an SP 8875 automatic sampler and an SP 8810 isocratic pump from Spectra-Physics France (Les Ullis, France), an injection valve with a 20-μl sampling loop and an SP 8430 differential refractometric detector from Spectra-Physics.

Gradient elution HPLC separations were performed with a system supplied by Kratos, consisting of a Spectroflow 450 solvent programmer which controlled two Spectroflow 400 pumps. Analytical gradient HPLC was performed using a Hypersil C_{18}, 5 μm (150 mm × 4.6 mm I.D.) column from Interchim (Montluçon, France). The flow-rate was 1 ml min^{-1} and the composition of the acetonitrile–water eluent was programmed from 2 to 70% acetonitrile in 48 min. For sample injection, a Rheodyne Model 7128 valve with a 20-μl sample loop was used. For detection, a DDL 21 evaporative light-scattering (ELS) detector (mass detector) from Cunow (Cergy Saint Christophe, France) was used. For preparative gradient HPLC, a 600-μl sampling loop was used. The column was an Ultrabase C_{18} (250 mm × 10 mm I.D.) from SFCC-Shandon. The flow-rate was 2.5 ml min^{-1} and the composition of the acetonitrile–water eluent was programmed from 50 to 85% acetonitrile in 40 min followed by a 15-min isocratic elution step. Separation was monitored by UV detection (UV 490 spectrophotometer from Waters, Milford, MA, USA) at 210, 233 and 268 nm.

Data acquisition in liquid and gas chromatography was done with an HP 1000/A600 system (Hewlett-Packard, Sunnyvale, CA, USA).

Nuclear magnetic resonance (NMR) spectroscopy

^{1}H and ^{13}C NMR spectroscopy were performed with an MSL 400 spectrometer (Bruker, Karlsruhe, Germany). Trimethylsilane was used as an internal standard. The solvent used was $C^{2}H_{3}O^{2}H$. In ^{13}C NMR, the gate inverse sequence was used. For ^{1}H NMR, one- and two-

dimensional NMR (COSY) techniques were used in chemical group identification.

RESULTS AND DISCUSSION

Production of sophorolipid mixtures with different compositions by varying the fermentation conditions

Sophorolipids present in fermentation broths of *Candida bombicola* are a mixture, the structures of which are presented in Fig. 1. The main structural forms identified are lactones acetylated or not in positions 6′ and 6″ of sophorose and acids acetylated or not in position 6′ of sophorose [5]. Each of these forms constitutes a structural class of compounds. Individual homologous compounds in each class differ in their hydroxy fatty acid moiety, which varies in chain length (ranging mostly from C_{16} to C_{20}), in the number of unsaturations (between 0 and 2) and in the position of the hydroxyl group ($\omega - 1$ or ω). All classes of sophorolipids discussed here are listed in Table I. The shorthand notation adopted to designate classes and individual sophorolipids is also presented.

In a previous study, sophorolipid production from glucose and rapeseed ethyl esters under optimized conditions was described in detail [8]. Further work was devoted to the investigation of the influence of the supply mode of these substrates on the composition of the sophorolipids produced.

The structural classes of the sophorolipid mixtures produced using different fermentations

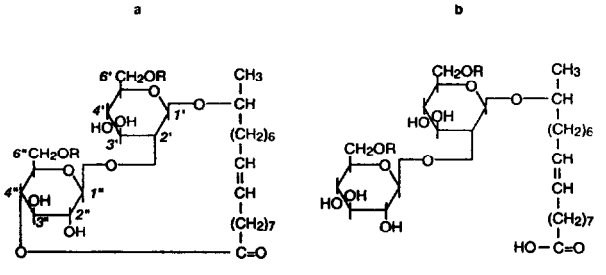


Fig. 1. Structural classes of sophorolipids. Main classes are represented for the case of the usually most abundant individual compound where the constitutive hydroxy fatty acid is 17-hydroxyoctadecenoic acid (17-hydroxyoleic acid). R = H or CH_3CO. (a) 1′,4″-Lactone classes; (b) acid classes.

TABLE I

SOPHOROLIPIDS DISCUSSED AND NOTATION USED

Structural class	Notation used[a]
1′,4″-Lactone 6′6″-diacetate[b]	Sl-4″L6′a6″a
1′,4″-Lactone 6′-monoacetate[c]	Sl-4″L6′a
1′,4″-Lactone 6″-monoacetate[b]	Sl-4″L6″a
1′,4″-Lactone[b]	Sl-4″L
1′,6′-Lactone[b]	Sl-6′L
1′,6″-Lactone[b]	Sl-6″L
Acid 6′6″-diacetate[c]	Sl-A6′a6″a
Acid 6′-monoacetate[b]	Sl-A6′a
Acid 6″-monoacetate[c]	Sl-A6″a
Acid[b]	Sl-A

[a] Notation: Sl, sophorolipid; L, lactone; A, acid; a, acetyl. Methyl ester derivatives, discussed in the text, are denoted mE. Individual sophorolipids are designated by adjunction of the notation for the constituent hydroxy fatty acid, *e.g.*, Sl-(17-OH C_{18}:1) 4″L6′a6″a represents (17-hydroxyoctadecenoic)-1′,4″-lactone 6′,6″-diacetate sophorolipid.
[b] Structures already described in the literature.
[c] Structures detected in this study.

conditions were determined after separation by TLC with reference to the characterization by Asmer *et al.* [5]. The relative compositions of the fermentation mixtures were obtained by scraping off the spots and determination using the anthrone method. Two different procedures of substrate supply allowing strongly preferential production of either lactonic or acidic forms of sophorolipids were developed. As shown in Table II, when glucose was supplied in excess throughout the fermentation, a sophorolipid mixture with a high content in lactonic forms (sample A) was obtained. In contrast, when glucose was only supplied to the culture in the growth phase, acidic forms were largely predominant (sample B). These two samples were used in this work for identification of the various sophorolipid components.

Resolution of sophorolipid classes by HPLC

Separation of the sophorolipid mixtures produced by fermentation was investigated using HPLC with isocratic elution and refractometric detection. After preliminary tests to select a stationary and a mobile phase, the resolution of the main components was obtained with two

TABLE II

RESOLUTION OF THE SOPHOROLIPID MIXTURES FROM FERMENTATIONS A AND B AS DETERMINED BY TLC

Sophorolipid classes			Composition (%)	
TLC spot[a]	R_F	Structure present	Sample A	Sample B
SL-1	0.55	Sl-4″L6′a6″a	51	3
SL-2	0.43	Sl-4″L6″a	3	1
SL-3	0.33	Sl-4″L	18	10
SL-4a, b	0.19	Sl-6′L + Sl-6″L	<1	<1
SL-5 + SL-6	0.10	Sl-A6′a + Sl-A	28	86

[a] Terminology according to Asmer *et al.* [5].

columns in series (a C_{18} and a C_8) and an eluent consisting of acetonitrile–water. Fig. 2 shows the chromatogram obtained for sample A. The lactonic forms were eluted separately at the end of the chromatographic run, but the acidic forms which were eluted ahead were not clearly separated.

In order to improve the separation of the acidic forms, an elution gradient obtained by varying the acetonitrile-to-water ratio was tested. The refractometric detector, which could not be used with gradient elution, was replaced with an ELS detector, which gave satisfactory results.

With gradient elution HPLC, a general strategy for identification of the individual sophorolipids contained in the fermentation mixture was elaborated (Fig. 3). The sophorolipid mixtures were separated into their structural classes by TLC and each isolated class (sample A1) was submitted to gradient HPLC. The elution orders with gradient mode and isocratic mode HPLC were similar. The acidic forms were eluted ahead, followed successively by the non-acetylated lactones and then the monoacetylated and diacetylated lactones. Separation of acetylated and non-acetylated acidic classes in TLC

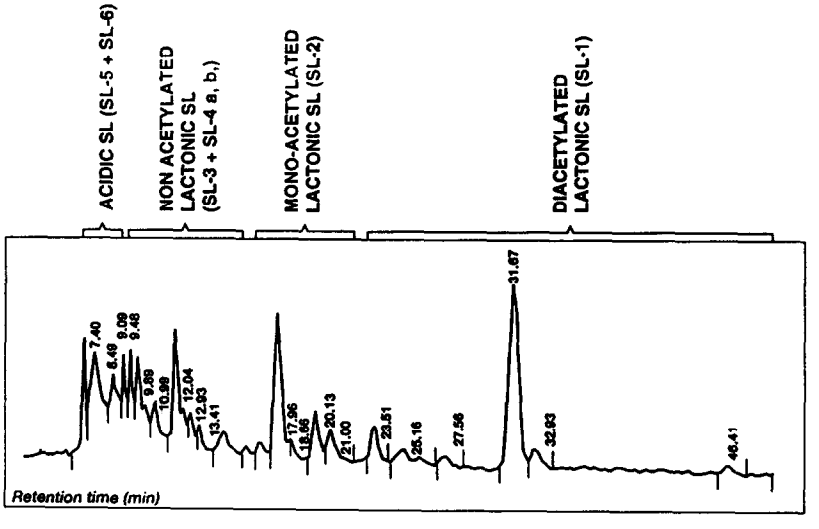


Fig. 2. Separation of a mixture of sophorolipids (sample A) by isocratic HPLC with refractometric detection. For conditions, see Experimental. The locations on the chromatogram of the elution zones for the spots separated in TLC (see Table II) are indicated.

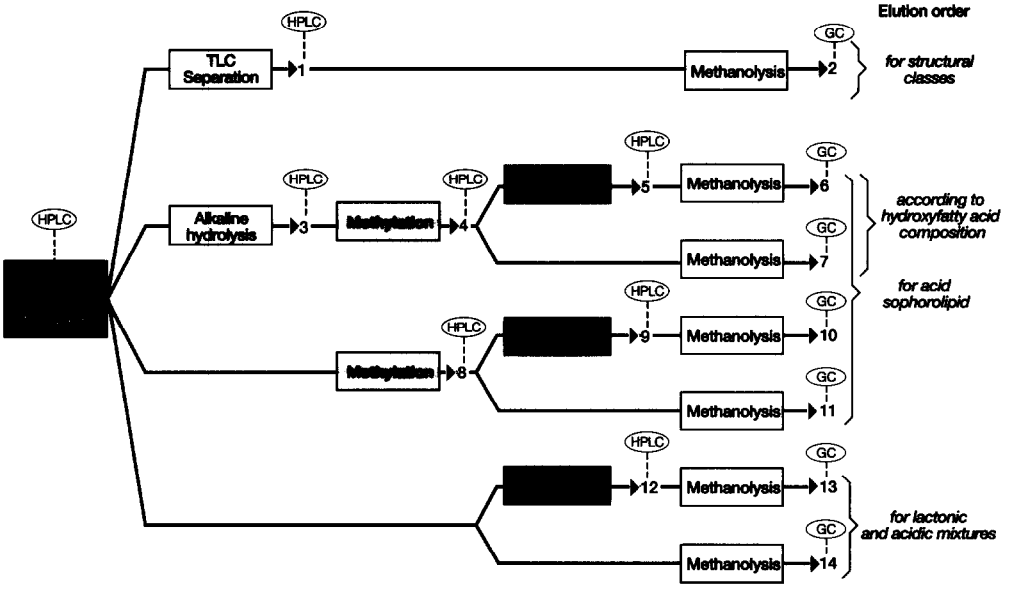


Fig. 3. General strategy for identification of individual sophorolipids. The type of information obtained from the various samples regarding elution order of sophorolipids in HPLC is indicated.

was not clear and therefore their identification in HPLC was ambiguous.

Separation of individual sophorolipids by HPLC: assignment of hydroxy fatty acid moieties

With gradient elution HPLC, it was observed that each class of the mixture isolated by TLC was resolved into several individual components putatively corresponding to homologous sophorolipids varying in their hydroxy acid moieties. The approach adopted to test this hypothesis was to compare on a qualitative and quantitative basis the distribution of sophorolipids obtained in HPLC with that of the corresponding hydroxy fatty acids obtained by GC. For this purpose, a single class, deacylated acidic sophorolipids (Sl-A), which could be obtained in good conditions, was first used (sample A3). It was prepared by alkaline hydrolysis and submitted to HPLC. A sample of Sl-A was also converted into methyl ester derivatives, Sl-mE (sample A4), because methyl esters exhibited a better separation than the corresponding acids in HPLC. Catalytic hydrogenation of the double bonds of the hydroxy fatty acid moieties was then performed on a portion of Sl-mE to yield sample A5. Samples A4 and A5 were submitted to HPLC. For GC analysis, hydroxy fatty acid moieties of samples A4 and A5 were split by methanolysis, yielding samples A7 and A6, respectively. Identification of the hydroxy fatty acids was performed after silylation by coupled GC–MS. The distribution of sophorolipid methyl esters obtained in HPLC (samples A4 and A5) could then be compared with that of the corresponding hydroxy fatty acid methyl esters obtained in GC (samples A7 and A6). Thus the comparison also extended to the effects of catalytic hydrogenation on both types of compounds. Fig. 4 shows that before catalytic hydrogenation the number of compounds was similar according to HPLC or GC. Hydrogenation of the double bonds of hydroxy fatty acids led to analogous changes in the HPLC and GC traces. Assuming the specific responses for all individual sophorolipids to be similar in ELS detection, the relative amounts of sophorolipids were calculated from HPLC and compared with GC data. Table III shows that the distributions of compounds resulting from HPLC and GC determinations could be matched correctly both before and after hydrogenation. Further, the

TABLE III

IDENTIFICATION OF HOMOLOGOUS SOPHOROLIPIDS BY COMPARISON OF THE DISTRIBUTION OF INDIVIDUAL SOPHOROLIPIDS AND OF THEIR CONSTITUTIVE HYDROXY FATTY ACID MOIETIES

Constitutive hydroxy fatty acid	Relative amount before hydrogenation (%)		Relative amount after hydrogenation (%)	
	By GC[a]	By HPLC[c]	By GC[b]	By HPLC[d]
15-OH C_{16}:0	1.7	<1	1.8	1.3
16-OH C_{16}:0	2.2	<1	2.4	2.2
17-OH C_{18}:2	6.3	6.3	–	–
17-OH C_{18}:1	60.6	55.7	–	–
17-OH C_{18}:0	3.2	4.1	69.2	66.7
18-OH C_{18}:2	13.2	18.3	–	–
18-OH C_{18}:1	12.8	15.5	–	–
18-OH C_{18}:0	<0.1	<1	26.6	29.8

[a] Distribution of hydroxy fatty acid methyl esters obtained by methanolysis of deacetylated sophorolipid methyl esters (sample A7).
[b] Similar data for hydrogenated sample (A6).
[c] Distribution of deacetylated sophorolipid methyl esters (sample A4) ordered by matching to the corresponding GC data.
[d] Similar data for hydrogenated sample (A5).

amounts of saturated sophorolipids produced by hydrogenation were globally equal to the sum of the corresponding unsaturated compounds, a similar situation holding also for hydroxy fatty acids. In this way, assignment of a definite hydroxy fatty acid could be done for each individual sophorolipid, thus allowing identification of individual sophorolipid methyl esters.

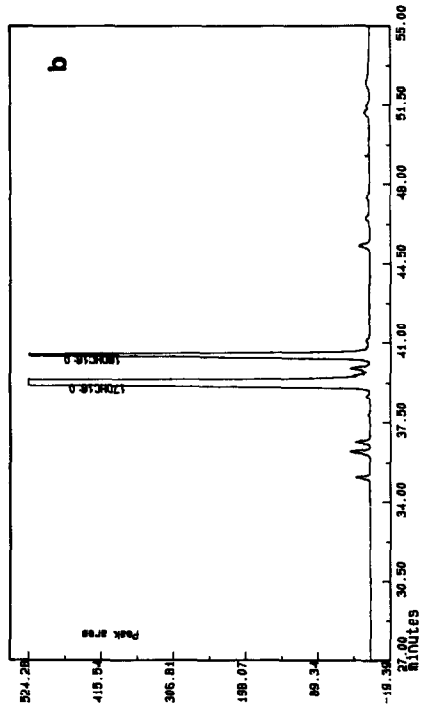
Influence of the nature of hydroxy fatty acid moieties in HPLC of esters and lactones

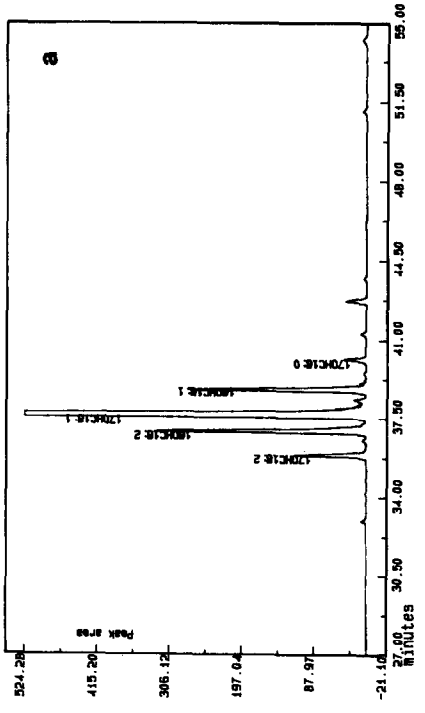
Interpretation of Fig. 4 also allows several observations to be made with respect to the influence of the nature of the hydroxy fatty acid moieties on the elution of sophorolipid methyl esters in HPLC. A first point is that sophorolipids were discriminated according to the degree of unsaturtion of their hydroxy fatty acid moieties and that the more saturated the hydroxy acid, the later it was eluted. Moreover, for an identical fatty acid chain, hydroxy acids bearing the hydroxy group on the terminal (ω) carbon were eluted after their homologs bearing the hydroxy group on the subterminal ($\omega - 1$) position. With more concentrated samples a third rule for elution which had priority over the two first ones was observed: it concerned the number of carbons of the fatty acid chain, the lighter compounds being eluted first (data not shown).

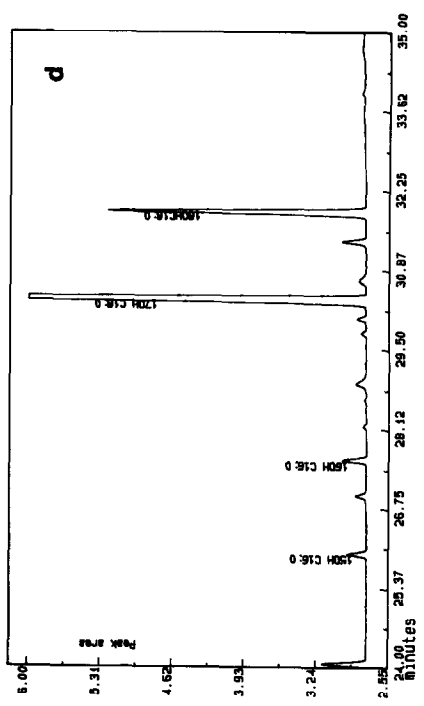
The HPLC elution pattern of individual sophorolipids was then investigated for lactones. The main sample used was obtained by crystallization of the lactonic forms (monoacetylated and diacetylated lactones) according to Tulloch *et al.* [4] from a fermentation mixture. The identity of individual sophorolipids was assigned by matching the sophorolipid and corresponding hydroxy fatty acid distributions as in the case of esters. Elution rules related to hydroxy fatty acid moieties established for esters were usually found to apply also to lactonic forms, with a few exceptions that will be discussed later.

Assessment by HPLC of the separation in TLC of lactonic sophorolipids

Identification of individual sophorolipids allowed the separation by TLC of sophorolipids in structural classes to be assessed. The technique of matching the distributions in HPLC and GC of corresponding compounds was again used. The case of acidic structural classes that are not resolved in TLC (see Table II) will not be discussed. Concerning lactonic forms, more peaks were detected in HPLC than in GC







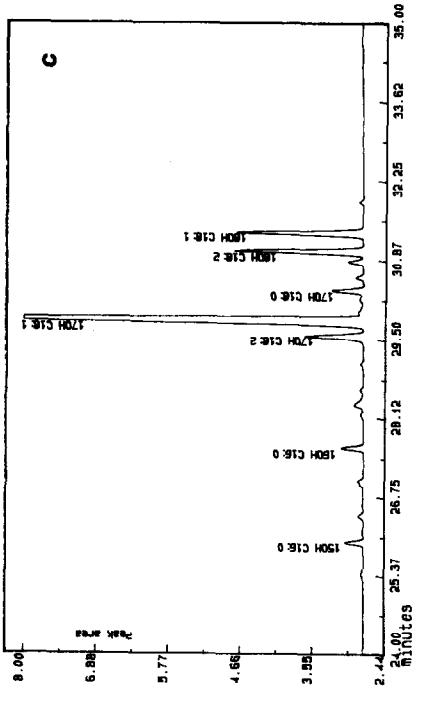


Fig. 4. Identification of homologous sophorolipid methyl esters with different fatty acid compositions. Separation by HPLC of deacetylated sophorolipid methyl esters (Sl-mE). (a) Sample A4; (b) sample A5. Separation by GC of hydroxy acid methyl esters obtained by methanolysis of Sl-mE. (c) Sample A7; (d) sample A6.

analysis for the spots of monoacetylated lactones (SL-2) and non-acetylated lactones (SL-4a, b) (data not shown). This suggested that the separation of classes by TLC was not absolute and that some spots might be constituted by several related classes. For spot SL-4a, b it was found, as already suggested by Asmer *et al.* [5] and indicated in Table II, that it was composed of 1′, 6′ and 1′,6″-lactones (Sl-6′L and Sl-6″L). Further, spot SL-2 appeared here to be constituted of two classes of monoacetylated lactones, two main peaks were observed in HPLC and only one main hydroxy fatty acid (17-OH C_{18}:1) was detected by GC in sophorolipids of SL-2. Therefore, SL-2 was likely to contain, in addition to class Sl-4″L6″a as reported by Asmer *et al.* [5], the related class Sl-4″L6′a. This point was confirmed by kinetic experiments on alkaline hydrolysis under controlled conditions, which showed the disappearance of both sophorolipids at different rates, one of them increasing temporarily (with concomitant hydrolysis of Sl-4″6′a6″a) before decreasing. Structure 4″L6″a, already identified in sophorolipid mixtures and most abundant in our preparation, was tentatively assigned to the most stable class.

Separation of the acidic forms of sophorolipids in HPLC

Sample A and derivatives allowed the identification and characterization of the elution pattern first of esters and then of lactone forms of sophorolipids. A similar study was completed for the acidic forms of sophorolipids using sample B. The deacylated acidic form was prepared (sample B3). To improve the resolution in HPLC, both samples B and B3 were methylated to give samples B8 and B4, respectively. The HPLC traces of these four samples were compared. Table IV shows that acetylated acids were eluted before non-acetylated acids and that the various classes of acetylated sophorolipids were not resolved in HPLC in the case of free acidic sophorolipids. In the present instance, addition of small amounts of acetic acid did not improve the resolution. When acidic forms were methylated (Table IV), the resolution was satisfactory and the elution order was reversed, non-acetylated methyl esters being eluted before

TABLE IV

ELUTION ORDER OF ACIDIC SOPHOROLIPIDS IN HPLC

HPLC separation was performed on a mixture of predominantly acidic sophorolipids (sample B), deacetylated acidic sophorolipids Sl-A (sample B_3), methyl esters of predominantly acidic sophorolipids (sample B_8) and deacetylated sophorolipid methyl esters Sl-mE (sample B_4).

Individual sophorolipid	Retention time (min)	
	Before methylation	After methylation
Sl-(17-OH C_{18}:1) A	32.9	37.3
Sl-(18-OH C_{18}:1) A	34.0	38.5
Sl-(17-OH C_{18}:1) A6′a	31.8	41.1
Sl-(18-OH C_{18}:1) A6′a	32.3	42.4
Sl-(17-OH C_{18}:1) A6″a	31.8	40.1
Sl-(18-OH C_{18}:1) A6″a	32.3	42.0
Sl-(17-OH C_{18}:1) A6′a6″a	31.8	45.0
Sl-(18-OH C_{18}:1) A6′a6″a	32.3	45.7

acetylated compounds. Three series of peaks corresponding to non-acetylated, monoacetylated and diacetylated forms of acidic sophorolipids were observed after esterification, distributed in a very similar way to those of the corresponding lactones. Two classes of monoacetylated forms were present and their identities (Sl-A6″a and Sl-A6′a) were tentatively assigned by analogy with the elution pattern of lactones.

Identification of individual sophorolipids was performed as described previously for sample A4 by combining catalytic hydrogenation and quantitative comparison of sophorolipids with the corresponding hydroxy fatty acid methyl esters obtained by methanolysis. For non-esterified acids, the elution order of homologous sophorolipids in each class was found the same as for esters.

Identification of individual sophorolipids in fermentation mixtures

Direct analysis of fermentation mixtures containing both acidic and lactonic forms of sophorolipids was studied. One such case was sample A from which samples A_{12}, A_{13}, A_{14}

were prepared. The HPLC and GC results for these samples confirmed the previous results.

UV detection was also used in HPLC analysis to confirm the location of unsaturated sophorolipids.

The most important compound of sample A, Sl-(17-OH C_{18}:1) 4″L6′a6″a, was isolated by preparative HPLC. ^{13}C and ^{1}H NMR analysis confirmed the identity of sophorose as the sugar moiety and the presence of all chemical groups constituting the molecule and their respective positions [4,21]. It allowed the purity of the sample analysed to be established as 95% (presence of 5% of the 18-OH C_{18}:1 homologue).

A quasi-complete identification of the individual sophorolipids present in fermentation products is presented for sample A in Fig. 5. The most important unknown compound is located in the Sl-4″L6′a6″a class. This compound has not been identified but several lines of evidence suggest it to be a derivative of 17-OH octadecenoate. As it is different from Sl-(17-OH C_{18}:1) 4″L6′a6″a, it is likely to belong to a structural class differing somehow from 4″L6′a6″a. Minor unknown peaks can also be observed in Fig. 5. Some of them can probably be accounted for by the presence of minor hydroxy fatty acids. Fatty acids such as C_{20}:0, C_{20}:1 and C_{22}:1 are present in rapeseed esters and hydroxy fatty acids such as 19-OH C_{20}:O and 19-OH C_{20}:1 have been reported in sophorolipids [2,22,23].

As already indicated, the elution patterns of homologous sophorolipids according to their hydroxy fatty acid moieties observed for esters also held in most instances for lactones and acids. One of the few exceptions can be seen in Fig. 5. Such cases occurred when the elution times for a given class were markedly different from those of the corresponding esters. Here, for class 4″L6′a6″a, 17-OH C_{18}:2 and 18-OH C_{18}:2 sophorolipids were not resolved and were eluted before their 15-OH C_{16}:0 and 16-OH C_{16}:0 homologues.

Finally, the data on the characterization of individual sophorolipids by HPLC obtained from samples of fermentations with rapeseed ethyl esters were also confirmed and extended by similar investigations with samples from fermentations with other lipidic carbon sources such

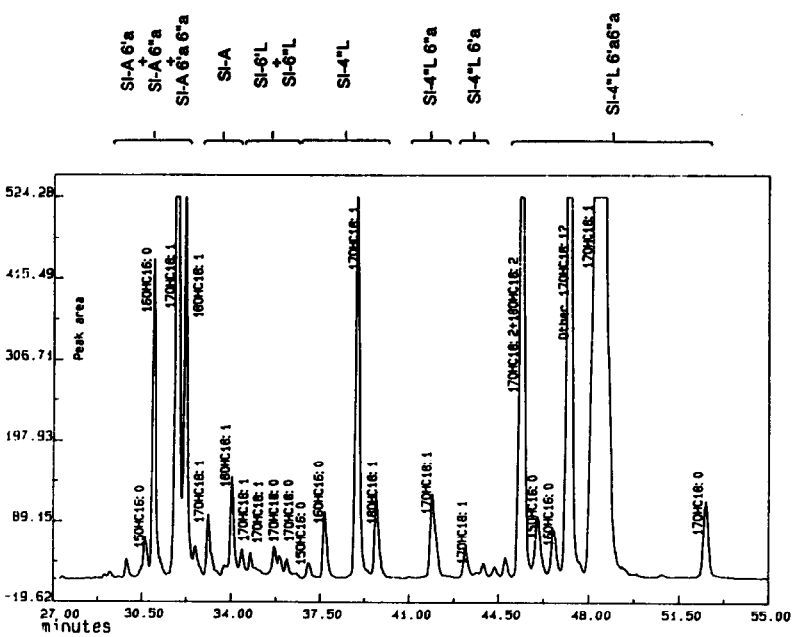


Fig. 5. Elution order in gradient elution HPLC of a mixture of sophorolipids obtained by fermentation (sample A). The identification of structural classes and of individual sophorolipids is presented.

as hexadecane and sunflower oil, which yielded sophorolipids with different hydroxy fatty acid compositions.

CONCLUSIONS

Gradient elution HPLC with ELS detection proved to be a powerful and rapid tool for qualitative and quantitative analysis of sophorolipids, as a single chromatographic analysis of a complex mixture allowed almost all significant individual compounds (over 20) present in the mixture to be identified and determined without prior preparation of the sample.

The ELS detector was found able to detect with excellent sensitivity all sophorolipids present. Within the concentration range utilized, corresponding to injected amounts of 50–400 μg of sophorolipid mixture, linearity of response was observed. The results are in line with the opinion that this detector can be considered as universal for non-volatile compounds [18].

Isolation and direct identification were not done for most of the compounds [only Sl-4″L6′a6″a and Sl-A classes were isolated and structure of the major compound Sl-(17-OH C_{18}:1) 4″L6′a6″a confirmed by NMR]. However, for most other sophorolipids, structure assignment resulted from mutually confirmative lines of evidence and can be conferred a high degree of confidence.

Several sophorolipids classes not yet reported were identified at least tentatively in this study, namely Sl-4″L6′a, Sl-A6″a and Sl-A6′a6″a, and the existence of more of them may be suspected.

The strong dependence of sophorolipid composition on both the nature of the lipidic substrate and on the fermentation conditions has already been mentioned or illustrated here. The technique developed in this study opens up this area to systematic investigation.

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REFERENCES

1 P.A.J. Gorin, J.F.T. Spencer and A.P. Tulloch, *Can. J. Chem.*, 39 (1961) 846.
2 J.F.T. Spencer, D.M. Spencer and A.P. Tulloch, in A.H. Rose (Editor), *Economic Microbiology*, Vol. 3, Academic Press, London and New York, 1979, p. 523.
3 D.F. Jones, *J. Chem. Soc. C*, (1967) 479.
4 A.P. Tulloch, A. Hill and J.F.T. Spencer, *Can. J. Chem.*, 46 (1968) 3337.
5 H.J. Asmer, S. Lang, F. Wagner and V. Vray, *J. Am. Oil Chem. Soc.*, 65 (1988) 1640.
6 D.F. Jones and R. Howe, *J. Chem. Soc. C*, (1968) 2801.
7 V. Klekner, N. Kosaric and Q.H. Zhou, *Biotechnol. Lett.*, 13 (1991) 345.
8 A.-M. Davila, R. Marchal and J.-P. Vandecasteele, *Appl. Microbiol. Biotechnol.*, 38 (1992) 6.
9 T.H. Mourey and L.E. Oppenheimer, *Anal. Chem.*, 56 (1984) 2427.
10 L.E. Oppenheimer and T.E. Mourey, *J. Chromatogr.*, 323 (1985) 297.
11 P.B. Stockwell and B.W. King, *Int. Chromatogr. Lab.*, 7 (1991) 4.
12 A. Stolyhwo, M. Martin and G. Guiochon, *J. Liq. Chromatogr.*, 10 (1987) 1237.
13 A. Stolyhwo, H. Colin and G. Guiochon, *Anal. Chem.*, 57 (1985) 1342.
14 W.W. Christie, *J. Lipid Res.*, 26 (1985) 507.
15 J. Becart, C. Chevalier and J.P. Biesse, *J. High Resolut. Chromatogr.*, 13 (1990) 126.
16 L. Breton, B. Serkiz, J.P. Volland and J. Lepagnol, *J. Chromatogr.*, 497 (1989) 243.
17 T.L. Mounts, S.L. Abidi and K.A. Rennick, *J. Am. Oil Chem. Soc.*, 69 (1992) 438.
18 W.W. Christie, *Rev. Fr. Corps Gras*, 38, No. 516 (1991) p. 155.
19 G.R. Bear, *J. Chromatogr.*, 459 (1988) 91.
20 D. Herbert, P.J. Philips and E. Strange, in J.R. Norris and D.W. Ribbons (Editors), *Methods in Microbiology*, Vol. 5B, Academic Press, London, 1971, p. 209.
21 L. Weber, J. Stach, G. Haufe, R. Hommel and H.-P. Kleber, *Carbohydr. Res.*, 206 (1990) 13.
22 A.P. Tulloch, J.F.T. Spencer and P.A.J. Gorin, *Can. J. Chem.*, 40 (1962) 1326.
23 A.P. Tulloch, in L.A. Wittling (Editor), *Glycolipid Methodology*, American Oil Chemists Society, Champaign, IL, 1976, p. 329.