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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY OF RED ALGAL BROMOPHENOLS

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SUMMARY

Red algal bromophenols have been analyzed by reversed-phase high-performance liquid chromatography using ion-suppression and a column stationary phase modifier. Isocratic elution produced good separation of a wide variety of bromophenols containing many types of functional groups. Aspects of the development of the method are discussed.

INTRODUCTION

Since the time of their initial discovery[1], halophenols have received increasing interest from marine natural products chemists. These compounds have been isolated from a number of algae representing several orders of the *Rhodophyta*[2–14].

Paper chromatography[15,16], thin-layer chromatography[3,5,11,16–21], column chromatography[6,8,9,12,14,17], gas–liquid chromatography (GLC)[11,13,19] and combined GLC–mass spectrometry (MS)[7,10,19,21–24] have been used extensively for the separation leading to subsequent structural elucidation of this group of phenols. Perhaps the most useful information regarding the array of halophenols within each alga so far examined has come from GLC–MS analyses. Unfortunately, the expense of installation and upkeep of such complex instruments make their use generally prohibitive. However, recent advances in the development of high-performance liquid chromatography (HPLC) systems make them comparatively inexpensive and therefore more widely available for the screening of organisms for various groups of compounds. Accurate and sensitive detection systems, when coupled to modern LC equipment, make possible the separation and detection of micro- to nanogram quantities of most compounds.

Because of the ease of sample preparation (derivatization as in GLC is not necessary) and the resolution and sensitivity achieved by this method, we chose to apply it to the determination of bromophenols from three species of red algae in British Columbia. *Rhodomela larix* (Turner) C. Agardh, having received the scrutiny of several previous chemical investigations[5,17], was chosen as a model organism on which to test the effectiveness of the HPLC method. This alga contains a variety of bromophenols, some of which, however, are thought to be artefacts of isolation procedures[5,7].

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EXPERIMENTAL

Chemicals

Standards of 2,3-dibromo-4,5-dihydroxybenzyl alcohol (2), (lanosol), 3-bromo-4,5-dihydroxybenzaldehyde (3), 3,5-dibromo-4-hydroxybenzyl alcohol (5), 3,5-dibromo-4-hydroxybenzoic acid (6) and 3,5-dibromo-4-hydroxybenzyl methyl ether (14) were kindly provided by Dr. J. S. Craigie (Atlantic Regional Lab., Halifax, Canada). Tetramethylammonium chloride (TMA) and 3,4-dihydroxybenzaldehyde were purchased from Aldrich, Milwaukee, WI, U.S.A. and HPLC-grade acetonitrile from Fisher Scientific, Pittsburgh, PA, U.S.A. All other chemicals and solvents were from various sources and were of the highest quality obtainable.

Synthesis of other bromophenols

The following syntheses were performed on a microscale to provide additional standards for comparison with crude extracts. Since purification of the products was not attempted due to the small quantities involved, the identity of many of the standards remains tentative. However, the synthetic methods utilized are generally well-known and for the most part produce a single product.

3-Bromo-4,5-dihydroxybenzyl alcohol (1). Approximately 1.0 mg (30 μM) sodium borohydride was slowly added to 2.0 mg (1.0 μM) of compound 3. When the reaction had ceased, 0.1 ml water was added to hydrolyze the hydroborane and free the alcohol.

3-Bromo-4,5-dihydroxybenzyl methyl ether (4). To 0.3 ml of the reaction mixture of compound 1 above, two drops of 2 *N* hydrochloric acid (HCl) were added. After heating for 1 h on a steam bath, a mixture of unreacted compound 1 and its benzyl methyl ether was found.

2,3-Dibromo-4,5-dihydroxybenzaldehyde (7). 1.0 mg (0.4 μM) of compound 2 was dissolved in 0.3 ml dimethyl sulfoxide and 0.2 ml acetic anhydride and heated on a steam bath for 15 min[25] to yield a single product. When possible (*i.e.*, for the supplied standards) verification of compound identity in algal extracts (see below) was achieved by co-chromatography with the authentic standard at different solvent strengths and flow-rates.

2,5-Dibromo-3,4-dihydroxybenzaldehyde (9). The detailed procedure for the preparation of this compound has been given by Lundgren *et al.*[12]. 1.8 g (0.13 *M*) freshly sublimed 3,4-dihydroxybenzaldehyde was used as starting material. Concentration of the reaction solvent produced white crystals which were recrystallized from ethanol (yield = 0.38 g, 21%), m.p. 178–180°C. Infrared and proton magnetic resonance spectroscopy agreed with those previously reported[12].

3,5-Dibromo-4-hydroxybenzaldehyde (10). 1.0 mg (0.5 μM) of compound 5 was worked up as in 7 above. However, this reaction produced three products as monitored by HPLC. In addition to compound 10 the other products are thought to be the hemiacetal (12) and acetal (13) of the starting material. Aqueous acid addition and continued heating caused total conversion to the aldehyde. An alternative oxidation procedure which yielded the aldehyde as sole product consisted of heating 1.0 mg of the alcohol (5) and 2 mg manganese dioxide in 0.5 ml methanol for 15 min.

2,3-Dibromo-4,5-dihydroxybenzyl ethyl ether (11). The procedure was the same as in the preparation of compound 8 except that methanol was replaced by ethanol.

Chromatography

HPLC was performed on a Varian Model 5000 liquid chromatograph linked to a Variscan 634 S UV–VIS spectrophotometer at a constant wavelength of 280 nm. The elution solvent consisted of 40% acetonitrile (in glass-distilled water) containing 10 m*M* each of TMA and dibasic sodium phosphate (buffer). The pH of the solvent was adjusted to 3.2–3.5 with concentrated HCl.

Initially, gradient elution with 80% acetonitrile and water (both containing additives) was used to determine optimal solvent strength for best resolution of the standards in the shortest time. Once established, isocratic elution with the 40% acetonitrile solvent proved the most useful and economical.

Octadecylsilane columns of Micropak MCH-10 (Varian) were used for separations in the reversed-phase mode. All compounds analyzed as standards were used in concentrations of about 1 mg/ml in methanol to give roughly equivalent detector responses. Injection volumes were typically 1–10 μl. When possible (*i.e.*, for the supplied standards) verification of compound identity in algal extracts (see below) was achieved by co-chromatography with the authenic standard at different solvent strengths and flow-rates.

Evaluation of the chromatographic method

Three modified solvent systems and a mixture of compounds 5 and 6 were used (in addition to the solvent system described above) for the evaluation of column selectivity and efficiency: (A) acetonitrile–water (4:6) only; (B) A plus 10 m*M* buffer (pH 3.2); (C) A plus 10 m*M* TMA; (D) acetonitrile–water (4:6) solvent containing both additives. Each system used independently gave an indication of the effectiveness of an individual component to the method as a whole. From these measurements four chromatographic parameters were calculated: capacity factor (k'), relative retention (α), number of theoretical plates (N) and resolution (R)[26].

Collection and extraction of algae

Rhodomela larix. Approximately 2 kg fresh weight of algae were collected by scuba off Bath Island, British Columbia, Canada. Visible epiphytes having been removed, the plants were washed in fresh water and immediately deep frozen at −80°C. Following lyophilization, the algae were ground in a Wiley mill to pass through a 2-mm mesh screen (yield = 447 g dry weight). Soxhlet extraction of the dried material using a series of solvents [light petroleum (b.p. 30–60°C), chloroform, ethyl acetate and methanol] gave four fractions. Each fraction was reduced in volume *in vacuo* (<40°C) to about 50 ml. The large amount of solid (42.9 g) deposited on concentration of the methanol extract was removed by filtration and identified as the dipotassium sulfate salt of lanosol[5]. 1.0 ml of each of four fractions was taken to dryness, redissolved in 1.0 ml methanol and analyzed by HPLC for bromophenols.

Ceramium washingtoniense Kylin and Prionitis lyallii Harvey. 10.0 g of each alga were ground in a Waring blender in boiling 80% methanol and refluxed for 1 h on a steam bath. The extracts were acidified to pH 2 with 1 *N* HCl, warmed to 60°C for 15 min and filtered. The methanol was removed *in vacuo* and the remaining water extracted three times with ethyl acetate. The ethyl acetate layers were combined and the solvent removed *in vacuo*. The residues were taken in 5.0 ml methanol and used for HPLC analysis.

RESULTS AND DISCUSSION

Separation of standard compounds

Fig. 1 illustrates the separation of 14 bromophenol standards at a flow-rate of 1 ml/min (pressure 1120 p.s.i.). Retention times, capacity factors and relative retention data for these compounds are given in Table I.

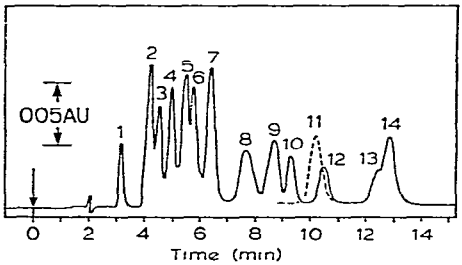

Fig. 1. Reversed-phase HPLC separation of fourteen bromophenol standards. The relative position of compound 11 is shown by a dashed line. Compounds 1, 4 and 7–13 are tentatively identified.

TABLE I

RETENTION TIMES, CAPACITY FACTORS AND RELATIVE RETENTIONS OF STANDARD BROMOPHENOLS SEPARATED BY REVERSED-PHASE HPLC

Flow-rate, 1.0 ml/min. Dead volume (t_0), 1.8 min.

Compound	t_R *(min)*	k'	α
(1) 3-Bromo-4,5-dihydroxybenzyl alcohol*	3.1	0.72	
(2) 2,3-Dibromo-4,5-dihydroxybenzyl alcohol	4.15	1.13	1.82
(3) 4-Bromo-4,5-dihydroxybenzaldehyde	4.55	1.53	1.17
(4) 3-Bromo-4,5-dihydroxybenzylmethyl ether*	4.9	1.72	1.12
(5) 3,5-Dibromo-4-hydroxybenzyl alcohol	5.4	2.0	1.16
(6) 3,5-Dibromo-4-hydroxybenzoic acid	5.8	2.22	1.11
(7) 2,3-Dibromo-4,5-dihydroxybenzaldehyde*	6.3	2.5	1.13
(8) 2,3-Dibromo-4,5-dihydroxybenzyl methyl ether*	7.7	3.28	1.31
(9) 2,5-Dibromo-3,4-dihydroxybenzaldehyde*	8.6	3.78	1.15
(10) 3,5-Dibromo-4-hydroxybenzaldehyde*	9.25	4.14	1.10
(11) 2,3-Dibromo-4,5-dihydroxybenzyl ethyl ether*	10.0	4.67	1.13
(12) Hemiacetal of 7 above*	10.4	4.78	1.02
(13) Acetal of 7 above*	12.7	6.06	1.27
(14) 3,5-Dibromo-4-hydroxybenzyl methyl ether	12.9	6.17	1.02

* Identification tentative.

The elution order of the compounds is, as expected, the reverse of that of a normal phase separation. The more polar alcohols and acids elute first, followed by aldehydes and ethers. *Ortho*-dihydroxy functions increase polarity while an *ortho*-dibromo function reduces polarity. Methyl and ethyl ethers are rendered quite non-polar compared to their free hydroxyl counterparts. The ethyl ether of lanosol (11) is included to show the effect of extended chain length on retention time. Also of interest is the decreased polarity of the *para*- (9) *versus ortho*-dibromoaldehyde (7).

Evaluation of the chromatographic method

Initial attempts at the separation of bromophenols by reversed-phase LC met with little success: lanosol was irreversibly retained on the column, acids showed no retention and aldehydes gave long retention times with very broad and tailing peaks. Some authors have noted that repeated injections of an irreversibly retained compound eventually give results[27]; however, the level of the compound retention is probably increased to the point that all column reactive sites are filled before elution occurs. Such gross contamination must be considered undesirable since it inevitably shortens column life, not to mention its effect on efficiency.

In an effort to reduce these problems, suitable solvent modifiers were sought which would improve selectivity and efficiency while maintaining capacity. Since most phenols are quite acidic ($K_a = 10^{-10}$) and are easily converted to phenoxide anions in solution, the first method chosen to improve retention capability was ion suppression. This technique is commonly used for the LC of phenols[27] as it generally improves peak symmetry by suppressing tailing.

For many compounds, however, there is still the problem of irreversible retention, which can be overcome simply and effectively by adding a phase altering reagent to the solvent. TMA has been used for this purpose[28]. This modifier is thought to selectively bind to unblocked silanol groups in the column packing material; the quartenary ammonium salt probably interacting ionically with the silanol group. Compounds contacting the stationary phase on a reversed-phase column thus modified are then exposed to a more complete nonpolar environment. The possibility of hydrogen bonding to silanol functions in the packing, as we believe to occur in the case of lanosol, is therefore negated.

The combination of ion suppression and TMA addition improves separation capability considerably. The results of the evaluation of each component taken separately and then in combination are shown in Table II. As noted from this table, TMA triples column selectivity and also increases column efficiency in the separation of compounds 5 and 6, a closely related alcohol and acid. Selectivity is reduced by addition of the buffer, although in the dual component system, efficiency is still maintained at a high level. Selectivity can easily be increased by reducing solvent strength or flow-rate.

TABLE II

RESOLUTION (R) AS A FUNCTION OF COLUMN EFFICIENCY (N), SELECTIVITY (α) AND CAPACITY (k') USING DIFFERENT SOLVENT SYSTEMS IN THE SEPARATION OF 3,5-DIBROMO-4-HYDROXYBENZYL ALCOHOL AND THE CORRESPONDING ACID

The retention time of the alcohol is used for calculation of k' values.

Solvent system	N	α	k'	R
(A) Acetonitrile–water (4:6)	557	No separation		
(B) A plus 10 m*M* buffer	304	1.20	2.04	0.49
(C) A plus 10 m*M* TMA	340	3.76	1.95	2.22
(D) B plus 10 m*M* TMA	321	1.11	2.0	0.30

Analysis of Rhodomela extracts

The test of a chromatographic system comes when it is applied to the separation of a complex mixture such as a crude extract. The separation achieved for the

four fractions of the Soxhlet extract of *Rhodomela larix* are shown in Fig. 2. Most of the standard compounds appear in trace amounts in the light petroleum fraction. Lanosol is the major component in the chloroform fraction, and the aldehyde (7) in the ethyl acetate fraction. Methyl ethers (4, 8 and 14) appear only in the methanol extract.

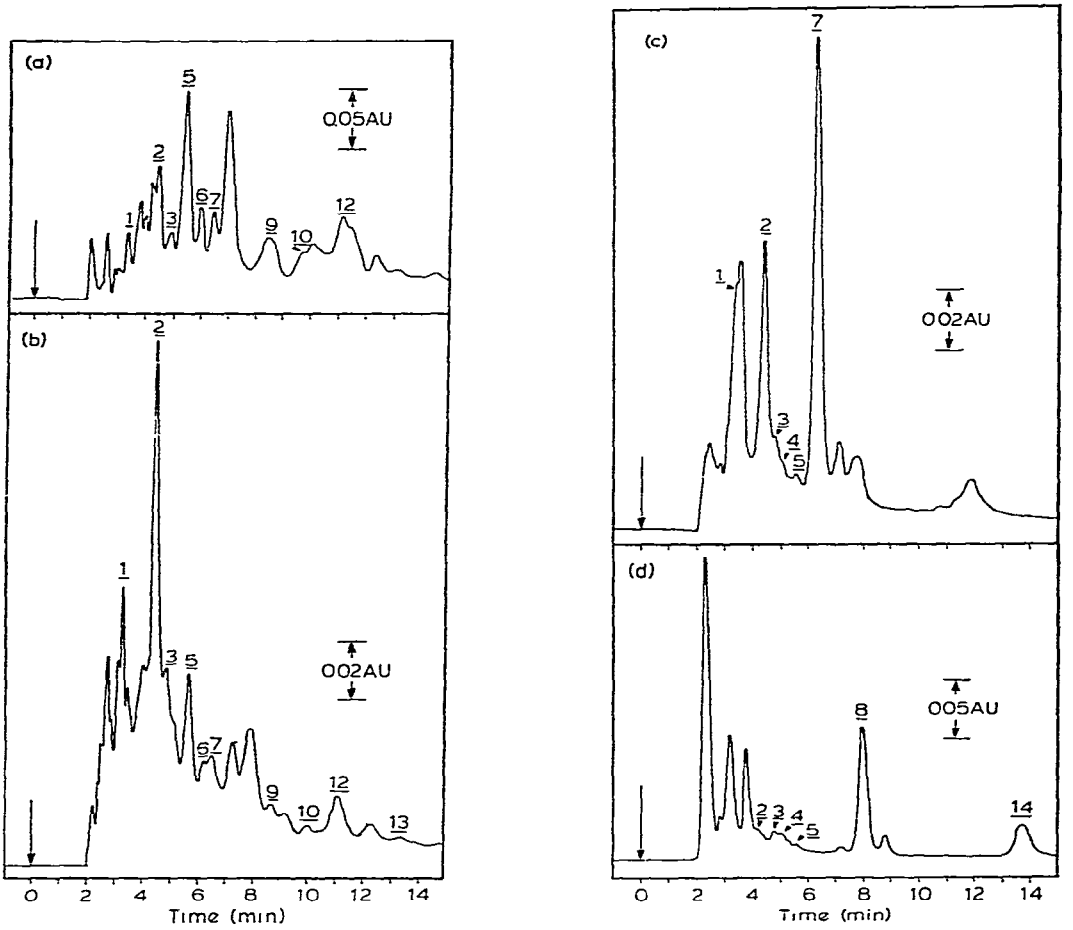

Fig. 2. Isocratic separation of bromophenols in four fractions from the large scale (Soxhlet) extraction of *Rhodomela larix*. (a) Light petroleum; compounds 1, 7, 9, 10 and 12 are tentatively identified. (b) Chloroform; compounds 1, 7, 9, 10, 12 and 13 are tentatively identified. (c) Ethyl acetate; compounds 1 and 4 are tentatively identified. (d) Methanol; compounds 4 and 8 are tentatively identified.

In previous studies the aldehydes and methyl ethers of lanosol and related compounds have been considered to be artefacts of isolation procedures. There is little doubt that ethyl and methyl ethers fall into this category; however, the presence of aldehyde (7) throughout all of the four fractions causes us to believe that such compounds are natural constituents of the algae. Even the most mild extraction procedures[10,29] show the presence of these compounds. The possibility of their existence in other species examined so far should, therefore, be re-evaluated.

Chromatographic analyses of other red algae

In this study two other algal species, *Ceramium washingtoniense* and *Prionitis lyallii*, were examined for the presence of bromophenols. The LC traces for the extracts of these two species appear in Fig. 3. Both algae contain bromophenols, *Ceramium* containing only one major compound (2).

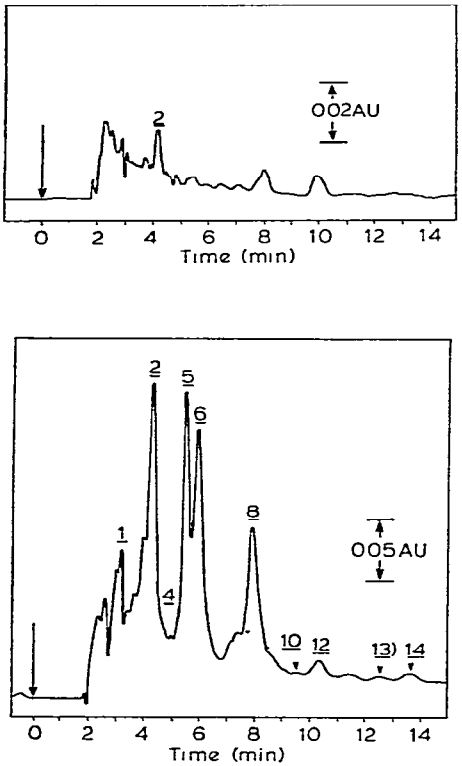

Fig. 3. Isocratic HPLC analysis of bromophenols extracted from *Prionitis lyallii* (lower trace) and *Ceramium washingtoniense* (upper trace). Compounds 1, 4, 8, 10, 12 and 13 are tentatively identified.

Prionitis, like *Rhodomela*, contains a variety of bromophenols, the most abundant being lanosol and the *meta*-dibromo acid (6) and alcohol (5). A more careful extraction of both *Prionitis* and *Ceramium* may lead to the identification of an even wider array of phenolic compounds.

In conclusion, HPLC is a rapid and sensitive method for the determination of bromophenolic compounds in algae. Column selectivity and efficiency in reversed-phase separations can be improved by the addition of modifiers which alter the nature of the stationary phase or suppress ionization when, for example, the separation of acidic metabolites is necessary. Isocratic elution with solvents containing modifiers produces adequate separation of a wide variety of bromophenols containing many types of functional groups. Under these conditions the need for chromatographs equipped with complex gradient forming devices is reduced.

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