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Evaluation of high-performance liquid and capillary gas chromatography for analysis of sesquiterpene lactones of the Melampodiinae

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SUMMARY

The capacity of reversed-phase high-performance liquid chromatography (HPLC) and capillary gas chromatography (GC) to separate 37 sesquiterpene lactones of the Melampodiinae has been investigated. The HPLC system employed a Hypersil ODS column and methanol–water as eluent for isocratic and gradient separations. The GC system utilized a RSL-150 bonded phase capillary column with flame ionization detection. The previously characterized melampolide melnerin B was determined by both HPLC and GC to be a mixture of two isomers, containing a 2-methylbutanoate (melnerin B) or a 3-methylbutanoate (melnerin B′) substituent at C-8. The HPLC and GC methods developed have been applied to the analysis of a crude extract of *Melampodium cinereum*.

INTRODUCTION

Sesquiterpene lactones are a structurally diverse group of over 3000 natural products isolated primarily from plants of the Asteraceae. Many have important biocidal and pharmaceutical activities[1,2]. The leaves of feverfew, *Tanacetum parthenium*, have yielded several sesquiterpene lactones which inhibit prostaglandin synthesis and are used for the treatment of migraine headaches[3,4]. A highly potent molluscicide, 7α-hydroxy-3-desoxyzaluzanin C has been isolated from *Podachaenium eminens* (Asteraceae)[5]. Dihydroparthenolide, a constituent of common ragweed, *Ambrosia artemisiifolia*, stimulates germination of the devastating parasitic weed *Striga asiatica* at nanomolar levels[6].

Less work has been done on the ecological functions of these compounds[2,7]. However, because of their high alkylating power, which is due to the α-methylene-γ-lactone moiety which most possess, along with α,β-unsaturated carbonyl and epoxide moieties in certain compounds, it may be expected that these compounds will exhibit toxic effects against many organisms and play an important role in the chemical

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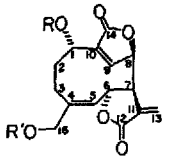
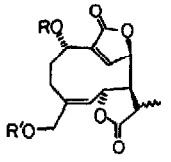
(a) LEUCANTHOLIDES

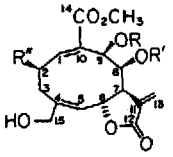


	R	R'
(1) Melampodin B	Ac	H
(2) Melampodin B Acetate	Ac	Ac
(3) Cinerenin	C_2H_5	H
(4) Cinerenin Acetate	C_2H_5	Ac
(5) Melampodin C	A	H
(6) Melampodin C Acetate	A	Ac
(7) Melampodin D	C	H



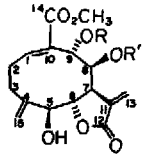
	R	R'
(8) 11,13-Dihydrocinerenin	C_2H_5	H

CIS,CIS-GERMACRANOLIDES



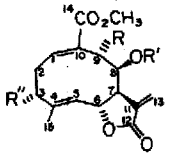
	R	R'	R''
(9) Melcanthin A	Ac	D	H
(10) Melcanthin B	Ac	D	OH
(11) Melrosin A	B	B	OH
(12) Longicornin B	A	A	H

REPANDOLIDES

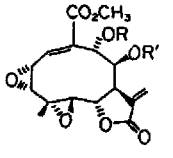


	R	R'
(13) Repandin A	E	A
(14) Repandin B	E	C

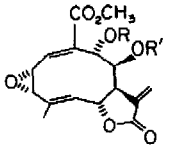
MELAMPOLIDES



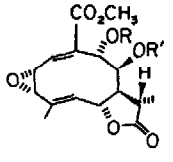
	R	R'	R''
(15) Melampolidin	H	I	H
(16) Leucanthinin	OAc	H	OH
(17) Desacetoxyleucanthinin	H	H	OH
(18) Leucanthinin Acetate	OAc	H	OAc



	R	R'
(19) Leucanthin B	Ac	H
(20) Desacetylleucanthin B	H	H



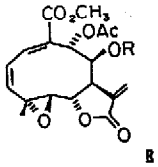
	R	R'
(21) Melampodin A	H	H
(22) Melampodinin A	Ac	I
(23) Melampodinin B	C	I



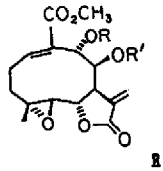
	R	R'
(24) 11,13-Dihydromelampodin A 9-α-[2-methylbutanoate]	C	H

Fig. 1.

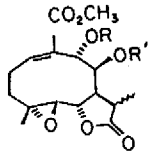
(b)



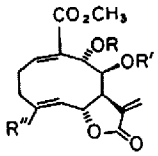
	R
(25) Leucanthin A	H



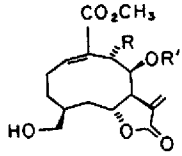
	R	R'
(26) Longipilin	H	D
(27) Enhydrin	Ac	H
(28) Desacetylenhydrin	H	H



	R	R'
(29) 11,13-Dihydroenhydrin	Ac	H



	R	R'	R''
(30) Longipin	H	H	CH_2OAC
(31) Tetrahelin C	C	I	CH_3
(32) Tetraludin C	F	G	CH_3



	R	R'
(33) Melnerin A	H	A
(34) 9-∝-Acetoxymelnerin A	OAc	A
(35) Melnerin B	H	C
(36) Melnerin B'	H	J
(37) 9-∝-Acetoxymelnerin B	OAc	C

ESTER SIDE CHAINS

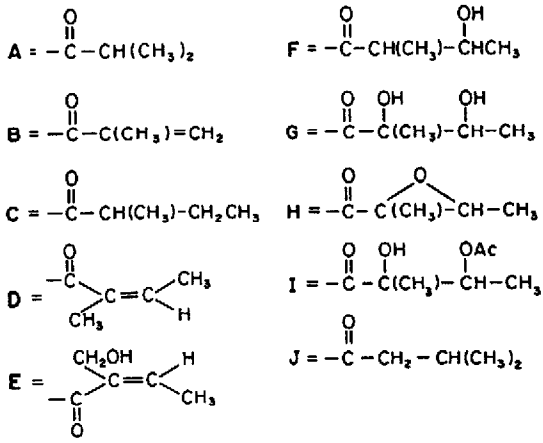


Fig. 1. Sesquiterpene lactones of the Melapodiinae. Ac = CH_3CO; OAc = CH_3COO.

defenses of plants which contain them. Evidence is accumulating that sesquiterpene lactones deter insect feeding[8,9], reduce the survival of insect larvae and adults[10,11], and inhibit germination and growth of other plants[12,13].

Melampolide-type sesquiterpene lactones (Fig. 1) are characteristic of plant species in the subtribe Melampodiinae of the Asteraceae[14]. The restricted distribution of many of these compounds has been used to clarify evolutionary relationships in this subtribe. There is evidence that melampodin A (**21**) and melampodinin A (**22**) deter insect feeding and inhibit insect growth[15].

The development of rapid, quantitative analytical methods for plant natural products is essential to studies of chemosystematics, chemical ecology and the monitoring of their production in cell and hairy root cultures[16]. Analytical high-performance liquid chromatography (HPLC) has been applied successfully to the separation of 33 pseudoguaianolides and xanthanolides of the genus *Parthenium*[17] and to the analysis of 21 pseudoguaianolides of *Arnica chamissonis*[18], as well as other compounds[19,20]. Acetonitrile–water[17] or methanol–water[18] gradients have been employed using reversed-phase columns, with detection at 210–225 nm to provide sensitivity. Successful separation of sesquiterpene lactones from *Helianthus* has been obtained with an isocratic methanol–water system[19]. Capillary gas chromatography (GC) has also been applied with success for certain underivatized sesquiterpene lactones[18,21]. Micro-sampling techniques developed by Spring *et al.*[20] have demonstrated that it is possible to directly sample the glandular trichomes of leaves and flower heads, where sesquiterpene lactones are sequestered.

The application of reversed-phase HPLC and capillary GC to sesquiterpene lactones of the Melampodiinae is reported in this paper. We have applied these methods to the analysis of a crude terpenoid extract of *Melampodium cinereum*.

EXPERIMENTAL

HPLC apparatus

HPLC analyses were performed on a Hewlett-Packard 1090 liquid chromatograph equipped with a diode array detector and auto-injector (25-μl syringe). Detection channels were set at 210, 220, 230 and 254 nm, with a bandwidth of 16 nm. Chromatograms were recorded and analyzed on a Hewlett-Packard HPLC Chemstation (Series 300 computer). The column was a 5-μm Hypersil ODS (150 × 4.6 mm I.D., Hewlett-Packard, Mt. View, CA, U.S.A.). Analyses were performed at ambient temperature with a flow-rate of 1.0 ml/min.

Elution

Two solvents were used: (A) HPLC-grade methanol and (B) distilled, deionized water. Compounds were eluted isocratically with 50% A. The gradient elution profile was 0–18 min, 20% A (isocratic); 18–36 min, 20–50% A (linear gradient); 36–46 min, 50–70% A (linear gradient); and 46–50 min, 70% A (isocratic).

GC equipment and experimental conditions

The GC system consisted of a Hewlett-Packard 5890 gas chromatograph equipped with a split/splitless injector system and a flame ionization detector. Nitrogen was the carrier gas and the carrier gas pressure was 20 p.s.i. Injections were

made in the split mode with a splitting ratio of 4:1. A 45 m × 0.25 mm I.D. fused-silica capillary column with a bonded polymethylsilicone stationary phase (RSL-150, Alltech, Deerfield, IL, U.S.A.) of 0.2-μm film thickness was used for the separations. Two temperature gradients were employed. For the first (G1), the initial temperature of 95°C was held for 2 min, increased to 285°C at 15°C/min and held at 285°C for 10 min. For the second (G2), the initial temperature of 125°C was held for 1 min, increased to 260°C at 15°C/min and held at 260°C for 15 min. The injection volume was 1 μl unless noted otherwise.

Samples

Compounds were obtained from the sample collection in our laboratory. A crude terpenoid extract of *Melampodium cinereum* var. *cinereum* was prepared by standard procedures[22] (Fischer No. 3792). Dried plant material was extracted with dichloromethane. After solvent removal by evaporation, the residue was taken up in 95% aqueous ethanol (100 ml/100 g plant material). To this solution an equal volume of 5% aqueous lead acetate was added and left overnight. The precipitated phenolics and chlorophyll were removed by filtration through Celite and the filtrate evaporated *in vacuo* to remove most of the ethanol. The residual material was extracted exhaustively with dichloromethane and this solvent evaporated to obtain the crude extract. Solutions of the sesquiterpene lactones and the extract were prepared in methanol to contain 1 mg/ml. Partial chemical change of a few compounds occurred over the several weeks they were kept in methanol solution. This was evidenced by the appearance of additional peaks and altered HPLC and GC retention times. These included melampodin B (**1**), melampodin C (**5**), longicorin B (**12**) and longipin (**30**).

RESULTS AND DISCUSSION

HPLC separations

HPLC (and GC) retention times of the 37 sesquiterpene lactones examined are listed in Table I. HPLC separations were performed both isocratically and using a gradient. Most compounds separated from one another with the isocratic system. The gradient developed increased the separation of the most polar compounds, allowing confirmation of their identities in plant extracts. Because of the presence of a restricted number of sesquiterpene lactones in any one species, there is only a slight likelihood of overlapping peaks in a given extract.

Of the various structural types of sesquiterpene lactones included in this study, the leucantholides were the least strongly retained, probably due to the added polarity given by the second lactone ring. The elution order of the eight individual leucantholides correlates well with the lipophilicity of the compounds. For example, elution of melampodin B (**1**, OR = acetate), cinerenin (**3**, OR = ethoxy), melampodin C (**5**, OR = 2-methylpropanoate) and melampodin D (**7**, OR = 2-methylbutanoate) was in the order **1** < **3** < **5** < **7**. One notable exception was 11,13-dihydrocinerenin (**8**), which eluted prior to cinerenin. Interestingly, Leven and Willuhn[18] also found that 11,13-dihydro compounds eluted before the homologous sesquiterpene lactones with an exocyclic methylene group.

The elution order of sesquiterpene lactones from the other structural classes examined also correlates well with the lipophilicity of the compounds. The presence of

TABLE I

HPLC AND GC RETENTION TIMES OF SESQUITERPENE LACTONES OF THE MELAMPODIINAE

t_R = Retention time; t_0 = dead time; na = data not available; I = injection volume 6 μl; T = severely tailed peak; X = decomposed.

No.	*Substance*	*Mol. wt.*	*HPLC isocratic* t_R *(min)*	*HPLC gradient* t_R *(min)*	*GC gradient 1* t_R *(min)*	*GC gradient 2* t_R *(min)*
			t_0 = 0.60	t_0 = 0.60	t_0 = 2.17	t_0 = 2.33
Leucantholides						
1	Melampodin B	334	1.30	6.96	15.84 TI	13.02 TI
2	Melampodin B acetate	376	1.94	27.98	16.51	14.02
3	Cinerenin	320	1.76	19.71	15.72 T	12.97 T
4	Cinerenin acetate	362	3.09	33.74	15.80	13.02
5	Melampodin C	362	2.06	27.29	15.70 TI	12.87 TI
6	Melampodin C acetate	404	4.02	36.50	16.97	14.97
7	Melampodin D	376	2.98	32.97	na	na
8	11,13-Dihydrocinerenin	322	1.48	9.99	15.17	12.32
cis,cis-Germacranolides						
9	Melcanthin A	448	5.44	39.38	16.81	14.65
10	Melcanthin B	464	3.38	35.91	17.76 X	15.89 X
11	Melrosin A	476	4.82	38.66	16.40 I	14.01 I
12	Longicornin B	464	12.89	43.14	16.58	14.26

Repandolides						
13	Repandin A	492	5.17	na	na	14.14 I
14	Repandin B	507	9.19	41.71	17.41	15.02 I
Melampolides						
15	Melampolidin	451	7.14	40.07	16.50	14.14
16	Leucanthinin	464	3.24	35.16	13.88 X	10.12 X
17	Desacetoxyleucanthinin	406	3.03	34.21	14.64	11.71
18	Leucanthinin acetate	522	6.46	39.86	16.65	14.36
19	Leucanthin B	478	5.30	38.37	– X	– X
20	Desacetylleucanthin B	436	1.75	20.23	17.42	15.62
21	Melampodin A	420	2.66	31.85	15.94	13.15
22	Melampodinin A	522	4.05	36.83	16.75 X	14.52 X
23	Melampodinin B	565	19.17	44.67	17.56 X	16.07 X
24	11,13-Dihydromelampodin A 9-α-[2-methylbutanoate]	507	24.72	45.75	16.72 X	15.00 X
25	Leucanthin A	462	3.92	36.46	– X	– X
26	Longipilin	406	3.86	35.68	16.02	13.40
27	Enhydrin	464	4.25	36.98	16.42	13.97
28	Desacetylenhydrin	422	3.86	35.69	16.03	13.42
29	11,13-Dihydroenhydrin	466	4.28	36.94	16.36	13.89
30	Longipin	464	2.85	33.68	17.16	15.21
31	Tetrahelin C	551	>30.00	47.31	17.83	16.62
32	Tetraludin C	525	7.06	40.38	16.78	14.53
33	Melnerin A	380	7.62	40.72	15.34	12.56
34	9-α-Acetoxymelnerin A	438	3.44	36.06	16.20	13.60
35	Melnerin B	394	13.63	43.38	15.80	13.12
36	Melnerin B′	394	12.52	42.96	16.05	13.42
37	9-α-Acetoxymelnerin B	452	3.45	36.01	16.17	13.62

hydroxyl substituents on a molecule decreased the HPLC retention times. Melcanthin B (**10**), with hydroxyl groups at C-2 and C-15, eluted before melcanthin A (**9**), wich lacks the C-2 hydroxyl. Compounds in which a hydroxyl group occurred in place of an acetate or other ester eluted before the corresponding ester; for example, melampodin B (**1**), cinerenin (**3**), melampodin C (**5**), leucanthinin (**16**), desacetylleucanthin B (**20**), and desacetylenhydrin (**28**) all eluted before their corresponding acetates. In some cases, the reasons for the magnitude of these differences in retention time are unclear. Replacement of the C-9 hydroxyl by acetate increases the isocratic retention time by 0.39 min for desacetylenhydrin (**28**), but 3.55 min for desacetylleucanthin B (**20**). A possible reason may be differences in the way these compounds are solvated in the mobile phase.

One surprising reversal of relative elution based on polarity considerations is leucanthin B (**19**), which elutes after leucanthin A (**25**). Leucanthin B contains an epoxide rather than a double bond at the C-2,3 position and behaves as the slightly more polar compound in normal-phase chromatography on silica gel. This anomaly may be due to interactions with underivatized silanol groups on the stationary phase.

Presence of the 2-methylbutanoate substituent (–OR; R = **C**) generally increased retention times markedly. Thus the isocratic retention times for melnerin B (**35**), which has the 2-methylbutanoate substituent, were 6 min longer than for melnerin A (**33**), which has an 2-methylpropanoate substituent in its place. The most strongly retained compounds, melampodinin B (**23**), 11,13-dihydromelampodin A 9-α-[2-methylbutanoate] (**24**) and tetrahelin C (**31**) all contain the 2-methylbutanoate ester side chain.

GC separations

With the non-polar methyl silicone chemically bonded column used for the GC separations, retention times normally increase with boiling point. Within groups of structurally similar compounds, it may be expected that this should be correlated with molecular weight. Although no general trend of this type is apparent, this behavior is observed when the compounds are grouped by structural types. The leucantholides exhibit this behavior, with the exception of melampodin C (**5**) and melampodin B (**1**), which both gave severely tailed peaks. The structurally similar melampolides longipilin (**26**), enhydrin (**27**), desacetylenhydrin (**28**) and 11,13-dihydroenhydrin (**29**), also eluted according to molecular weight.

Several of the sesquiterpene lactones under study decomposed, either in the injector port, which was at 270°C, or in the column itself. This was evidenced by multiple, poorly shaped peaks. The presence of a hydroxyl group at C-3, as in leucanthinin (**16**) and desacetoxyleucanthinin (**17**), or at C-2, as in melcanthin B (**10**) and melrosin A (**11**), made these compounds thermally unstable. However, if the acetate, rather than the alcohol, is present at C-3, such as in leucanthinin acetate (**18**), the compound did not decompose. Leucanthin A (**25**), leucanthin B (**19**), melampodinin A (**22**), melampodinin B (**23**) and 11,13-dihydromelampodinin A 9-α-[2-methylbutanoate] (**24**), all of which contain an epoxide in the 2,3- or the 4,5-position also decomposed. However, desacetylleucanthin B (**20**) and melampodin A (**21**) did not decompose, obscuring this correlation between structure and stability. In addition, within the leucantholides, the presence of a hydroxyl group at C-4 caused distorted peak shapes, such as tailing. This tailing was reduced in cinerenin (**3**) and 11,13-dihydrocinerenin (**8**), which both have an ethoxy substituent at C-1 rather than an ester group at this carbon. This trend does not hold for compounds of other structural types.

Comparison of HPLC and GC separations

For specific pairs of compounds, markedly better separation was often obtained by one of the two chromatographic methods. Cinerenin (**3**) and desacetylleucanthin B (**20**) co-eluted by HPLC but were easily separated by GC, with retention times of 15.72 and 17.42 min, respectively, on GC gradient 1. Cinerenin acetate (**4**) and melnerin B (**35**), on the other hand, did not separate by capillary GC under the conditions employed. These two compounds were easily distinguished by HPLC, with isocratic retention times of 3.09 and 13.63 min, respectively.

Several pairs of compounds were not resolved by either HPLC or capillary GC. These include enhydrin (**27**) and 11,13-dihydroenhydrin (**29**), longipilin (**26**) and desacetylenhydrin (**28**), and the 9-α-acetoxymelnerins A (**34**) and B (**37**). Use of ultraviolet spectra obtained with the diode array detector to confirm HPLC peak purity and/or positively identify these compounds is of limited utility. This is because of the high degree of similarity of the spectra of the sesquiterpene lactones in this study, most of which exhibit only end absorption. In cases where these compounds occur together in a given plant extract, optimization of gradient parameters or the use of longer HPLC columns should be possible. The initial separation of the 9-α-acetoxymelnerins A and B from a extract of *Melampodium leucanthum* was by preparative reversed-phase HPLC[23]. Marchand *et al.*[17] have also reported better resolution by preparative HPLC.

A sample of melnerin B (**35**), previously characterized and thought to be pure[23], was determined by both HPLC and GC to be a mixture of two isomers in approximately a 2:1 ratio. High field (400 MHz) NMR spectra of this sample clearly showed a mixture of melnerin B, which contains the 2-methylbutanoate substituent at C-8, and the corresponding compound with the 3-methylbutanoate ester (melnerin B′, **36**) in a 2:1 ratio, based on integration of the methyl signals. This information was used to assign the HPLC and GC retention times of the major (melnerin B) and minor (melnerin B′) components of the mixture.

Application to a crude plant extract

The joint application of HPLC and GC to analysis of an extract of *Melampodium cinereum* is illustrated in Figs. 2 and 3. HPLC indicated the presence of major amounts of cinerenin (**3**) or desacetylleucanthin B (**20**) (peak 1), along with 9-α-acetoxymelnerin A (**34**) or 9-α-acetoxymelnerin B (**37**) (peak 3). GC confirms the presence of 9-α-acetoxymelnerin A or B as a major constituent of the extract, while the presence of desacetylleucanthin B is ruled out. The absence of a major peak for cinerenin by GC is not unexpected because this compound gives small, tailed peaks. The presence of melcanthin A (**9**), melnerins A (**33**) and B (**35**), longicornin B (**12**) and leucanthinin acetate (**18**) is indicated as minor components of the extract. The presence of longipin (**30**) and enhydrin (**27**) or 11,13-dihydroenhydrin (**29**) in the extract, as indicated by HPLC peaks 2 and 4, was not confirmed by GC. Cinerenin, melcanthin A and the melnerins A and B have previously been reported from this species[14].

In conclusion, use of the GC and HPLC systems described here in conjunction with one another is a powerful technique for the rapid analysis of plant samples for these sesquiterpene lactones.

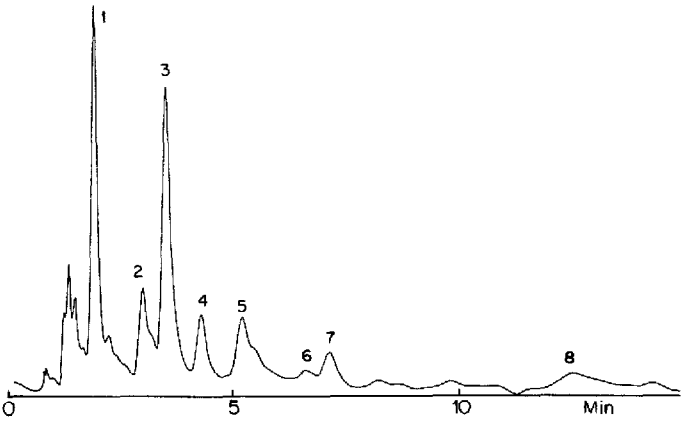


Fig. 2. HPLC (210 nm) of crude terpenoid extract of *Melampodium cinereum* var. *cinereum*. Peaks: 1 = cinerenin; 2 = unidentified; 3 = 9-α-acetoxymelnerin A or B; 4 = unidentified; 5 = melcanthin A; 6 = leucanthenin acetate; 7 = melnerin A; 8 = melnerin B and longicorin B.

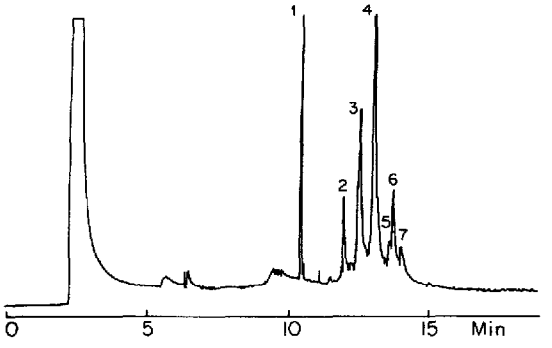


Fig. 3. GC of crude terpenoid extract of *M. cinereum* var. *cinereum*. Peaks: 1 = unidentified; 2 = melnerin A; 3 = melnerin B; 4 = 9-α-acetoxymelnerin A or B; 5 = longicorin B; 6 = leucanthenin acetate; 7 = melcanthin A.

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