ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
29 (2002) 1053–1060

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

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GC–MS analysis of the lipophilic principles of *Echinacea purpurea* and evaluation of cucumber mosaic cucumovirus (CMV) infection

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Received 6 June 2001; received in revised form 13 November 2001; accepted 13 November 2001

Abstract

An analytical GC–MS method based on nonpolar fused silica capillary column was developed to analyze the lipophilic constituents, mainly alkamides, from the root extracts of *Echinacea purpurea* (L.) Moench. In particular, the proposed method was applied to evaluate the phytochemical impacts of cucumber mosaic cucumovirus (CMV) infection on the plant's lipophilic marker phytochemicals. Methanolic (70% v/v) extracts, obtained from root materials by ultrasonic treatments, were subjected to liquid–liquid extraction with *n*-hexane-ethyl acetate (1:1 v/v) to recover the lipophilic, volatile to semivolatile, principles. Seventeen components, including the 11 alkamides known to *E. purpurea* roots, were identified in the GC–MS traces of the analyzed fractions and efficiently separated in a turnaround time of 25 min. CMV infection was found to be responsible for significant variations in the relative compositions of the major constituents, in particular germacrene D, Dodeca-2E, 4E, 8Z, 10Z(E)-tetraenoic acid isobutylamide *cis*/*trans* isomers, Undeca-2Z, 4E-diene-8, 10-diynoic acid isobutylamide and Dodeca-2E, 4Z-diene-8, 10-diynoic acid isobutylamide.

Keywords: *Echinacea purpurea* (L.); Alkamides; GC–MS analysis; Nonpolar capillary column; Cucumber mosaic cucumovirus

1. Introduction

Echinacea purpurea (L.) Moench (also known as 'purple cone flower') is one of the most important medicinal plants, used worldwide as herbal drug for its immunostimulant, bacteriostatic, anti-inflammatory and woundhealing properties that mediate its indications mainly for upper respiratory tract diseases and infections [1–4]. The plant is indigenous to northern America and is one of the most investigated *Echinacea* species (*E. purpurea*, *E. angustifolia*, and *Echinacea pallida*) highly cultivated in Europe (mainly in Germany) and USA as a natural drug and/or food supplement. Many polar and apolar compound fractions were so far isolated and characterized from the roots or top parts of the plant [5–8]. These in-

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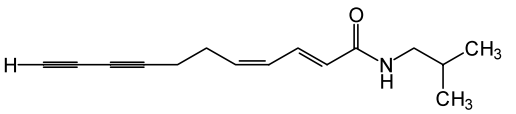
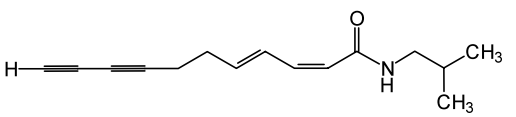
0731-7085/02/$ - see front matter
PII: S0731-7085(02)00146-2

cluded mainly alkamides (or alkylamides), polyphenolic caffeic acid derivatives, polysaccharides, alkaloids, essential oils and many other miscellaneous structures. Although claimed to be mostly related to the former two fractions (alkamides and caffeic acid derivatives) in addition to the polysaccharides, the different activities of the plant, in particular immunostimulating, are not yet assigned to a specific constituent and most clinical studies are still controversial to precisely determine the active component(s). Different analytical studies, mainly by Bauer et al., were conducted to fingerprint and analyze the hydro- and/or lipo-philic (alkamides and polyacetylenes) fractions of the plant, most of which were based on liquid chromatography by HPLC with UV (DAD) [5,8,9] or MS [10] detection. Other recent studies used micellar electrokinetic chromatography for the analysis of caffeic acid derivatives (free and conjugated) [11], and GC–MS on polar carbowax-bonded capillary columns [12] for lipophilic fraction analysis.

The lipophilic fraction, represented mainly by the alkamides, was widely investigated and 11 structures were identified in the roots of *E. purpurea* (Fig. 1) [5]. Other lipophilic constituents included some polyenic derivatives and essential oil components (mainly germacrene D) and were principally available in the top herbal parts of the plant [12,13]. Effect of viral infection on the plant



Undeca-2E, 4Z-diene-8, 10-diynoic acid isobutylamide **(1)**



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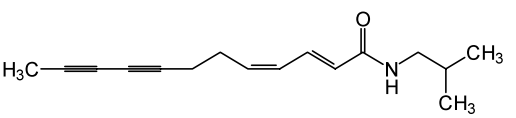

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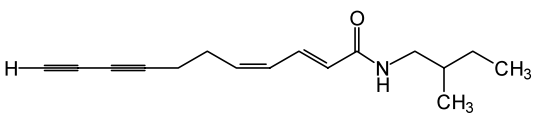
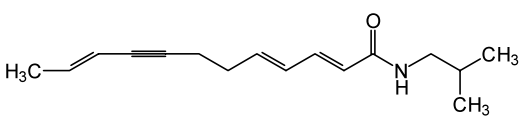
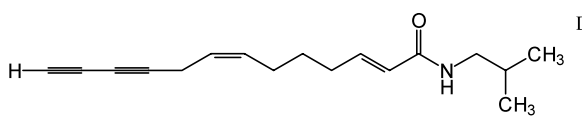


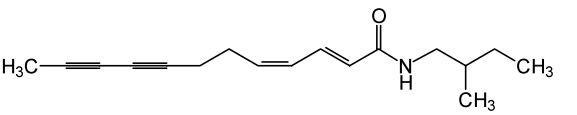
Undeca-2E, 4Z-diene-8, 10-diynoic acid 2-methylbutylamide **(4)**



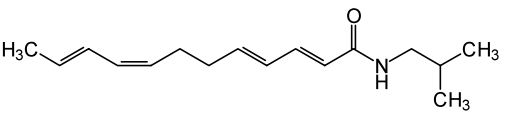
Dodeca-2E, 4E, 10E-triene-8-ynoic acid isobutylamide **(5)**



Trideca-2E, 7Z-diene-10, 12-diynoic acid isobutylamide **(6)**



Dodeca-2E, 4Z-diene-8, 10-diynoic acid 2-methylbutylamide **(7)**



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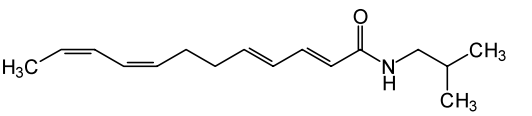
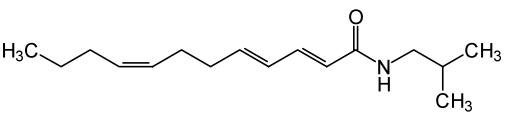
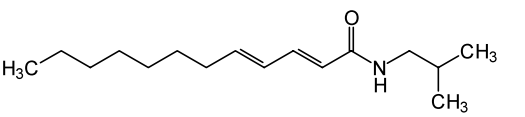


Dodeca-2E, 4E, 8Z, 10Z-tetraenoic acid isobutylamide **(9)**



Dodeca-2E, 4E, 8Z-trienoic acid isobutylamide **(10)**



Dodeca-2E, 4E-dienoic acid isobutylamide **(11)**

Fig. 1. Chemical structures of major alkamides from *E. purpurea*. Compounds numbering as denoted by Bauer and Remiger [5].

chemical composition (mainly active components) was rarely investigated [14] and to our knowledge no study reported the impact of virus infection on the marker phytochemicals of *E. purpurea* roots. In the present study, a GC–MS method based on nonpolar (BP-5 equivalent) column with an integrated liquid–liquid extraction procedure, was developed to separate and analyze the lipophilic constituents of *E. purpurea* extracts obtained from roots by ultrasonic extraction in methanol (70% v/v). The developed method was applied to evaluate the chemical effects of the infection by cucumber mosaic cucumovirus (CMV) on the quality of extracts from of *E. purpurea* plants grown in the Herb Garden of Casola Valsenio (Ravenna, Italy). In particular, the infection effect on the relative percentages of the main lipophilic fraction constituents (alkamides) was evaluated.

2. Experimental

2.1. Plant materials (collection and CMV-identification)

Before effecting the collection procedure, healthy and infected plants of *E. purpurea* grown in the open field at the Herb Garden of Casola Valsenio (Ravenna, Italy) were clustered and labelled by visual inspection of their aerial parts. The infection by CMV was associated with symptoms on both leaves and flowers. The most characteristic symptoms are yellow mosaic, ring and line-patterns on crinkled and deformed leaves that drop prematurely. The flowers, which may be smaller than normal, show color breaking with white or pale stripes on red petals. Shortening of the internodes is also very common, giving the plant a bushy appearance known as stunting. In Italian environmental conditions, these symptoms are best visible in the summer. On the other hand, plants that appeared symptom-free were collected as healthy material.

To identify the virus infecting symptomatic plants, double antibody sandwich–enzyme-linked immunosorbent assay (DAS–ELISA) method was applied as according to Clark and Adams [15]. The commercial kit containing polyclonal antibodies to CMV was obtained from Loewe Biochemica GmbH (Germany). Wells of ELISA microplates contained extraction buffer, healthy and symptomatic tissue extracts, and homologous antigen as control. DAS–ELISA identified the virus infected symptomatic *E. purpurea* as CMV.

About 1–2 kg entire plant materials (Roots and tops) of healthy and CMV-infected *E. purpurea* plants (evaluated by DAS–ELISA) were collected in October, 2000 at almost the full growth of roots. Root materials were separated, washed, cut into small parts, air-dried and ground into small particles (0.5 mm) just before extraction.

2.2. Samples preparation

2.2.1. Ultrasonic extraction

About 1 g accurately weighed amounts of dried ground root materials from either healthy or infected plants were extracted, in an ultrasonic bath (for 5–7 min at room temperature), three times with 7–8 ml solvent (70% v/v methanol in water). The extracts ($n = 3$) were collected, filtered through 0.20 μm membrane filter and the volume was completed up to 25 ml to a final concentration equivalent to 0.04 g roots/ml.

2.2.2. Liquid–liquid extraction

To recover the lipophilic fractions, a 10 ml volume from the hydro-alcoholic extract was subjected to a three fold extraction with 20 ml *n*-hexane-ethyl acetate (1:1 v/v). The organic phases were collected, concentrated under vacuum, and a 50 μl volume of the internal standard working solution (propyl-parahydroxy benzoate, 0.1% w/v in *n*-hexane-ethyl acetate (1:1 v/v)) was added before completing the volume up to 5 ml with *n*-hexane-ethyl acetate (1:1 v/v). The final solution, corresponding to 0.08 g roots/ml and containing 10 μg/ml of the internal standard, was dried over anhydrous sodium sulphate before GC–MS analysis.

2.3. GC–MS analysis

About 1 μl aliquots ($n = 5$) of the final solutions prepared as described above without further modification, were injected into a TRACE GC 2000

SERIES (ThermoQuest CE Instruments, Austin TX) gas chromatograph equipped with a split-splitless injector (Split ratio of 20:1). The column was an Rtx®-5MS (30 m × 0.25 mm, fused silica capillary column, 0.25 μm film thickness) consisting of Crossbond® (5% diphenyl 95% dimethyl polysiloxane). Helium (He) was the carrier gas at a flow rate of 1.0 ml/min. The GC was interfaced with a GCQ plus (ThermoQuest, Finnigan) mass detector operating in the EI mode (70 eV) with a 1.5 min filament delay. The mass spectra were generally recorded over 40–650 amu full-scan mode that provided the total ion current (TIC) chromatograms. A linear temperature program was adapted to separate the different extracts components as follows: the column temperature, from an initial value of 100 °C, was raised at 10 °C/min up to 200 °C at which maintained for 20 min, a second ramp was then applied at 3 °C/min up to final temperature of 250 °C at which maintained isothermal for 5 min. Temperatures of the injector base, transfer line, and ion source were maintained at 250, 250 and 200 °C, respectively. Percentage contents of the various components, identified in the TIC GC–MS traces, were calculated by integrating their corresponding peak areas (relative to the internal standard area) and assuming a unity response by all the components.

2.4. Liquid chromatography (HPLC) analysis

HPLC analysis was performed with a HP Ti series 1050 (Hewlett Packard) liquid chromatograph, equipped with a photodiode array detector (HP series 1050). All solvents were of analytical chromatographic grade (ROMIL Chemistry, Pure Cambridge, GB). Methanolic extracts from *E. purpurea* roots were subjected to reversed phase HPLC analysis carried out on a Phenomenex Kromasil C_{18} (5 μm, 15 × 4.6 mm I.D.) column, using a gradient elution from acetonitrile/water 40/60 (v/v) to 80/20 (v/v) in 40 min, at a flow rate of 1.0 ml/min. The injector was a Rheodyne valve with a 20-μl loop. UV detection at two wavelengths (210 and 254 nm) was used.

Eluate fractions from HPLC (peaks 1–11, Fig. 2) were collected and subsequently subjected to GC–MS analysis.

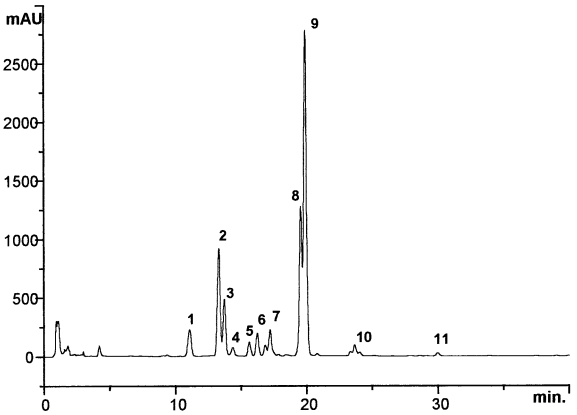


Fig. 2. A typical HPLC chromatogram of a methanolic root extract from *E. purpurea* (healthy) showing the 11 major alkamides. Numbers (**1**–**11**) as in Fig. 1. For chromatographic conditions see Section 2.

2.5. Compounds identification

2.5.1. Alkamides and polyenes

The structures of these components were identified by their relative retention times on polar columns (Carbowax 20 M) and by comparing their recorded mass spectra with internal MS-library compiled from literature data [8,12,16]. In addition, the different alkamide fractions (# **1**–**11**, Fig. 2) separated by HPLC (replicate runs were carried out to increase the alkamide amount in each fraction) and identified by their UV-data (DAD) and retention times [5], were individually extracted as described in Section 2.2.2. The final concentrated extract from each single fraction was then subjected to GC–MS analysis as a reference to confirm the peak identity in the TIC-traces of the tested samples. Moreover, for better confirmation of the GC–MS peaks corresponding to the smallest HPLC peaks (minor alkamide fractions, e.g. # **5**, **6**, **10**, and **11**, Fig. 2), selective ion monitoring GC–MS chromatograms were reconstructed by monitoring the masses of the base-peak ion and other principal characteristic MS-ions of these alkamides.

2.5.2. Sesquiterpenes

The chemical identities of the sesquiterpene components (e.g. germacrene D) were determined

by matching their recorded mass spectra with the data bank mass spectra (General Purpose and Terpene libraries 'ThermoQuest') provided by the instrument software, and by comparing their calculated RI values with literature values measured on columns with identical polarity [17].

3. Results and discussion

3.1. Method development

The hydro-alcoholic (methanol) extracts obtained from *E. purpurea* were subjected to liquid–liquid extraction, using *n*-hexane-ethyl acetate (1:1 v/v), to recover the lipophilic, semi to nonpolar, fractions readily analyzable by GC–MS. GC separations were based on BP-5 equivalent (Rtx-5MS) apolar capillary column. Under the selected temperature program, the apolar column was found to be advantageous over the usual polar carbowax one [12,16], providing efficient separation with better resolution and good peak shape and purity for all the components in a relatively shorter analysis time (Fig. 3). The GC–MS analysis afforded the separation of 17 components (Fig. 3 and Table 1), most of which were identified using their combined MS and retention parameters in addition to the HPLC fingerprints for alkamides (Fig. 2). In particular the identity was confirmed by comparison with the retention parameters and MS spectra of the HPLC fractions, whose structural assignments were, so far, established [5,8]. As the alkamide standards were not commercially available and as the present study concerned with the comparative quantitative profiles between healthy and infected plants, the percentage (as % composition) of each component in the whole-analyzed fraction was calculated on the basis of percent of total area (Table 1).

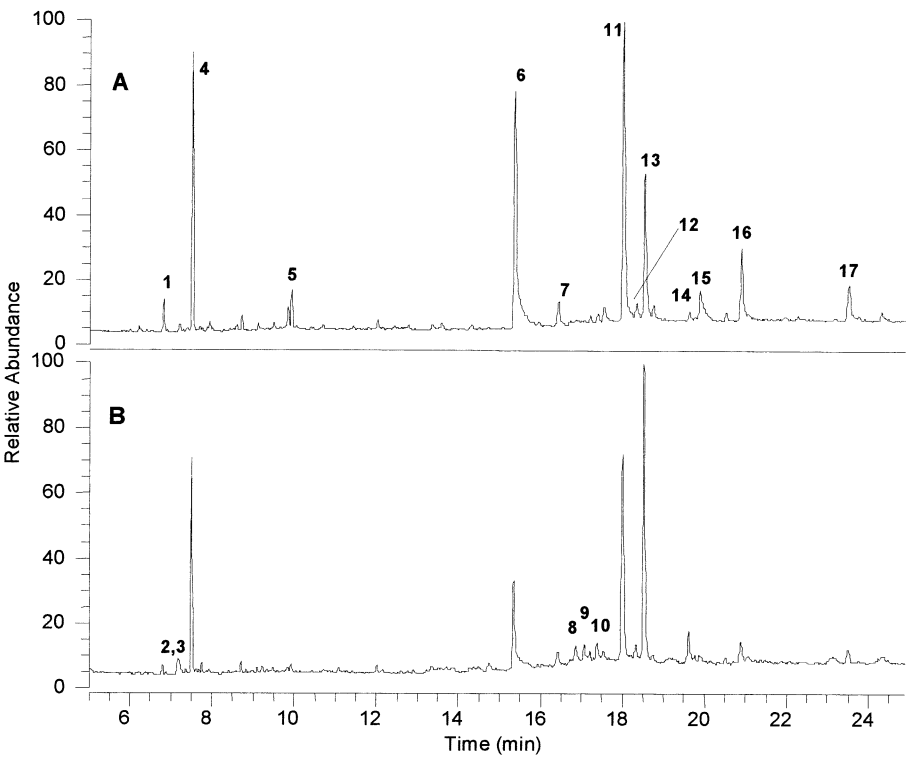


Fig. 3. Typical GC–MS chromatograms of lipophilic fraction of *E. purpurea* roots from (A) Healthy and (B) CMV-infected plants. For peaks identification refer to Table 1. For chromatographic conditions see Section 2.

Table 1
Lipophilic constituents of *E. purpurea* roots analyzed by GC–MS (Fig. 3)

Peak no.	Rt	Compound	% content (±S.D.)*		*P*
			Healthy	Infected	
1	6.70	B-caryophyllene	1.57 (0.13)	0.59 (0.06)	<0.0001[a]
2	7.05	1,8-Pentadecadiene	0.46 (0.03)	0.69 (0.06)	<0.0001[a]
3	7.20	1,8,11-Pentadecatriene	0.22 (0.02)	0.33 (0.04)	0.0006[a]
4	7.40	Germacrene D	9.63 (0.70)	5.91 (0.31)	<0.0001[a]
5	9.82	Germacrene-4-ol	2.03 (0.05)	0.47 (0.04)	<0.0001[a]
6	15.19	Alkamide **2**	19.43 (0.89)	13.23 (0.54)	<0.0001[a]
7	16.24	Alkamide **1**	2.14 (0.18)	2.36 (0.12)	0.0623[n]
8	16.66	Alkamide **10**	0.70 (0.06)	2.69 (0.19)	<0.0001[a]
9	16.86	Alkamide **11**	0.66 (0.05)	2.02 (0.12)	<0.0001[a]
10	17.20	Alkamide **5**	1.33 (0.08)	1.89 (0.13)	<0.0001[a]
11	17.82	Alkamide **9**	29.37 (0.13)	23.24 (0.73)	<0.0001[a]
12	18.12	Alkamide **8**/**9** derivative (unkn.)[s]	2.36 (0.12)	2.15 (0.08)	0.0116[b]
13	18.32	Alkamide **8**	15.07 (0.40)	31.89 (0.46)	<0.0001[a]
14	19.42	Alkamide **6**	0.40 (0.02)	3.50 (0.19)	<0.0001[a]
15	19.67	Alkamide **4**	3.48 (0.17)	2.36 (0.10)	<0.0001[a]
16	20.67	Alkamide **3**	6.77 (0.25)	3.83 (0.10)	<0.0001[a]
17	23.27	Alkamide **7**	4.68 (0.30)	2.65 (0.07)	<0.0001[a]
	8.88	Propylparaben (internal standard)			

Rt: Retention time according to Fig. 3, unkn.: unknown structure from MS and retention data. *P*: probability value from unpaired *t*-test. Alkamides (1–11) are according to Fig. 1.

* Mean percentage of five determinations ± standard deviation (S.D.).

[a] Extremely significant variation.

[b] Significant variation.

[n] Non-significant variation.

[s] MS, 70 eV, 200 °C, *m*/*z*(rel. int.): 247[M]$^+$(7), 167(81), 152(62), 147(23), 128(46), 115(33), 100(28), 96(15), 91(19), 81(53), 79(100), 68(76), 57(47), 40(20).

3.2. Analysis of healthy and CMV-infected E. purpurea extracts

Extracts from healthy and infected plants were analyzed and first the yield of the lipophilic components from the both plant materials was evaluated. Using the internal standard methodology, the mean total area of all components in the infected plant was found to be inferior to its corresponding value in healthy material. These observations proved a lower yield of the lipophilic components to be provided by the infected plants (83.65% relative to healthy), a difference that was also found to be significant between both materials ($P = 0.003$). The percentage composition of the individual components was then determined and the obtained results are summarized in Table 1. Using the profile of healthy plants (Fig. 3A) for general chemical characterization, the lipophilic components of *E. purpurea* roots could be classified into three groups; these are the isobutylamide acid derivatives (known as alkamides), sesquiterpenoids (mainly hydrocarbons), and polyenes. Alkamides constituted the main fraction (86.13% of total) and included principally all the 11 compounds so far reported by Bauer [5,8] to occur in the roots of *E. purpurea* (Fig. 3). Using the numerical system by Bauer [5] (Fig. 1), the *trans*/*cis* (**8**/**9**) alkamides were the principal alkamides (29.37% of **9** and 15.07% of **8**). An additional minor unknown peak was found to occur between the two isomers (compound **12**, Table 1), most possibly a third **8**/**9** isomer as it has a similar MS spectrum (Table 1 and Fig. 4). Alkamide **2** (MS spectrum in Fig. 4) was the second principal alkamide corresponding to about 19.43% of the

total. Other main alkamides included alkamide **3** (6.77%), **7** (4.68%), **4** (3.48%), and **1** (2.14%). The alkamide percentages were found to be in substantial agreement with those reported by many researchers [18–20].

Sesquiterpes were the second major lipophilic fraction (13.19%) and included mainly hydrocarbons such as germacrene D (9.63%) and B-caryophyllene (1.57%), in addition to a lower level of the oxygenated derivative germacrene-4-ol (2.03%). The latter was found mostly corresponding to the germacrene alcohol, reported by Bauer [21] to occur in the fresh aerial parts of *E. purpurea*, whose level (together with other terpenoids) was also reported to be dependent upon the dry state and the type of the plant tissue [13,19,21]. Finally, polyenes were found in our extracts in trace levels (0.68%) and included compounds # **2** and **3** (Table 1) as the most possible structures.

On the other hand, as indicated in Table 1 and Fig. 3, infection of *E. purpurea* by CMV was found associated with considerable variations in the levels of almost all the lipophilic components, in particular, alkamides **8**/**9** and **2** and germacrene D. These variations were tested (by unpaired *t*-test) to be very significant for most of the components, mainly the major ones (Table 1). The inverted relative compositions of alkamides **8**/**9** between healthy and infected plants, mainly as a result of the double increase in **8** (31.89% in infected compared to 15.07% in healthy), was the most evident and important variation. Germacrene D as well as the other sesquiterpene hydrocarbon (B-caryophyllene) showed almost 50% reduction in their levels in the infected plant extracts, while almost 75% reduction was observed for the oxygenated derivative (germacrene-4-ol). These latter variations, in particular for germacrene D, were also reported as preliminary observations in the composition of the essential oil derived from the top parts of infected *E. purpurea* plants [22]. Reduced levels were also observed for the alkamides **2**, **3**, **4**, and **7** as shown in Table 1. While comparable levels were observed for the alkamides **1** and **8**/**9**-derivative (compounds # **7** and **12**, respectively, Table 1), significant peak enrichments were obtained for the two polyenic compounds and the remaining alkamides (**5**, **10**, **11** and **6**), in particular alkamide **6**, which showed almost 10 times elevation in its level in the infected plant.

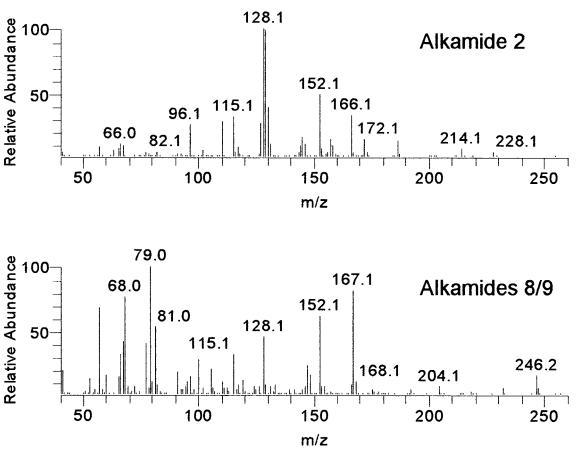


Fig. 4. Typical mass spectra of the principal alkamides (# **2** and **8**/**9**) of *E. purpurea* registered online from the reconstructed TIC-GC–MS chromatogram (Fig. 3). For alkamide structures and numbers refer to Fig. 1, for MS analytical conditions see Section 2.

4. Conclusions

GC–MS analysis based on nonpolar capillary column (BP-5 equivalent) proved to be an effective and convenient method for fingerprinting and/or analysis of the lipophilic constituents of *E. purpurea*. The good resolution and rapid analysis together with the simple liquid–liquid extraction provided an effective analytical procedure to evaluate any qualitative and/or quantitative variations, on the apolar components of this plant species, brought about via different environmental, cultivation and/or disease conditions. The natural infection of *E. purpurea* plants with CMV was found to induce significant reduction in the yield of the lipophilic fraction and sever changes in the relative composition of the marker components such as the alkamides (**8**/**9**) tetraene stereoisomers, alkamide **2** and germacrene D. Therefore, as the plant activities were so far reported to correlate, even in part, with the princi-

pal lipophilic constituents (mainly alkamides), routine physical inspection (for plant diseases) and chemical control of *Echinacea* herbs and/or extract preparations using valid analytical methods are highly recommended to ensure uniformity of the drug chemical constituents and thus clinical efficacy.

Acknowledgements

Thanks are due to Sauro Biffi from the Herb Garden of Casola Valsenio for his technical assistance in plant identification and collection.

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