JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

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# Chromatographic Methods for Metabolite Profiling of Virus- and Phytoplasma-Infected Plants of *Echinacea purpurea*

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**ABSTRACT:** This study was focused on the effects of virus and phytoplasma infections on the production of *Echinacea purpurea* (L.) Moench secondary metabolites, such as caffeic acid derivatives, alkamides, and essential oil. The identification of caffeic acid derivatives and alkamides was carried out by means of high-performance liquid chromatography−diode array detection (HPLC-DAD), HPLC−electrospray ionization−mass spectrometry (ESI-MS), and $MS^2$. Quantitative analysis of these compounds was carried out using HPLC-DAD. The results indicated that the presence of the two pathogens significantly decreases ($P < 0.05$) the content of cichoric acid, the main caffeic acid derivative. Regarding the main alkamide, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, a significant decrease ($P < 0.05$) in the content of this secondary metabolite was observed in virus-infected plants in comparison with healthy plants, while in the phytoplasma-infected sample the variation of this secondary metabolite was not appreciable. The % relative area of the *E*/*Z* isomers of this alkamide was also found to change in infected samples. The gas chromatography (GC) and GC-MS analysis of *E. purpurea* essential oil enabled the identification of 30 compounds. The main significant differences ($P < 0.05$) in the semiquantitative composition were observed for three components: limonene, *cis*-verbenol, and verbenone. The results indicate that the presence of virus and phytoplasma has an appreciable influence on the content of *E. purpurea* secondary metabolites, which is an important issue in defining the commercial quality, market value, and therapeutic efficacy of this herbal drug.

**KEYWORDS:** *Echinacea purpurea*, caffeic acid derivatives, alkamides, essential oil, HPLC, GC, virus, phytoplasma

## INTRODUCTION

Among the species belonging to the genus *Echinacea* (Asteraceae), *Echinacea purpurea* (L.) Moench, *Echinacea angustifolia* DC. var. *angustifolia*, and *Echinacea pallida* (Nutt.) Nutt. have shown potential pharmacological activity[1,2] and are traditionally employed in the formulation of dietary supplements used as immunostimulants mainly in the prevention and treatment of inflammatory and viral diseases. The drug consists of *E. purpurea*, *E. angustifolia*, and *E. pallida* roots, but, in the case of *E. purpurea*, aerial parts are also commonly used in phytotherapy.

The chemical composition of the plants of the genus *Echinacea* is extremely complex, including caffeic acid derivatives,[3,4] alkamides, polyacetylenes and polyenes,[5−7] polysaccharides, [8] and glycoproteins,[9,10] which exhibit diverse pharmacological activity. Recent research has concluded that the pharmacological activity of *Echinacea* preparations may depend on the combined activities of several plant constituents.[11]

Regarding caffeic acid derivatives, several compounds have been identified in the hydrophilic fractions of *Echinacea* extracts: caftaric acid, chlorogenic acid, caffeic acid, cynarin, echinacoside, and cichoric acid.[4] The main biological activities of these phenolic compounds include antiviral, anti-inflammatory, and antioxidant effects.[1,2]

Alkamides have been isolated and characterized only from *E. purpurea* and *E. angustifolia*,[12,13] while *E. pallida* was found to contain polyacetylenes and polyenes.[6,7] A series of pharmacological studies have shown that *Echinacea* alkamides have anti-inflammatory and immunomodulatory properties.[14] It has also been demonstrated that alkamides significantly bind the $CB_2$ receptors, and this is considered to be a possible molecular mechanism of action of these compounds as immunomodulatory agents.[14]

The market demand and the medicinal properties are responsible for the considerable interest in this genus, and this has led to a rapid expansion of *Echinacea* cultivation worldwide. Besides genetic chemodiversity, it is generally known that secondary metabolism in plants can be altered in response to several factors, including exposure to pathogens, such as viruses and phytoplasmas, which are responsible for various diseases affecting several hundred species in the world, including medicinal and aromatic plants. Different phytoplasmas have recently been identified in plants of pharmaceutical interest, such as *Hypericum perforatum*, *Digitalis lanata*, and *Grindelia robusta*.[15−17] The phytoplasmas, prokaryotes with no cell wall and not cultured in artificial media,

**Received:** March 28, 2011
**Accepted:** August 10, 2011
**Published:** August 10, 2011


**Figure 1.** Healthy (A) and phytoplasma-infected (B) plants of *E. purpurea*. Picture B clearly shows the development of floral parts into leafy structures (phyllody).

inhabit the phloem of affected plants and are responsible for severe diseases, affecting several hundred species with morpho-functional alterations of plant metabolism. Recent developments have dramatically improved the knowledge of the physiochemical relationships between phytoplasma and host plants; however, very little data exist regarding the effects that phytoplasma infections have on plant secondary metabolism, in particular on the quality of cultivated medicinal plants.[15−17]

During the spring−summer of 2009, plants grown in an experimental field of *E. purpurea* were found to be infected with *cucumber mosaic virus* (CMV) and with a phytoplasma belonging to 16SrIX-C ribosomal subgroup. Considering the severity of the infections, a detailed phytochemical investigation was undertaken for the first time by means of the development and application of reliable chromatographic methods to evaluate the effect of both pathogens on the content of *E. purpurea* secondary metabolites (caffeic acid derivatives, alkamides, and essential oil), enabling the definition of their impact on the market value and contributing to quality certification of this medicinal plant.

## MATERIALS AND METHODS

**Chemicals.** Caftaric acid and cichoric acid were purchased from Chromadex (Santa Ana, CA). High-performance liquid chromatography (HPLC)-grade methanol (MeOH), acetonitrile (ACN), *n*-hexane, ethyl acetate (EtOAc), chloroform ($CHCl_3$), formic acid (HCOOH), phosphoric acid ($H_3PO_4$), sodium sulfate ($Na_2SO_4$), protein-A, protein A-alkaline phosphate conjugate, and *Taq* polymerase were from Sigma-Aldrich (Milan, Italy). Water ($H_2O$) was purified using a Milli-Q Plus185 system from Millipore (Milford, MA).

**Plant Material.** During the spring-summer of 2009, virus and phytoplasma infections were observed in an experimental field of *E. purpurea* (L.) Moench, located at the University of Bologna (Emilia-Romagna region, Northern Italy) in its second year of cultivation. The soil (heavy clay and well-drained) had been fertilized with cattle manure before planting the seedlings and then every year at the end of the flowering period. Virus-infected plants showed stunting; leaves with yellow mosaic, ring, line patterns and malformations; small flowers with pale stripes on red petals. Regarding the phytoplasma disease, almost 60% of the plants showed severe flower abnormalities (phyllody symptoms) (Figure 1B) and, in some cases, purplish reddening of the basal leaves.

*E. purpurea* plants showing virus or phytoplasma symptoms and other asymptomatic ones were labeled at the same development stage (at blooming) after visual inspection of the aerial parts. In relation to the severity of the symptoms, the collected plant material was homogeneous. Each fresh plant was analyzed using PAS-ELISA and nested-PCR, then uprooted and air-dried in a dark greenhouse. Plants affected by the virus alone were combined to obtain a representative virus-infected sample, while the phytoplasma-infected sample was composed of plants infected by the phytoplasma alone. The healthy sample was represented by virus- and phytoplasma-free plants, properly chosen after the serological and molecular biology assays, respectively. The collected plant material was stored at a low temperature, protected from light and humidity, until required for chemical analysis.

**Virus Identification by PAS-ELISA.** To detect the presence of the virus,[18] the plates were first coated with 1 $\mu$/mL protein-A in carbonate buffer (pH 9.6). A portion (2 g) of each *E. purpurea* fresh plant (leaves and/or flowers) was homogenized in phosphate buffer saline (PBS) (pH 7.2), containing 0.05% Tween-20, 2% polyvinyl pyrrolidone (PVP, MW 24000) and 2% powdered chicken albumin. Polyclonal antisera were added at 1:500 dilution in PBS-Tween. Protein A-alkaline phosphate conjugate was diluted at the concentration of 1 $\mu$g/mL in PBS-Tween (pH 7.4). The serological reaction was considered positive when the absorbance value at 405 nm was higher than 1.0 and exceeded that of the negative control (mixed leaves of *Nicotiana* species) by a factor of 3. The UV absorbance was measured with a Dynatech MR 7000 spectrophotometer (Chantilly, VA, USA). The polyclonal antiserum of CMV (PVAS-30, from *Comellina diffusa*

Burn.) was obtained from the American Type Culture Collection (ATCC) (Manassas, VA, USA).

**Phytoplasma Identification by Nested-PRC and RFLP.** The DNA was extracted from 1 g of each *E. purpurea* fresh plant (leaf midribs and flowers) using a $CHCl_3$/phenol procedure.[19] Nucleic acid samples, used as a positive control for both nested-polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) analyses, were extracted from phytoplasmas belonging to ribosomal subgroup 16SrI-A (CHRY), 16SrI-B (AYW, AY2192, CCH, EAY, and KAY), 16SrI-C (GY and PPT), and 16SrI-F (CVB). The DNA was diluted in TE buffer [10 mM Tris-HCl and 1 mM EDTA (pH 8.0)] to a final concentration of 20 ng/$\mu$L for each PCR reaction. The reaction mixture (25 $\mu$L total volume) contained 1 $\mu$L of DNA, 200 $\mu$mol of deoxynucleotide triphosphate (dNTP), 0.8 U Taq polymerase, 0.4 $\mu$mol of each primer, PCR buffer [10 mM TRIS buffer (pH 8.3) and 50 mM HCl], 1.5 mM $MgCl_2$, and 0.001% (w/v) gelatin. The first set of primers was P1/P7,[20,21] followed by nested-PCR with the primers R16F2/R2.[22] The PCR amplification[22] was performed in a Biometra thermal cycler (Goettingen, Germany). A negative control (without DNA) was included in each set of reactions. The PCR products (6 $\mu$L) were detected by 1% agarose gel electrophoresis followed by ethidium bromide staining and UV observation.

In RFLP analyses, 100−200 ng of R16F2/R2 product (1240 bp) was digested with *Tru*I at 65 °C and *Hha*I at 37 °C for at least 16 h, following the manufacturer's instructions (Fermentas, Vilnius, Lithuania). The restriction patterns were compared with those of phytopasma control strains after electrophoresis on a 5% polyacrylamide gel and ethidium bromide staining and were photographed under UV at 312 nm using a transilluminator.

**Isolation of Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-Tetraenoic Acid Isobutylamide Standard.** The alkamide dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide was isolated from *E. angustifolia* DC. var. *angustifolia* roots,[5] and it was used as a standard to quantify the alkamides. Powdered dried roots of *E. angustifolia* (360 g, CRA-Forest and Range Management Research Institute, Villazzano, Trento, Italy) were extracted in a Soxhlet apparatus for 8 h using *n*-hexane (2.5 L). The extract was evaporated to dryness under vacuum to give a yellow oil (2.5 g). The extract was then dissolved in a small amount of *n*-hexane and EtOAc (2:1, v/v), subjected to silica gel flash column chromatography on Kieselgel 60 (40−63 $\mu$m, Merck, Darmstadt, Germany), and eluted with *n*-hexane/EtOAc (2:1, v/v), affording 130 fractions of 10 mL each. The collected fractions were analyzed by thin-layer chromatography (TLC) and RP-HPLC and combined into four fractions (A−D), according to the chromatographic profile. Dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide was purified from fraction C (0.24 g) by reversed-phase flash column chromatography on LiChroprep RP-18 (40−63 $\mu$m, Merck), using ACN/$H_2O$ (60:40, v/v) as the mobile phase, to give 60 fractions of 2 mL each, which were analyzed by TLC and RP-HPLC. The combined fractions 25−28 were then concentrated under reduced pressure to give an aqueous residue, which was extracted with $CHCl_3$ (3 × 15 mL). The combined organic phases were dried over anhydrous $Na_2SO_4$; the resulting anhydrous solution was then filtered and concentrated under vacuum to give a white powder, which was dissolved in *n*-hexane and recrystallized as needles (11.5 mg, 0.003% yield).

The degree of purity of the isolated compound was 98% as determined by RP-HPLC with a diode array detector (DAD) (Agilent Technologies). The structure elucidation was carried out on the basis of UV (recorded online by HPLC-DAD), IR (recorded on a 1600 Series FT-IR instrument, Perkin-Elmer, Norwalk, CT), NMR, including 1D and 2D NMR experiments, such as $^1H-^1H$ gCOSY, gHSQC-DEPT, gHMBC, gNOESY (recorded on a FT-NMR AVANCE 400 spectrometer, Bruker, Rheinstetten, Germany), and MS data [recorded online by HPLC−electrospray ionization−mass spectrometry (ESI-MS) with an ion trap mass analyzer, Agilent Technologies].[12] The purified compound was stored under argon atmosphere at a low temperature (−80 °C), protected from light and humidity.

**Sample Preparation for HPLC Analysis of Caffeic Acid Derivatives and Alkamides.** Dried roots (82 g for the healthy, 96 g for the virus-infected, and 31 g for the phytoplasma-infected sample, respectively) were used for the determination of caffeic acid derivatives and alkamides in *E. purpurea*. The pulverized roots were macerated on the ratio 0.5 g sample:10 mL of MeOH−0.1% $H_3PO_4$ in $H_2O$ (70:30, v/v) for 20 min at room temperature under magnetic stirring. After centrifugation for 5 min at 4000 rpm, the supernatant solution was filtered in a vacuum, and the residue was re-extracted. The filtrates of the two extractions were combined, and the solvent was added to 25 mL of final volume. The extracts were filtered through a 0.45 $\mu$m PTFE filter into a HPLC vial and capped. All the sample preparations were carried out in duplicate.

**HPLC-UV/DAD Conditions.** Chromatography was performed using an Agilent Technologies modular model 1100 system, consisting of a vacuum degasser, a quaternary pump, an autosampler, a thermostatted column compartment, and a DAD. The chromatograms were recorded using an Agilent ChemStation for LC and LC-MS systems (Rev. B.01.03). The analyses were carried out on an Ascentis $C_{18}$ column (250 mm × 4.6 mm i.d., 5 $\mu$m, Supelco, Bellefonte, PA). The mobile phase was composed of (A) 0.1% HCOOH in $H_2O$ and (B) ACN. The gradient elution was modified as follows: initial 15% B; 0−10 min linear gradient from 15 to 30% B; 10−18 min from 30 to 65% B, 18−25 min from 65 to 80% B, 25−30 min from 80 to 90% B, and 30−32 min 90% B. The postrunning time was 3 min. The flow rate was 1.0 mL/min. The column temperature was set at 30 °C. The sample injection volume was 10 $\mu$L. The DAD acquisition range was 190−450 nm, and peak integration was performed at 254 nm for alkamides and 330 nm for caffeic acid derivatives. Three injections were performed for each sample.

**HPLC-ESI-MS and $MS^2$ Conditions.** The analyses were performed using an Agilent Technologies modular 1200 system, equipped with a vacuum degasser, a binary pump, an autosampler, a thermostatted column compartment, and a 6310A ion trap mass analyzer with an ESI ion source (Agilent Technologies). The HPLC column and the chromatographic conditions were the same as reported for the HPLC-DAD system. The flow rate was split 6:1 before the ESI source. For ESI-MS and $MS^2$ experiments, the parameters were set as follows: the capillary voltage was 3.5 kV, the nebulizer ($N_2$) pressure was 20 psi, the drying gas ($N_2$) temperature was 300 °C, the drying gas flow was 9 L/min, and the skimmer voltage was 40 V. The data were acquired by Agilent 6300 Series Ion Trap LC/MS system software (version 6.2). The mass spectrometer was operated in the negative ion mode during the first 17 min of analysis and then switched to the positive ion mode for the remainder analysis time (17−32 min). The full scan acquisition was performed in $m/z$ range 100−1000. $MS^2$ spectra were automatically performed in the $m/z$ range 50−1000 with He as the collision gas, by using the SmartFrag function.

**HPLC-UV/DAD Method Validation.** The linearity was tested by preparing calibration curves for caftaric acid and cichoric acid, which were commercially available, and for dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, which was isolated and purified from *E. angustifolia*. To an amount of 0.3−3.0 mg of each standard compound (caftaric acid, cichoric acid, and dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide), MeOH−0.1% $H_3PO_4$ in $H_2O$ (70:30, v/v) was added to reach a final volume of 5 mL. The external standard calibration curve was generated using six data points for caffeic acid derivatives and five for the reference alkamide, at the following concentrations: 3.1, 7.7, 15.3, 30.7, 76.7, and 153.4 $\mu$g/mL for caftaric acid, 12.0, 24.0, 48.0, 120.0, 240.0, and 600.0 $\mu$g/mL for cichoric acid, and 2.6, 5.1, 12.8, 25.6, and 51.2 $\mu$g/mL for dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide. Ten microliter aliquots of each standard

solution were used for the HPLC analysis. The injections were performed in triplicate for each concentration level. The calibration curve was obtained by plotting the peak area of the compound at each level versus the concentration of the sample. The amount of caffeic acid derivatives and alkamides in *E. purpurea* root samples was determined by using the above-mentioned calibration curves, when the standard was available. All of the other alkamides identified in the *E. purpurea* samples, which share the same chromophore based on a 2,4-diene moiety and displayed a strong UV absorption at 254 nm, were quantified with the above-mentioned calibration curve, and their amounts were corrected by using the molecular weight ratio.[23,24]

The limit of quantification (LOQ) of the method was evaluated considering the analyte concentration that would yield a signal-to-noise (S/N) value of 10; the limit of detection (LOD) represents the analyte concentration that would yield a S/N value of 3. The LOQ and LOD values were experimentally verified by injections of standard solutions of the compounds at the LOQ and LOD concentrations.

The accuracy of the analytical procedure was evaluated using the recovery test: This involved the addition of known quantities of reference standard compounds to half the sample weight of *E. purpurea* healthy plant material. The fortified samples were then extracted and analyzed using the proposed HPLC method. The results were expressed as percentage recovery values.

The precision of the chromatographic system was tested by performing intra- and inter-day multiple injections of an *E. purpurea* extract obtained from healthy plants and then checking the % RSD of retention times and peak areas. Five injections were performed each day for 3 consecutive days.

The stability was tested with an *E. purpurea* extract that was stored in amber glass flasks at 4 °C and at room temperature (about 25 °C) and analyzed every 12 h.

**Extraction of Essential Oil.** Steam distillation was performed to extract *E. purpurea* essential oil from the dried plants, using a commercial Clevenger apparatus. Raw material (aerial parts) (750 g for the healthy, 518 g for the virus-infected, and 166 g for the phytoplasma-infected sample, respectively) was subjected to steam distillation for 40 min after the mixture reached boiling point. The essential oil was kept in amber vials at 4 °C before analysis.

**GC and GC-MS Analysis of Essential Oil.** GC analysis of *E. purpurea* essential oil was carried out on a model 5890 Hewlett-Packard gas chromatograph (Palo Alto, CA), equipped with a flame ionization detector (FID). The compounds were separated on a MetSil capillary column, fitted with a poly-5% diphenyl−95% dimethyl polysiloxane (12.5 m × 0.25 mm, 0.25 $\mu$m film thickness) (Perkin-Elmer, Monza, Italy). Splitless injection was performed with He (purity >99%) as the carrier gas, at a flow rate of 0.9 mL/min. The injector and detector temperatures were set at 280 and 250 °C, respectively. The oven temperature was programmed from 50 to 270 at 4 °C/min. The semiquantitative data were obtained by electronic integration of the peak area, without the use of response factor correction. Three injections were performed for each sample.

GC-MS analysis was performed using a 6890 Hewlett-Packard gas chromatograph, equipped with a 5973 single quadrupole mass spectrometer. The HP 6890 ChemStation was used. The mass spectrometer was operated at 70 eV, with a scan rate of 1 scan/s over the $m/z$ range 40−300 and an ion source temperature of 180 °C. The column and the chromatographic conditions were the same as described above.

A mixture of *n*-alkanes ($C_7$−$C_{25}$) was injected under the above-described conditions to calculate the retention indices ($R_i$). The *E. purpurea* essential oil constituents were identified by comparison of the chromatographic data with those of authentic reference standards, by comparison of the $R_i$ values with those reported in the literature[25] and by matching the mass spectra of the analytes with those of the mass spectral database (Nist98).

**Statistical Analysis.** The means and standard deviation (S.D.) data were calculated using Microsoft Office Excel 2003. The analysis of variance (ANOVA) was used to evaluate the statistical significance of the measured differences between the healthy, virus- and phytoplasma-infected samples; moreover, a posthoc test was performed using the Tukey "honest significant difference test" (HSD). For all these monovariate tests, the $P$ level was set at 0.05. All the statistical analyses were performed using Statistica 6.0 for Windows (StatSoft Italia, Vigonza, Italy).

## RESULTS AND DISCUSSION

**Virus and Phytoplasma Identification.** PAS-enzyme-linked immunosorbent assay (ELISA) enabled identification of the virus infecting *E. purpurea* as CMV, which was detected in all the symptomatic plants and also in some symptomless ones, which were removed. Nested PCR/RFLP analyses confirmed the presence of a phytoplasma belonging to the 16SrIX-C ribosomal subgroup in all of the symptomatic plants. None of the symptomless plants, and none of those showing virus symptoms, were found to be infected by phytoplasmas. It must be pointed out that all the phytoplasma-infected plants were affected by the 16SrIX-C phytoplasma alone and also those labeled as virus-infected were affected by CMV alone. The healthy sample was composed of *E. purpurea* plants which proved to be CMV-free in PAS-ELISA tests and phytoplasma-free in nested-PCR/RFLP analyses.

CMV (family *Bromoviridae*, genus *Cucumovirus*) is one of the most important worldwide viruses, infecting more than 1000 species (belonging to 100 families), transmitted in a nonpersistent manner by almost 75 aphid species and seed-transmitted in different host plants. In Italy, CMV is endemic in all horticultural and ornamental areas, and during the past decade, it has been detected in more than 30 different medicinal plant species[26] and also in *E. purpurea*.[26] Considering the constant presence of aphid infestations in horticultural and ornamental crops, medicinal plants infected with CMV, as well as their seeds, represent a good source of CMV inoculum for other cultivated species. The phytoplasmas belonging to ribosomal group 16SrIX are not commonly reported in European plants, while they are common and found to be associated with strong epidemics in almond and peach in Lebanon, in other middle eastern countries, and also in some cultivated species in tropical regions worldwide.[26] In Italy, 16SrIX phytoplasmas were in some cases reported to infect weeds located in vineyards or orchards.[26] In the case of *E. purpurea*, the damage caused by virus or phytoplasma infections appeared serious enough to apply control measures to prevent the spreading of these diseases, including the removal of infected plants from the field as well as weeds and aphids, the main vectors of virus infection.

**Method Development and Identification of Constituents in *E. purpurea* Extracts.** Regarding the extraction conditions, different solvent mixtures were tested, and dynamic maceration with MeOH−0.1% $H_3PO_4$ aqueous solution (70:30, v/v) provided the highest yield of constituents from *E. purpurea* roots. The chromatographic conditions were then optimized to obtain a good separation of adjacent peaks in a reasonable analysis time, thus allowing the simultaneous determination of both polar and nonpolar secondary metabolites. The HPLC-DAD chromatograms obtained from the analysis of healthy, virus-infected, and phytoplasma-infected *E. purpurea* samples are shown in Figure 2.

The chromatographic data and UV spectra, in comparison with reference standards, enabled the assignment of the main

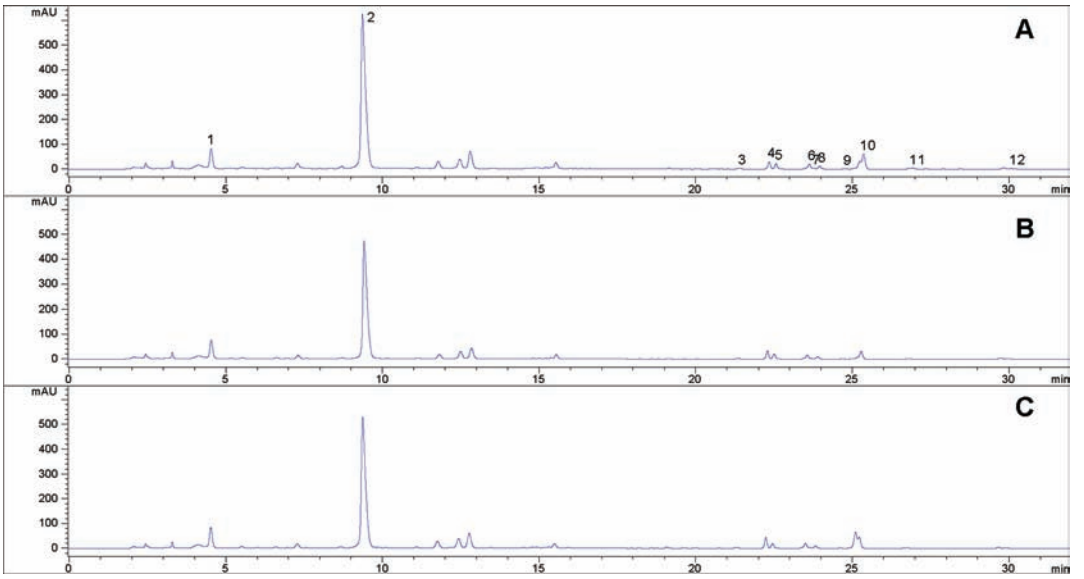


**Figure 2.** Chromatograms obtained from the HPLC-DAD analysis of *E. purpurea* root extracts at 254 nm: (A) healthy, (B) virus-infected, and (C) phytoplasma-infected sample, respectively. For peak identification, see Table 1.

classes of compounds occurring in *E. purpurea*, including caffeic acid derivatives and alkamides, since each class exhibits a characteristic UV−vis spectrum: in fact, the caffeic acid derivatives and alkamides displayed a high absorbance at 330 and 254 nm, respectively. In the chromatogram of Figure 2, the first two peaks correspond to caffeic acid derivatives, which, due to their high polarity, eluted first under reversed-phase conditions. The other peaks correspond to alkamides, which are less polar and, therefore, eluted later.

However, the retention time data and UV spectra alone did not provide sufficient information for the correct identification of the constituents in such a complex matrix. By using HPLC-ESI-MS and $MS^2$, it was possible to obtain the quasi-molecular ions and the product ions of the target analytes, which, in combination with retention times and UV data, made their identification very reliable. From the quasi-molecular ion, it was possible to select potential candidates, and among these, correct identification was possible on the basis of its product ions. When the reference standards were available, the identification was further confirmed by comparison of the MS and $MS^2$ data with those of the reference compounds. The MS and $MS^2$ data of the present study were also compared with the literature.[27−29]

The MS and $MS^2$ spectra of the samples analyzed indicated that the negative ion mode provided a higher level of sensitivity for caffeic acid derivatives, while the positive ion mode was more suitable for alkamide detection. Therefore, the MS detector was set in the negative ion mode for the first stage of the analysis (0−17 min); in the second stage (17−32 min), the detector was operated in the positive ion mode to facilitate the detection of alkamides.

The MS spectra of caffeic acid derivatives in the negative ion mode consisted of two main peaks, corresponding to the quasi-molecular ion $[M - H]^-$ and to a proton bound dimer $[2M - H]^-$ of these molecules. The proposed structures of the product ions of caffeic acid derivatives in the $MS^2$ spectra, obtained by fragmentation of the $[M - H]^-$ precursor ions, were assigned as shown in Table 1. The $MS^2$ spectra of caftaric acid and cichoric acid from the *E. purpurea* extracts analyzed in this study perfectly matched those obtained from the reference standards.

Alkamides originated $[M + H]^+$ and $[M + Na]^+$ precursor ions in the positive ion mode. A proton bound dimer $[2M + H]^+$ was also commonly observed. The $MS^2$ spectra of the $[M + H]^+$ precursor ions of alkamides showed that the main sites of fragmentation were the C−N bonds of the amide functional groups, originating fragments corresponding to the loss of the alkyl group attached to nitrogen and to the loss of the entire amine portion of the molecule. In this way, it was possible to distinguish between isobutylamides and 2-methylbutylamides.[28] In the case of the main alkamide, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, the losses of the isobutyl group (−56 u) and the isobutyl amine (−73 u) were observed. The $MS^2$ data of this compound in the *E. purpurea* extracts perfectly matched with those obtained from the analysis of the isolated secondary metabolite used as a reference. In the case of 2-methylbutylamides, such as dodeca-2*E*,4*Z*-diene-8,10-diynoic acid 2-methylbutylamide, the loss of the 2-methylbutyl group (−70 u) and the 2-methylbutyl amine (−87 u) occurred. In the $MS^2$ spectra of alkamides, other products ions were commonly observed, due to the loss of the amide portion (−101 u for isobutylamides and −115 u for 2-methylbutylamides). Other fragments were produced by the cleavage of various C−C bonds of the main carbon chain. The proposed structures of these fragments are shown in Table 1. In agreement with the literature,[29] polyacetylenic alkamides (compounds **3**−**9**) eluted first in the separation, followed by polyenic alkamides (compounds **10**−**12**).

Overall, the UV spectra, in combination with MS and $MS^2$ data, enabled the identification of two caffeic acid derivatives (caftaric acid and cichoric acid) and 10 different alkamides in the *E. purpurea* extracts analyzed. Table 1 lists the retention times ($t_R$), maximum absorbance wavelengths ($\lambda_{max}$), the precursor ions, and the products ions of the secondary metabolites identified.

**Method Validation.** The HPLC-UV/DAD method was validated to demonstrate its suitability for the determination of caffeic acid derivatives and alkamides in *E. purpurea* extracts. Good linearity was observed for caftaric acid, cichoric acid, and dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide between

**Table 1. Secondary Metabolites (Caffeic Acid Derivatives and Alkamides) Identified in *E. purpurea* Root Extracts by HPLC-DAD, HPLC-ESI-MS, and MS$^2$**

| peak no. | retention time (min) | compd | UV $\lambda_{max}$ (nm) | precursor ions ($m/z$) | product ions ($m/z$)[a] |
|---|---|---|---|---|---|
| 1 | 4.5 | caftaric acid | 298, 328 | 623.0 $[2M - H]^-$<br>311.0 $[M - H]^-$ | 179 (18) $[C_9H_7O_4]^-$, 149 (100) $[C_4H_5O_6]^-$, 135 (3) $[C_8H_7O_2]^-$, 113 (6) |
| 2 | 9.4 | cichoric acid | 300, 330 | 947.1 $[2M - H]^-$<br>473.0 $[M - H]^-$ | 340 (19), 311 (100) $[C_{13}H_{11}O_9]^-$, 293 (26) $[C_{13}H_9O_8]^-$, 179 (36) $[C_9H_7O_4]^-$, 149 (10) $[C_4H_5O_6]^-$, 135 (10) $[C_8H_7O_2]^-$ |
| 3 | 21.4 | undeca-2*E*,4*Z*-diene-8, 10-diynoic acid isobutylamide | 262 | 481.1 $[2M + Na]^+$<br>459.1 $[2M + H]^+$<br>252.1 $[M + Na]^+$<br>230.1 $[M + H]^+$ | 202 (4), 188 (12) $[M + H - C_3H_6]^+$, 174 (21) $[M + H - C_4H_8]^+$, 157 (14) $[M + H - C_4H_{11}N]^+$, 146 (9), 131 (100), 129 (100) $[M + H - C_5H_{11}NO]^+$, 116 (21), 103 (5) $[M + H - C_7H_{13}NO]^+$, 91 (76) |
| 4 | 22.3 | undeca-2*Z*,4*E*-diene-8, 10-diynoic acid isobutylamide | 260 | 481.1 $[2M + Na]^+$<br>459.1 $[2M + H]^+$<br>252.1 $[M + Na]^+$<br>230.1 $[M + H]^+$ | 188 (12) $[M + H - C_3H_6]^+$, 174 (79) $[M + H - C_4H_8]^+$, 157 (12) $[M + H - C_4H_{11}N]^+$, 146 (35), 129 (98) $[M + H - C_5H_{11}NO]^+$, 116 (16), 103 (4) $[M + H - C_7H_{13}NO]^+$, 91 (40) |
| 5 | 22.6 | dodeca-2*E*,4*Z*-diene-8, 10-diynoic acid isobutylamide[b] | 262 | 482.2 $[2M + H]^+$<br>266.1 $[M + Na]^+$<br>244.1 $[M + H]^+$ | 216 (11), 202 (6), 188 (18) $[M + H - C_4H_8]^+$, 171 (15) $[M + H - C_4H_{11}N]^+$, 161 (6), 145 (71), 143 (30) $[M + H - C_5H_{11}NO]^+$, 129 (9) $[M + H - C_6H_{13}NO]^+$, 117 (100) $[M + H - C_7H_{13}NO]^+$, 105 (18), 91 (14) $[M + H - C_9H_{15}NO]^+$ |
| 6 | 23.6 | trideca-2*E*,7*Z*-diene-10, 12-diynoic acid isobutylamide | 260 | 280.1 $[M + Na]^+$<br>258.2 $[M + H]^+$ | 230 (3), 216 (3), 202 (33) $[M + H - C_4H_8]^+$, 185 (10) $[M + H - C_4H_{11}N]^+$, 157 (31) $[M + H - C_5H_{11}NO]^+$, 142 (23) $[C_8H_{16}NO]^+$, 131 (53) $[M + H - C_7H_{13}NO]^+$, 129 (72), 117 (100) $[M + H - C_8H_{15}NO]^+$, 105 (29), 103 (5) $[M + H - C_9H_{17}NO]^+$, 91 (24), 79 (5) |
| 7 | 23.8 | dodeca-2*Z*,4*E*-diene-8, 10-diynoic acid isobutylamide[b] | 260 | 482.2 $[2M + H]^+$<br>266.1 $[M + Na]^+$<br>244.1 $[M + H]^+$ | 188 (6) $[M + H - C_4H_8]^+$, 171 (19) $[M + H - C_4H_{11}N]^+$, 160 (3), 145 (34), 129 (6) $[M + H - C_6H_{13}NO]^+$, 117 (100) $[M + H - C_7H_{13}NO]^+$, 105 (10), 91 (5) $[M + H - C_9H_{15}NO]^+$ |
| 8 | 24.0 | dodeca-2*E*,4*Z*-diene-8, 10-diynoic acid 2-methylbutylamide | 262 | 515.1 $[2M + H]^+$<br>280.1 $[M + Na]^+$<br>258.2 $[M + H]^+$ | 230 (4) $[M + H - C_2H_4]^+$, 188 (32) $[M + H - C_5H_{10}]^+$, 171 (18) $[M + H - C_5H_{13}N]^+$, 145 (92), 143 (49) $[M + H - C_6H_{13}NO]^+$, 130 (15), 117 (100) $[M + H - C_8H_{15}NO]^+$, 105 (12), 91 (5) $[M + H - C_{10}H_{17}NO]^+$, 79 (4) |
| 9 | 24.8 | dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide | 202, 234 | 268.1 $[M + Na]^+$<br>246.2 $[M + H]^+$ | 190 (3) $[M + H - C_4H_8]^+$, 173 (56) $[M + H - C_4H_{11}N]^+$, 155 (6), 147 (100), 145 (48) $[M + H - C_5H_{11}NO]^+$, 131 (31), 119 (24) $[M + H - C_7H_{13}NO]^+$, 105 (27), 93 (3) $[M + H - C_9H_{15}NO]^+$, 91 (14), 74 (4) |
| 10 | 25.4 | dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide | 236, 259 | 295.3 $[2M + H]^+$<br>270.1 $[M + Na]^+$<br>248.2 $[M + H]^+$ | 192 (3) $[M + H - C_4H_8]^+$, 175 (12) $[M + H - C_4H_{11}N]^+$, 167 (8) $[M + H - C_6H_9]^+$, 147 (27) $[M + H - C_5H_{11}NO]^+$, 149 (82), 142 (100), 133 (16), 121 (24) $[M + H - C_7H_{13}NO]^+$, 107 (53), 100 (20), 93 (30), 86 (16), 81 (7) $[C_6H_9]^+$, 74 (3) $[C_4H_{12}N]^+$ |
| 11 | 27.4 | dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide | 260 | 499.2 $[2M + H]^+$<br>272.1 $[M + Na]^+$<br>250.1 $[M + H]^+$ | 194 (6) $[M + H - C_4H_8]^+$, 177 (8) $[M + H - C_4H_{11}N]^+$, 167 (100) $[M + H - C_6H_{11}]^+$, 152 (20), 149 (7) $[M + H - C_5H_{11}NO]^+$, 135 (12), 121 (19), 109 (34), 107 (30), 97 (3) $[M + H - C_9H_{15}NO]^+$, 95 (53), 81 (26), 74 (4) |
| 12 | 30.1 | dodeca-2*E*,4*E*-dienoic acid isobutylamide | 224, 278 | 503.3 $[2M + H]^+$<br>274.1 $[M + Na]^+$<br>252.2 $[M + H]^+$ | 210 (10) $[M + H - C_3H_6]^+$, 196 (30) $[M + H - C_4H_8]^+$, 179 (60) $[M + H - C_4H_{11}N]^+$, 168 (11) $[C_{10}H_{18}NO]^+$, 151 (10) $[M + H - C_5H_{11}NO]^+$, 154 (20) $[C_9H_{16}NO]^+$, 151 (10) $[M + H - C_5H_{11}NO]^+$, 133 (9), 119 (29), 105 (40), 95 (100), 91 (22), 81 (38), 74 (6) |

[a] The MS$^2$ data were obtained from the fragmentation of the $[M - H]^-$ and $[M + H]^+$ precursor ions of caffeic acid derivatives and alkamides, respectively. Relative intensities of product ions are in parentheses. [b] *E*/*Z* stereochemistry is indicated in agreement with the literature,[5,12] but it is not possible to conclusively distinguish between the two isomers without conformational NMR spectra.



dx.doi.org/10.1021/jf2025677 |J. Agric. Food Chem. 2011, 59, 10425–10434

**Table 2. Content of Caffeic Acid Derivatives and Alkamides (mg/g) in the Roots of Healthy and Infected Samples of *E. purpurea* by HPLC-DAD[a]**

| peak no. | compd | healthy | virus-infected | phytoplasma-infected |
|---|---|---|---|---|
| 1 | caftaric acid | 2.47 ± 0.04 a | 2.34 ± 0.08 b | 2.77 ± 0.03 c |
| 2 | cichoric acid | 18.35 ± 0.13 a | 13.31 ± 0.21 b | 15.38 ± 0.38 c |
| 3 | undeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide | <LOQ | <LOQ | <LOQ |
| 4 | undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide | 0.10 ± 0.01 a | 0.13 ± 0.01 b | 0.16[b] c |
| 5 | dodeca-2*E*,4*Z*-diene-8,10-diynoic acid isobutylamide[c] | 0.07 ± 0.01 a | 0.08 ± 0.01 b | 0.06[b] a |
| 6 | trideca-2*E*,7*Z*-diene-10,12-diynoic acid isobutylamide | 0.09 ± 0.01 ab | 0.08 ± 0.01 a | 0.09[b] b |
| 7 | dodeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide[c] | <LOQ | <LOQ | <LOQ |
| 8 | dodeca-2*E*,4*Z*-diene-8,10-diynoic acid-2 methylbutylamide | 0.03[b] a | 0.03 ± 0.01 a | 0.02[b] b |
| 9 | dodeca-2*E*,4*E*,10*E*-triene-8-ynoic acid isobutylamide | <LOQ | <LOQ | <LOQ |
| 10 | dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide | 0.53 ± 0.01 a | 0.23 ± 0.04 b | 0.60 ± 0.04 c |
| 11 | dodeca-2*E*,4*E*,8*Z*-trienoic acid isobutylamide | <LOQ | <LOQ | <LOQ |
| 12 | dodeca-2*E*,4*E*-dienoic acid isobutylamide | <LOQ | <LOQ | <LOQ |
| | total caffeic acid derivatives | 20.82 ± 0.17 a | 15.65 ± 0.28 b | 18.15 ± 0.40 c |
| | total alkamides | 0.81 ± 0.03 a | 0.55 ± 0.08 b | 0.94 ± 0.05 c |

[a] Data are expressed as means ($n = 6$) ± SDs. The same letter in the same row indicates no significant differences ($P < 0.05$). [b] SD < 0.005. [c] *E*/*Z* stereochemistry is indicated in agreement with the literature,[5,12] but it is not possible to conclusively distinguish between the two isomers without conformational NMR spectra.

peak areas and concentrations over the tested ranges ($r > 0.999$). The LOQ value was 3.0 μg/mL for both caffeic acid derivatives and 1.3 μg/mL for dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide used as the alkamide standard. The LOD value was 0.9 μg/mL for caffeic acid derivatives and 0.4 μg/mL for the reference alkamide. With regard to accuracy, the percentage recovery values were found to be 89.3 ± 0.4% for caftaric acid, 85.4 ± 0.1% for cichoric acid, and 104.7 ± 0.2% for dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide. The low intra- and inter-day % RSD values for both retention times (% RSD < 0.3) and peak areas (% RSD < 1.2) indicate the high precision of the chromatographic system. Regarding stability, the analytes in solution did not show any appreciable change in the chromatographic profile over 72 h, and degradation products were not detected.

**Quantification of Constituents in *E. purpurea* Extracts.** The method developed was applied for the determination of the content of caffeic acid derivatives and alkamides in *E. purpurea* root extracts. The results are shown in Table 2, expressed as mg/g. All the samples analyzed displayed a common phytochemical profile, based on the presence of the same caffeic acid derivatives and alkamides. The content of caffeic acid derivatives and alkamides in healthy plants was found to be largely in agreement with the literature.[4,27] However, there was great variability in the concentration of the active constituents among the healthy and infected samples of *E. purpurea*. The RP-HPLC analysis revealed that the presence of the disease significantly ($P < 0.05$) influenced the content of cichoric acid, which was the main caffeic acid derivative present in *E. purpurea* extracts: The healthy individuals showed a cichoric acid content of 18.35 ± 0.13 mg/g, which was remarkably and significantly ($P < 0.05$) lower in virus-infected and in phytoplasma-infected samples (13.31 ± 0.21 and 15.38 ± 0.38 mg/g, respectively). Regarding caftaric acid, its content was slightly decreased in virus-infected plants (2.34 ± 0.08 vs 2.47 ± 0.04 mg/g), while in the phytoplasma-infected sample, there was a slight increase in the content of this phenolic compound (2.77 ± 0.03 mg/g). The total amount of caffeic acid derivatives followed the same trend as described for cichoric acid.

Regarding the main alkamide, dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, a decrease in the content of this secondary metabolite was observed in the virus-infected sample (0.23 ± 0.04 mg/g) in comparison with the healthy plants (0.53 ± 0.01 mg/g), while in the phytoplasma-infected sample, the variation in this secondary metabolite was not appreciable (0.60 ± 0.04). Another statistically significant variation ($P < 0.05$) was found for undeca-2*Z*,4*E*-diene-8,10-diynoic acid isobutylamide, whose content was increased in virus-infected and phytoplasma-infected plants (0.13 ± 0.01 and 0.16 mg/g, respectively) in comparison with the healthy sample (0.10 ± 0.01 mg/g). The overall alkamide content showed the same trend as described for dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide. Hudaib et al.[30] have already observed that infection of *E. purpurea* with CMV caused a significant reduction in the yield of the lipophilic fraction and several changes to the relative composition of hydrophobic components.

In the case of dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide, the ratio between the *E* and *Z* isomers was calculated using the % area values integrated from the HPLC chromatograms. Considering that the *E*/*Z* ratio of this alkamide was found to be 33:67 in the healthy sample, it was observed that the virus and phytoplasma infections had inverse effects on the relative composition of the two isomers: The first one decreased the percentage of the *E* isomer (16:84), while the second caused an increase (62:38). The % relative area of the *E*/*Z* isomers of the main alkamide could thus be a useful marker to detect a phytopathological condition in cultivated *E. purpurea* raw material.

The differences in the content of caffeic acid derivatives and alkamides between healthy and infected samples of *E. purpurea* observed in the present study may be ascribed to the profound histological modifications to the plant organs induced by the pathogens, which caused alterations to their physiological secondary metabolite production. A number of diseases can modify the morphology of structures that secrete and accumulate plant secondary metabolites at tissue and cell levels. This may have different relapses on biosynthetic pathways and change both the

**Table 3. Chemical Composition of the Essential Oil (Relative Area Percentage, RA%) in the Aerial Parts of Healthy and Infected Samples of *E. purpurea*[a]**

| compd | $R_i$[b] | healthy | virus-infected | phytoplasma-infected |
|---|---|---|---|---|
| α-pinene | 942 | 8.9 ± 0.4 a | 8.4 ± 0.2 a | 7.4 ± 0.1 b |
| camphene | 953 | 0.6[c] a | 0.5 ± 0.1 ab | 0.4[c] b |
| sabinene | 976 | 0.7[c] a | 0.5[c] b | 0.5 ± 0.1 b |
| β-pinene | 980 | 9.4 ± 0.7 a | 8.9 ± 0.7 a | 9.0 ± 0.5 a |
| verbene | 984 | 0.6[c] a | 0.5[c] a | 0.5 ± 0.1 a |
| myrcene | 986 | 0.5[c] a | 0.3[c] b | 0.4[c] a |
| α-phellandrene | 1003 | 9.0 ± 0.6 a | 8.7 ± 0.3 a | 8.2 ± 0.1 a |
| β-phellandrene | 1005 | 0.3[c] a | 0.3[c] a | 0.2[c] b |
| limonene | 1025 | 2.2 ± 0.4 a | 8.4[c] b | 4.4 ± 0.2 c |
| *p*-cymene | 1026 | 3.8 ± 0.4 a | 3.2 ± 0.1 a | 3.1 ± 0.1 b |
| pinocarvone | 1124 | 1.2 ± 0.1 a | 1.0 ± 0.1 b | 1.0 ± 0.1 b |
| *cis*-verbenol | 1140 | 1.8 ± 0.3 a | 3.4 ± 0.1 b | 5.7 ± 0.1 c |
| verbenone | 1204 | 2.7 ± 0.4 a | 7.1 ± 0.1 b | 11.6 ± 0.2 c |
| pinocarvol | 1224 | 0.9 ± 0.1 a | 0.8 ± 0.1 a | 0.7 ± 0.1 a |
| carvone | 1242 | 0.8 ± 0.1 a | 0.6 ± 0.1 a | 2.5 ± 0.2 b |
| *p*-menthan-1, 3,8-triene | 1286 | 3.1 ± 0.2 a | 2.0 ± 0.1 b | 1.9 ± 0.2 b |
| thymol | 1290 | 1.1 ± 0.1 a | 0.7 ± 0.1 b | 0.7 ± 0.1 b |
| isoledene | 1365 | 2.2 ± 0.3 a | 1.6[c] b | 1.5 ± 0.2 b |
| β-caryophyllene | 1418 | 2.0 ± 0.2 a | 1.8 ± 0.2 ab | 1.4 ± 0.1 b |
| α-humulene | 1440 | 0.6[c] a | 0.2[c] b | 0.5 ± 0.1 a |
| germacrene D | 1480 | 10.8 ± 0.5 a | 9.9 ± 0.1 b | 8.5 ± 0.1 c |
| δ-cadinene | 1524 | 1.8 ± 0.4 a | 1.7 ± 0.2 a | 1.4 ± 0.1 a |
| nerolidol | 1534 | 13.7 ± 0.4 a | 13.1 ± 0.3 ab | 12.4 ± 0.2 b |
| caryophyllene oxide | 1572 | 4.5 ± 0.1 a | 4.0 ± 0.1 b | 3.3 ± 0.1 c |
| spathulenol | 1575 | 4.4 ± 0.6 a | 3.8 ± 0.3 b | 3.2[c] c |
| linalyl acetate | 1580 | 2.0 ± 0.3 a | 0.9 ± 0.1 b | 1.9 ± 0.2 a |
| nor-copanon | 1582 | 2.1 ± 0.4 a | 1.8 ± 0.2 a | 1.5 ± 0.2 a |
| widrol | 1641 | 5.3 ± 0.1 a | 4.1 ± 0.1 b | 4.0 ± 0.3 b |
| valerenol | 1653 | 1.6 ± 0.2 a | 1.0 ± 0.1 b | 1.1 ± 0.1 b |
| α-cadinol | 1667 | 1.4 ± 0.1 a | 0.9 ± 0.1 b | 1.1[c] b |

[a] Data are expressed as means ($n = 3$) ± SDs. The same letter in the same row indicates no significant differences ($P < 0.05$). [b] Retention index. [c] SD < 0.05.

chemical structure of the active principles as well as their absolute and relative abundance. Whether this translates into an increase or a decrease of specific substances is not easily predictable, and mixed results have been reported in the literature.[15,16] In the case of *H. perforatum*, the phytoplasma-infected plants have shown a decrease in the content of total flavonoids and naphthodianthrones,[15] while the content of chlorogenic acid was doubled when compared with the healthy plants.[15] The analyses carried out on phytoplasma-infected samples of *D. lanata* demonstrated that the presence of this pathogen caused a decrease in the primary glycoside content (lanatosides A, B, and C),[16] which was paralleled by an increased amount of α-acetyldigoxin and deactyllanatoside C, the production of which has been related to the activity of hydrolytic enzymes.[16] In the case of *E. purpurea* and its secondary metabolites, a consistent decrease in the content of cichoric acid and dodeca-2*E*,4*E*,8*Z*,10*E*/*Z*-tetraenoic acid isobutylamide was observed in the infected plant material, particularly in the virus-infected sample: These compounds are likely to be produced in lower amounts or degraded in infected plants, and their carbon units recycled to a different use. In view of the remarkable properties of both phenolic compounds, which are mainly antiviral, anti-inflammatory, and antioxidant,[1,2] and alkamides, which are mainly anti-inflammatory and immunomodulatory,[14] the commercial value of the raw material and the biological activity of the extracts obtained from *E. purpurea* infected plants can be significantly affected.

**Analysis of the Essential Oil.** The chemical composition of the essential oil obtained from healthy and infected samples of *E. purpurea* was studied by GC and GC-MS, and the results are shown in Table 3. The analysis of the volatile compounds was focused on *E. purpurea* aerial parts, which are known to contain a higher amount of terpenoids in comparison with the root tissues.[31] In this study, the difference in the extraction yield of the essential oil between healthy and infected samples of *E. purpurea* was found to be not appreciable (0.029% w/w in the healthy, 0.028% w/w in the virus-infected and 0.026% w/w in the phytoplasma-infected sample, respectively). The GC and GC-MS analysis enabled the identification of a total of 30 compounds, and the qualitative composition was found to be in agreement with the literature.[31] In the healthy *E. purpurea* sample, the main monoterpenes were found to be α-pinene (8.9%), β-pinene (9.4%), and α-phellandrene (9.0%), while regarding sesquiterpenes the main constituents were germacrene D (10.8%) and nerolidol (13.7%). The comparison of the semiquantitative composition of the essential oils extracted from healthy and infected samples showed that the main significant discrepancies ($P < 0.05$) can be attributed to three components: limonene (2.2% in healthy, 8.4% in virus-infected, and 4.4% in phytoplasma-infected plants), *cis*-verbenol (1.8% in healthy, 3.4% in virus-infected, and 5.7% in phytoplasma-infected plants), and its parent ketone verbenone (2.7% in healthy, 7.1% in virus-infected, and 11.6% in phytoplasma-infected plants). Another minor enrichment occurred for the carvone content (from 0.8 to 2.5%) but only in plants affected by phytoplasma. An increase in the content of limonene in the essential oil extracted from phytoplasma-infected plants of *G. robusta* in comparison with that extracted from healthy plants has already been described in the literature.[17] The limonene content was also increased in CMV-infected plants of *Agastache anethiodora*.[32] As shown in Table 3, other compounds whose relative area percentages were significantly lower ($P < 0.05$) in *E. purpurea* infected samples are germacrene D, cariophyllene oxide, and spathulenol. In a previous study,[33] germacrene D was found to be the main constituent of *E. purpurea* essential oil, and its percentage was significantly reduced in a virus-infected sample. Regarding spathulenol, a decreased content of this compound has already been described in the essential oil extracted from phytoplasma-infected plants of *H. perforatum*.[15]

Plant pathogens are known to influence the yield and composition of essential oils,[15,17,32−34] suggesting that they can affect plant secondary metabolism, leading to losses in the commercial and therapeutic values of the marketed product. However, there have been few investigations into the effect of phytoplasmas on the chemical composition of essential oils from infected herbal crops[15,17] and none previously on *E. purpurea*. It is unclear whether the modifications to the composition of *E. purpurea* volatile constituents associated with virus and phytoplasma are a consequence of the morphological modifications induced by the pathogens or represent plant chemical defense to reduce the detrimental effects of the infections,[15,17,32,34] since the three

above-cited monoterpenes have already been reported to provide beneficial effects to plants against microbial and insect attacks.[17,35,36] In any case, this evidence clearly suggests that the phytopathological status should be a further variable, in addition to genetic variation, agronomic practices, and environmental conditions,[33] to be considered whenever a fluctuation in *E. purpurea* essential oil composition is observed.

The commercial value of a medicinal plant and the biological activity of its extracts are strictly correlated with its chemical composition and the relative abundance of its secondary metabolites. Caffeic acid derivatives and alkamides are responsible for the main biological activities attributed to *E. purpurea* extracts,[1,2,14] and therefore, their content is critical for both the quality of the raw material and the therapeutic effect of the plant extracts. Even though the biological activity of *E. purpurea* essential oil has not been properly investigated in the literature, it is likely that the volatile compounds determined in this study may contribute not only to the overall aroma, flavor, and market value of the plant material but also to the biological properties of the extracts.[31] The chromatographic techniques developed and applied in this study enabled a comparative phytochemical analysis of the plant material obtained from healthy and infected plants of *E. purpurea*. The results indicate that virus and phytoplasma infections influence the amount of secondary metabolites in *E. purpurea*, thus representing an important parameter in defining the commercial quality, market value, and therapeutic efficacy of this plant material. For this reason, in addition to routine growth stage control during the plant life cycle, attention should be paid to the disease status upon collection, to reduce damage due to parasite and pathogen attacks (including viruses and phytoplasmas) and obtain final plant material of good quality. From the determination of caffeic acid derivatives, alkamides (including also the % relative area of the *E*/*Z* isomers of the main alkamide) and essential oil, the quality of *E. purpurea* can be effectively controlled. In this context, the methods proposed can be considered highly suitable for metabolite profiling and quality assurance of *E. purpurea* plant material used for pharmaceutical purposes.

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### Funding Sources

We acknowledge the "Fondazione Cassa di Risparmio di Modena" for funding the HPLC-ESI-MS$^n$ system at the Centro Interdipartimentale Grandi Strumenti (CIGS) of the University of Modena and Reggio Emilia.

## ACKNOWLEDGMENT

We thank Dr. Diego Pinetti, CIGS, for his technical support and Dr. Katie Jane Henry for the linguistic revision.

## REFERENCES

(1) Barrett, B. Medicinal properties of *Echinacea*: A critical review. *Phytomedicine* **2003**, *10*, 66–86.

(2) Barnes, J.; Anderson, L. A.; Gibbons, S.; Phillipson, J. D. *Echinacea* species (*Echinacea angustifolia* (DC) Hell., *Echinacea pallida* (Nutt.) Nutt., *Echinacea purpurea* (L.) Moench): A review of their chemistry, pharmacology and clinical properties. *J. Pharm. Pharmacol.* **2005**, *57*, 929–954.

(3) Cheminat, A.; Zawatzky, R.; Becker, H.; Brouillard, R. Caffeoyl conjugates from *Echinacea* species: Structures and biological activity. *Phytochemistry* **1988**, *27*, 2787–2794.

(4) Pellati, F.; Benvenuti, S.; Magro, L.; Melegari, M.; Soragni, F. Analysis of phenolic compounds and radical scavenging activity of *Echinacea* spp. *J. Pharm. Biomed. Anal.* **2004**, *35*, 289–301.

(5) Bauer, R.; Reminger, P. TLC and HPLC analysis of alkamides from *Echinacea* drugs. *Planta Med.* **1989**, *55*, 367–371.

(6) Pellati, F.; Calò, S.; Benvenuti, S.; Adinolfi, B.; Nieri, P.; Melegari, M. Isolation and structure elucidation of polyacetylenes and polyenes from *Echinacea pallida*. *Phytochemistry* **2006**, *67*, 1359–1364.

(7) Pellati, F.; Calò, S.; Benvenuti, S. High-performance liquid chromatography analysis of polyacetylenes and polyenes in *Echinacea pallida* by using a monolithic reversed-phase silica column. *J. Chromatogr. A* **2007**, *1149*, 56–65.

(8) Wagner, H.; Stuppner, H.; Schäfer, W.; Zenk, M. Immunologically active polysaccharides of *Echinacea purpurea* cell cultures. *Phytochemistry* **1988**, *27*, 119–126.

(9) Classen, B.; Witthohn, K.; Blaschek, W. Characterization of an arabinogalactan-protein isolated from pressed juice of *Echinacea purpurea* by precipitation with the $\beta$-glucosyl Yariv reagent. *Carbohydr. Res.* **2000**, *327*, 497–504.

(10) Thude, S.; Classen, B. High molecular weight constituents from roots of *Echinacea pallida*: An arabinogalactan-protein and an arabinan. *Phytochemistry* **2005**, *66*, 1026–1032.

(11) Dalby-Brown, L.; Barsett, H.; Landbo, A. K.; Meyer, A. S.; Mølgaard, P. Synergistic antioxidative effects of alkamides, caffeic acid derivatives, and polysaccharide fractions from *Echinacea purpurea* on in vitro oxidation of human low-density lipoproteins. *J. Agric. Food. Chem.* **2005**, *53*, 9413–9423.

(12) Bauer, R.; Remiger, P.; Wagner, H. Alkamides from the roots of *Echinacea purpurea*. *Phytochemistry* **1988**, *27*, 2339–2342.

(13) Bauer, R.; Remiger, P.; Wagner, H. Alkamides from the roots of *Echinacea angustifolia*. *Phytochemistry* **1989**, *28*, 505–508.

(14) Woelkart, K.; Bauer, R. The role of alkamides as an active principle of *Echinacea*. *Planta Med.* **2007**, *73*, 615–623.

(15) Bruni, R.; Pellati, F.; Bellardi, M. G.; Benvenuti, S.; Paltrinieri, S.; Bertaccini, A.; Bianchi, A. Herbal drug quality and phytochemical composition of *Hypericum perforatum* L. affected by ash yellows phytoplasma infection. *J. Agric. Food Chem.* **2005**, *53*, 964–968.

(16) Pellati, F.; Bruni, R.; Bellardi, M. G.; Bertaccini, A.; Benvenuti, S. Optimization and validation of a high-performance liquid chromatography method for the analysis of cardiac glycosides in *Digitalis lanata*. *J. Chromatogr. A* **2009**, *1216*, 3260–3269.

(17) Bertaccini, A.; Contaldo, N.; Benni, A.; Curini, M.; Genovese, S.; Epifano, F.; Bellardi, M. G. Effetcs of "Candidatus phytoplasma asteris" on the volatile chemical content and composition of *Grindelia robusta* Nutt. *J. Phytopathol.* **2011**, *159*, 124–126.

(18) Edwards, M. L.; Cooper, J. I. Plant virus detection using a new form of indirect ELISA. *J. Virol. Methods* **1985**, *11*, 309–313.

(19) Prince, J. P.; Davis, R. E.; Wolf, T. K.; Lee, I.-M.; Mogen, B. D.; Dally, E. L.; Bertaccini, A.; Credi, R.; Barba, M. Molecular detection of diverse MLOs associated with grapewine yellows and their classification with aster yellow, X-disease, and elm yellow MLOs. *Phytopathology* **1993**, *83*, 1130–1137.

(20) Deng, S.; Hiruki, C. Amplification of l6S rRNA genes from culturable and nonculturable *Mollicutes*. *J. Microbiol. Methods* **1991**, *14*, 53–61.

(21) Schenider, B.; Seemuller, E.; Smart, C. D.; Kirkpatrick, B. C. Phylogenetic classification of plant pathogenic mycoplasmalike organisms or phytoplasmas. In *Molecular and Diagnostic Procedures in Mycoplasmology*; Razin, S., Tully, J. G., Eds; Academic Press: New York, NY, 1995; Vol. 2, pp 369−380.

(22) Lee, I.-M.; Bertaccini, A.; Vibio, M.; Gundersen, D. E. Detection of multiple phytoplasmas in perennial fruit trees with decline symptoms in Italy. *Phytopathology* **1995**, *85*, 728–735.

(23) Kim, H.-O.; Durance, T. D.; Scaman, C. H.; Kitts, D. D. Retention of alkamides in dried *Echinacea purpurea*. *J. Agric. Food Chem.* **2000**, *48*, 4187–4192.

(24) Binns, S. E.; Livesey, J. F.; Arnason, J. T.; Baum, B. R. Phytochemical variation in *Echinacea* from roots and flowerheads of wild and cultivated populations. *J. Agric. Food Chem.* **2002**, *50*, 3673–3687.

(25) Davies, N. W. Gas chromatographic retention indices of monoterpenes and sesquiterpenes on methyl silicone and carbowax 20M phases. *J. Chromatogr.* **1990**, *503*, 1–24.

(26) Bellardi, M. G.; Bertaccini, A. *Malattie delle Piante Officinali—Virosi e Fitoplasmosi*; L'informatore Agrario Ed.: Verona, Italy, 2005.

(27) Luo, X. B.; Chen, B.; Yao, S. Z.; Zeng, J. G. Simultaneous analysis of caffeic acid derivatives and alkamides in roots and extracts of *Echinacea purpurea* by high-performance liquid chromatography-photodiode array detection-electrospray mass spectrometry. *J. Chromatogr. A* **2003**, *986*, 73–81.

(28) Cech, N.; Eleazer, M. S.; Shoffner, L. T.; Crosswhite, M. R.; Davis, A. C.; Mortenson, A. M. High performance liquid chromatography/electrospray ionization mass spectrometry for the simultaneous analysis of alkamides ad caffeic acid derivatives from *Echinacea purpurea* extracts. *J. Chromatogr. A* **2006**, *1103*, 219–228.

(29) Spelman, K.; Wetschler, M. H.; Cech, N. B. Comparison of alkylamide yield in ethanolic extracts prepared from fresh versus dry *Echinacea purpurea* utilizing HPLC-ESI-MS. *J. Pharm. Biomed. Anal.* **2009**, *49*, 1141–1149.

(30) Hudaib, M.; Fiori, J.; Bellardi, M. G.; Rubies-Autonell, C.; Cavrini, V. GC-MS analysis of the lipophilic principles of *Echinacea purpurea* and evaluation of cucumber mosaic cucumovirus (CMV) infection. *J. Pharm. Biomed. Anal.* **2002**, *29*, 1053–1060.

(31) Mazza, G.; Cotrell, T. Volatile components of roots, stems, leaves, and flowers of *Echinacea* species. *J. Agric. Food Chem.* **1999**, *47*, 3081–3085.

(32) Bruni, R.; Bianchi, A.; Bellardi, M. G. Essential oil composition of *Agastache anethiodora* Britton (Lamiaceae) infected by cucumber mosaic virus (CMV). *Flavour Fragrance J.* **2007**, *22*, 66–70.

(33) Hudaib, M.; Cavrini, V.; Bellardi, M. G.; Rubies-Autonell, C. Characterization of the essential oil of healthy and virus infected *Echinacea purpurea* (L.) Moench plants. *J. Essent. Oil Res.* **2002**, *14*, 427–430.

(34) Bruni, R.; Bellardi, M. G.; Parrella, G.; Bianchi, A. Impact of alfalfa mosaic virus subgroup I and II isolates on terpene secondary metabolism of *Lavandula vera* D.C., *Lavandula* × *alardii* and eight cultivars of *L. hybrida* Rev. *Physiol. Mol. Plant Pathol.* **2006**, *68*, 189–197.

(35) Nerio, L. S.; Olivero-Verbel, J.; Stashenko, E. Repellent activity of essential oils: A review. *Bioresour. Technol.* **2010**, *101*, 372–378.

(36) Borden, J. H.; Sparrow, G. R.; Gervan, N. L. Operational success of verbenone against the mountain pine beetle in a rural community. *Arboriculture Urban For.* **2007**, *33*, 318–324.