ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
24 (2001) 517–526

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

LC coupled to ion-trap MS for the rapid screening and detection of polyphenol antioxidants from *Helichrysum stoechas*

Marina Carini [a,*], Giancarlo Aldini [a], Sandra Furlanetto [b], Rita Stefani [a], Roberto Maffei Facino [a]

[a] *Istituto Chimico Farmaceutico Tossicologico, Viale Abruzzi 42-20131, Milano, Italy*
[b] *Dipartimento di Scienze Farmaceutiche, Via G. Capponi, 9-50121, Firenze, Italy*

Received 1 April 2000; received in revised form 20 July 2000; accepted 6 September 2000

Abstract

Liquid Chromatography-Ion Trap Mass Spectrometry with an atmospheric pressure chemical ionization (APCI) interface in the negative and positive-ion modes in parallel to UV-diode-array detection (DAD), was applied for the rapid detection/characterization in crude extracts of the water-soluble antioxidant phenolics from *Helichrysum stoechas*. APCI-MS provides unequivocal molecular weight data of these compounds and useful information about their structures (diagnostic fragments ions), which were confirmed by the UV-DAD fingerprints. This combined approach allows the identification of ten constituents, including the three naturally occurring isomers of caffeoylquinic acid (CGAs), namely neo-chlorogenic acid, chlorogenic acid and crypto-chlorogenic acid, 2 isomeric dicaffeoyl quinic acids, 2 isomeric naringenin glucosides, quercetin, kaempferol and apigenin glucosides and a tetrahydroxychalcone-glucoside. The water-soluble extract from *H. stoechas*, standardized in both total polyphenol and kaempferol-3-glucoside content, exhibits strong antioxidant activity in vitro when tested in both artificial membrane systems (phosphatidylcholine liposomes) and in a cell model (rat erythrocytes).

Keywords: *Helichrysum stoechas*; Glucosyl flavonoids; Caffeoyl conjugates; Liquid Chromatography/Ion Trap Mass Spectrometry; Antioxidant activity

1. Introduction

The potential of liquid chromatography coupled to mass spectrometry for a rapid and direct screening of crude plant extracts for the search of products of benefit to human health has gained recently considerable interest. This is the case of

* Corresponding author. Tel.: +39-02-29510823; fax: +39-02-29514197.
E-mail address: marina.carini@unimi.it (M. Carini).

0731-7085/01/$ - see front matter
PII: S0731-7085(00)00431-3

polyphenols (phenolic acids, catechins, anthocyanins, flavonoids), which attract major interest because of their well-demonstrated anti-inflammatory effects, and of their potential anti-atherogenic properties, all based on their strong antioxidant capacity [1]. In previous studies we have demonstrated in both guinea pig and in man that the topical application of a purified water-soluble extract from *Helichrysum italicum* G. Don (Compositae) inhibits the inflammatory/erythematous reactions induced by UVB exposure [2] and further phytochemical investigations indicated that the effect is due to the radical scavenging activity of its main flavonoid constituents (naringenin-4-*O*-glucoside, kaempferol-3-*O*-glucoside, tetrahydroxychalcone-2′-*O*-glucoside), able to quench different reactive oxygen species induced by UV radiation [3,4].

Pursuing our interest on the antioxidant constituents from *H.* species (the genus *Helichrysum* is composed by at least 1000 species and the flowering tops of many of them, *H. arenarium*, *H. angustifolium*, *H. italicum*, *H. stoechas*, are known and used in folk medicine for their anti-inflammatory and anti-allergic properties), the aim of this work was to characterize the main polyphenol components of the polar fraction isolated from *H. stoechas*, by combining Liquid Chromatography-Ion Trap Mass Spectrometry with LC-UV-diode-array detection (DAD). This at the light of the following considerations: (a) *H. stoechas*, since its wide distribution in the Mediterranean area, is a source of easily available plant material; (b) very few studies report the phytochemical composition of this plant [5,6]; (c) notwithstanding the growing use in the last years of LC-MS in phytochemical analysis, no studies exist on its application to the characterisation of flavonoids from *Helichrysum* species. In the second part of this work we have done a preliminary in vitro evaluation of the antioxidant activity of the so far characterized fraction using acellular (phosphatidylcholine liposomes stressed by hydroxyl radicals) and cellular (rat erythrocytes exposed to cumene hydroperoxide) models.

2. Experimental

2.1. Chemicals

HPLC-grade and analytical-grade organic solvents were purchased from Merck (Bracco, Milan, Italy). HPLC-grade water was prepared with a Milli-Q water purification system.

The standard compounds chlorogenic acid, kaempferol-3-*O*-glucoside, naringenin-7-*O*-glucoside and quercetin-3-*O*-glucoside were purchased from Extrasynthese (Gigalabo, Milan, Italy); cumene hydroperoxide (CuOOH), Trolox (6-hydroxy-2,5,7,8-tetra-methylchroman-2-carboxylic acid) and soybean phosphatidylcholine (PC) from Fluka Chimica (Milan, Italy).

2.2. Plant material

Commercial *Helichrysum stoechas* capitula were kindly provided by Universal Flavors (Sesto S. Giovanni, Milan, Italy) and authenticated at the Department of Botany (University of Turin, Italy). The dried and finely minced flowering tops (50 g dry mass) were suspended in 350 ml of 70% ethanol (containing 0.1% ascorbic acid to prevent oxidation) and shaken for 3 h; the insoluble residue was re-extracted twice with the same solvent and the extracts combined. The ethanol was eliminated by evaporation under reduced pressure at 35°C and the aqueous residue washed with methylene chloride (1:1, v/v), to eliminate free aglycons and other not phenolic substances. The washed aqueous extract was concentrated under vacuum to dryness to obtain a pale-yellow powder (yield = 11%). The crude aqueous extract of *Helichrysum* was dissolved in water ($\approx$ 5 mg/ml), filtered through a 0.45 μm filter (Sartorius Minisart), and directly analyzed by LC-MS (5 μl injection); the same extract was used to test the antioxidant activity.

2.3. Instrumentation

LC-MS experiments were performed on a Finnigan MAT (San José, CA) LCQ ion trap mass spectrometer equipped with an APCI interface (source current 5 μA; capillary temperature

150°C; vaporizer temperature 600°C) and connected to a Waters (Vimodrone, Milan, Italy) 616 HPLC pump equipped with a gradient controller, a Waters 996 photodiode array detector and a Waters 717 automatic sample injection module. The mass spectrometer operated in negative-ion and positive-ion modes, with a scan range from m/z 150 to 800 (scan rate 0.5 scans/s).

The HPLC separation was done by reverse phase elution (Waters Symmetry LC-18 column, 250 × 4.6 mm, 5 μm) in the following conditions: concave gradient from 90% solvent A [ammonium formate buffer (pH 4.5; 30 mM)–CH_3CN, (95:5, v/v)] to 80% solvent B [CH_3CN–ammonium formate buffer (pH 4.5; 30 mM) (95:5, v/v)] in 30 min; flow rate 1 ml/min; UV-DAD detection (scan range 200–600 nm; rate 0.5 scan/s).

Spectrophotometric studies were performed with a computer aided Perkin Elmer lambda 16 spectrophotometer (Monza, Milan, Italy).

2.4. Standardization of the extract

The extract was standardized in both total polyphenol content, determined by the Prussian Blue Test and expressed as naringenin-glucoside [7] and in one of the main components (kaempferol-3-*O*-glucoside). Quantitation of kaempferol-3-*O*-glucoside (external standard method) was performed by LC-UV-DAD in the above described conditions with detection at 270 nm. Stock solution of kaempferol-3-glucoside [0.1 mg ml^{-1} in methanol–water (1:1, v/v)] was prepared and stored in the dark in a refrigerator. Working standard solutions were prepared daily by dilution with distilled water in the range 0.5–100 μg ml^{-1}; calibration curve was determined on five levels of concentration with three injections for each level. The precision of the method was estimated by measuring the repeatability and reproducibility for the analyte at three concentration levels (5, 25, 50 μg ml^{-1}) with three injections for each level and the relative standard deviations (RSDs) values were calculated.

The *Helichrysum* extract was dissolved in distilled water (0.5 mg ml^{-1}), filtered through a 0.45 μm filter (Sartorius Minisart), and 20 μl aliquots, corresponding to approximately 4.6 μg total polyphenols, injected for quantitation (five different preparations were tested and analyzed in duplicate).

2.5. In vitro antioxidant activity

The antioxidant activity of the extract was evaluated first in phosphatidylcholine liposomes (PCL) by determining its ability to inhibit, during the propagation phase of lipid peroxidation, the formation of conjugated dienes (CD) at 233 nm, as previously described [8]. The results were expressed as percentage inhibition of CD formation relative to the controls (means ± S.D. of 5 determinations), and used for determination of the IC_{50} value. The radical scavenging activity of the extract was then confirmed in a cellular model (1% rat erythrocyte suspensions) exposed to the free radical promoter cumene hydroperoxide (50 μM), and monitoring the time-course of hemolysis (turbidimetric determination at 710 nm) at 30 min intervals [9]. The protective effect of the extract (1–20 μg/ml) on CuOOH-induced hemolysis was compared to that of Trolox (1–20 μg/ml).

3. Results and discussion

3.1. LC-MS analysis

Fig. 1 and Fig. 2 show the HPLC-UV-DAD profile (270 nm) and the total-ion current traces in both positive- and negative-ion modes of the aqueous extract from *H. stoechas*.

The UV profile evidences at least 13 peaks with well distinct absorption maxima, due to different polyphenol species. Four of these (peaks A, B, E, F) have identical UV spectra, with absorption bands at 328–330 nm and 242 nm, and a sharp, diagnostic shoulder at 290–300 nm, typical of compounds containing a caffeoyl moiety. Their full negative-ion mass spectra exhibit intense $[M-H]^-$ ions and some diagnostic fragments (Table 1) which, as already reported [10], allow structure assignment. The structures of peaks A and B, with $[M-H]^-$ at m/z 353 (M.W. 354) and ions due to caffeic (m/z 179) and quinic acid (m/z 191) moieties, can be attributed to chlorogenic

acid isomers; those of peaks E and F, with $[M-H]^-$ at m/z 515 (M.W. 516) and fragments ions at m/z 179, 191 and 353 $[M-H-Caffeoyl]^-$, to isomeric dicaffeoyl esters of quinic acid (i.e. 3,4-*O*-dicaffeoyl, 4,5-*O*-dicaffeoyl, 3,5-*O*-dicaffeoyl or 1,5-*O*-dicaffeoyl esters), although in the absence of reference standards no definitive structure assignment can be done. Peak F (R.T. 12.62 min) co-elutes in part with peak G (R.T. 12.81), but both the UV (Fig. 1) and the MS specificity enables to identify two structurally unrelated compounds. The molecular weight and structure of peaks A, B, E, F were confirmed by the full scan positive-ion mass spectra (Table 1), showing quasi-molecular ions $[M+H]^+$ at m/z 355 (A, B) and m/z 517 (E, F) and by the selected ion chromatograms (SICs) of their $[M-H]^-$ ions (Fig. 3). The SIC for m/z 353 shows five peaks, two of them with retention times (4.09 and 6.56) identical to those of peaks A and B; the peaks with R.T. 10.03 and 12.62, corresponding to those in the SIC for m/z 515 (peaks E, F), arise by fragmentation of dicaffeoyl conjugates. The remaining peak of low intensity (A1, R.T. 5.71 min), not detectable in the UV-DAD profile, shows again in the full mass spectrum (inserted) a base peak at m/z 353. Hence peaks A, A1 and B, on the basis of order elution [10], were identified as neochlorogenic, chlorogenic and crypto-chlorogenic acids respectively. The ion trace chromatogram of m/z 191, $[\text{quinic acid}-H]^-$, a typical fragment ion of quinic acid esters, confirms the presence of five caffeoyl conjugates in the *H. stoechas* extract. These results agree with those reported by other authors, which demonstrate that mono- and dicaffeoylquinic acids, until now never identified in *H. stoechas*, but present in both *H. italicum* [11] and *H. bracteatum* flowers [12], are typical components of *Helichrysum* species. Peaks C and D show identical mass spectra, both in negative and positive-ion modes (Table 1): $[M-H]^-$ and $[M+H]^+$ ions at m/z 433/435 and, as main frag-

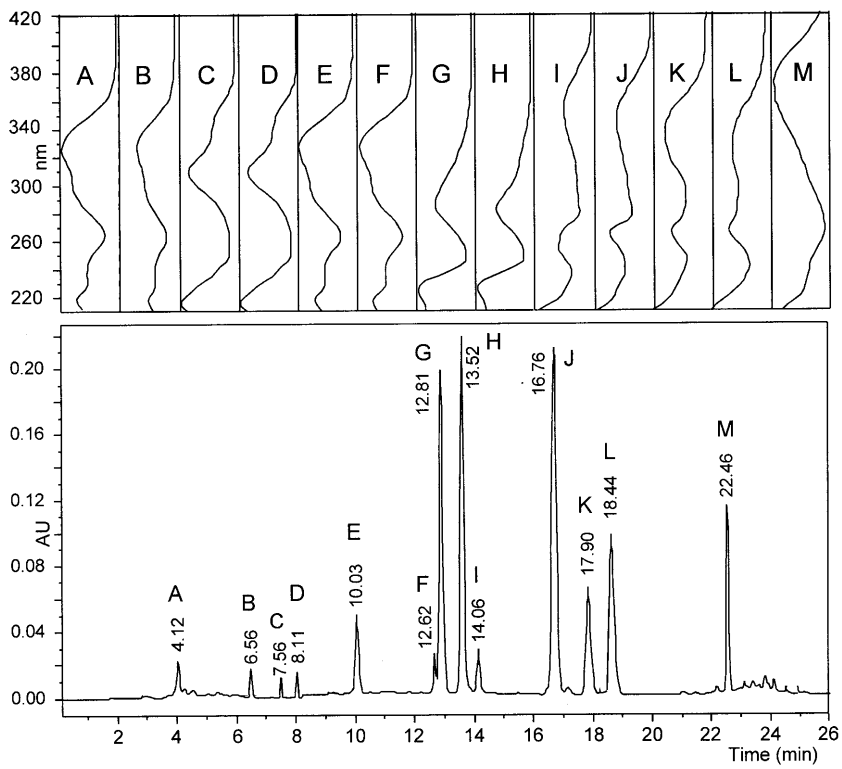

Fig. 1. LC-UV-DAD profile of *Helichrysum stoechas* extract (270 nm).

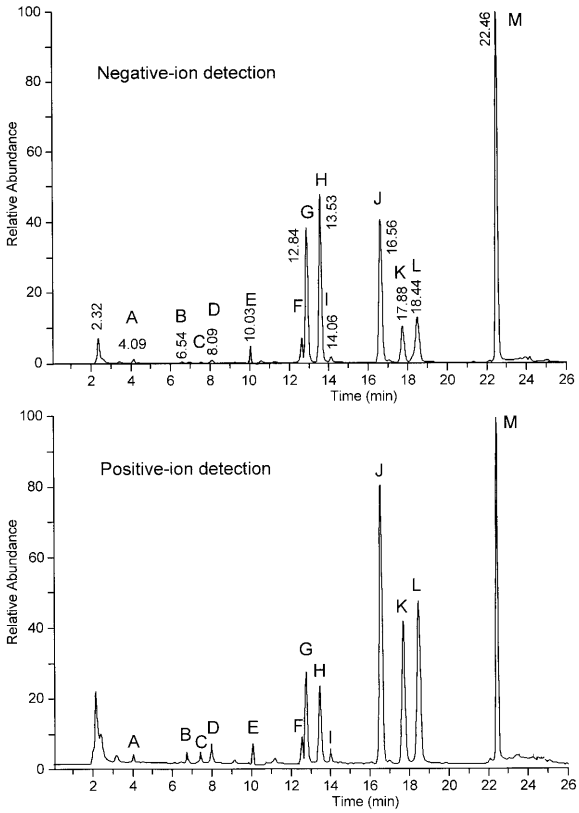

Fig. 2. Negative- and positive-ion LC-APCI-MS profiles of *Helichrysum stoechas* extract.

ment, the ions at m/z 271 $[M-H-162]^-$ or 273 $[M+H-162]^+$, due to loss of a glucosyl residue. This indicates for the aglycons a M.W. of 272 u, which could be attributed to flavanone or isomeric chalcone derivatives, but their UV-DAD profiles, with λ max at 309, shoulder at 355 and minimum at 260 nm (Fig. 1), seem to be non consonant with both the proposed structures (studies are in progress to elucidate the structures of these two minor components).

Peaks G and H show mass spectra (positive and negative-ion mode) superimposable to those of compounds C and D (Table 1), but UV-DAD absorption spectra with maxima at 224 and 288 nm, typical of flavanones: this combined information unequivocally identifies structural isomers of naringenin-glucoside, very likely naringenin-5-*O*-glucoside (helichrysine B) and naringenin-4′-*O*-glucoside (the most common derivatives in many *Helichrysum* species [3,13], since naringenin-7-*O*-glucoside was excluded by the LC behaviour of the reference standard, which elutes later in our chromatographic conditions (R.T. 15.30).

To peak I was assigned the structure of quercetin-3-*O*-glucoside on the basis of the UV-DAD fingerprint (λ max 256 and 356 nm, typical of flavonols), of the negative and positive-ion mode mass spectral information (Table 1) and finally by HPLC-UV-DAD behaviour of the reference compound (quercetin-3-*O*-glucoside has been already identified in *H. stoechas* capitula) [6].

Peak J shows the $[M-H]^-$ and $[M+H]^+$ ions at m/z 447/449 and the main fragment ion at m/z 285 $[M-H-Glu]^-$ and 287 $[M+H-Glu]^+$ (Table 1), to indicate an aglycon moiety of M.W. 286 u: i.e. kaempferol (flavonol) or luteolin (flavone). The UV-DAD profile (λ max 265, 296sh and 350 nm) is typical of kaempferol and not of luteolin (λ max 255, 267sh, 290sh, 350 nm). Kaempferol-3-*O*-glucoside (astragalin), the most probable candidate since already identified in both *H. graveolens* [13] and *H. italicum* [3], was confirmed by the comparison with the retention time of the reference standard.

The mass spectra of peaks K and L (Table 1) suggest glucosyl derivatives of flavones (M.W. 270), another class of compounds widely distributed in several *H.* species and their identical UV-DAD fingerprints, with λ max 267, 335/338 are consonant with the structure of isomeric apigenin glucosides, namely apigenin-7-*O*-glucoside and apigenin-4′-*O*-glucoside, already detected in *H. graveolens* [13], but until now never identified in *H. stoechas*. Finally, the UV spectrum of peak M shows a broad absorption band with λ max 369 nm and a shoulder at 250 nm, typical of chalcones; the mass spectrometric data (Table 1) in both negative and positive-ion mode indicate an isomeric structure of the flavanone naringenin, 4,2′,4′,6′-tetrahydroxychalcone. The M.W. of 434 was confirmed by the selected ion chromatogram (SIC) of the $[M-H]^-$ ions at m/z 433 (Fig. 4), showing five main peaks, with retention times corresponding to peaks C, D, G, H and M. Isosalipurposide (6′-*O*-glucoside) has been identified in *H. graveolens* [13] and the isomeric 2′-*O*-glucoside in *H. italicum* [3]: by comparison of the

chromatographic behaviour of peak M and of that of the chalcone derivative previously isolated from *H. italicum* and identified by us [3], to compound M was assigned the structure of 4,2′,4′,6′ - tetrahydroxychalcone - 2′ - *O* - glucoside. Fig. 4 reports also the SICs for the ions at *m/z* 431 and 447, which confirm molecular weight attribution to peaks J, K and L, and Fig. 5 the structures of the main polyphenol components identified in *H. stoechas* extract.

3.2. Determination of total/single polyphenols

Before testing the radical scavenging activity of the polar fraction of the drug, for standardization purposes, we evaluated the total polyphenol and kaempferol-3-*O*-glucoside content of some *Helichrysum* extracts ($n = 5$), all prepared according to the above reported procedure. The polyphenol content, determined by the Prussian Blue Test and expressed as naringenin-glucoside, was 46.1 ±

Table 1
Positive and negative-ion APCI mass spectra of the main constituents of the aqueous extract from *Helichrysum stoechas*[a]

Peak	R.T. min	Negative Ions		Positive Ions		Structure
		m/z (%)	Attribution	*m/z* (%)	Attribution	
A	4.12	353 (100)	$[M–H]^-$	355 (100)	$[M+H]^+$	
		191 (20)	$[\text{quinic acid}-H]^-$			Neo-chlorogenic acid
		179 (6.5)	$[\text{caffeic acid}-H]^-$			
A1	5.71	353 (100)	$[M-H]^-$	355 (100)	$[M+H]^+$	Chlorogenic acid
		191 (12)	$[\text{quinic acid}-H]^-$			
B	6.56	353 (100)	$[M-H]^-$	355 (100)	$[M+H]^+$	
		191 (27)	$[\text{quinic acid}-H]^-$			Cryptochlorogenic acid
		179 (5.5)	$[\text{caffeic acid}-H]^-$			
C	7.56	433 (100)	$[M-H]^-$	435 (100)	$[M+H]^+$	Not identified
		271 (60)	$[M-H-\text{Glu}]^-$	273 (64)	$[M+H-\text{Glu}]^+$	
D	8.11	433 (100)	$[M-H]^-$	435 (100)	$[M+H]^+$	Not identified
		271 (30)	$[M-H-\text{Glu}]^-$	273 (53)	$[M+H-\text{Glu}]^+$	
E	10.03	515 (100)	$[M-H]^-$	517 (100)	$[M+H]^+$	Dicaffeoylquinic acid
		353 (12.2)	$[M-H-\text{caff}]^-$			
		191 (7)	$[\text{quinic acid}-H]^-$			
		179 (1)	$[\text{caffeic acid}-H]^-$			
F	12.62	515 (100)	$[M-H]^-$	517 (100)	$[M+H]^+$	Dicaffeoylquinic acid
		353 (18.5)	$[M-H-\text{caff}]^-$			
		191(9)	$[\text{quinic acid}-H]^-$			
		179 (13.5)	$[\text{caffeic acid}-H]^-$			
G	12.81	433 (100)	$[M-H]^-$	435 (100)	$[M+H]^+$	Naringenin-glucoside
		271 (25)	$[M-H-\text{Glu}]^-$	273 (22)	$[M+H-\text{Glu}]^+$	
H	13.52	433 (100)	$[M-H]^-$	435 (100)	$[M+H]^+$	Naringenin-glucoside
		271 (31)	$[M-H-\text{Glu}]^-$	273 (18)	$[M+H-\text{Glu}]^+$	
I	14.06	463 (100)	$[M-H]^-$	465 (100)	$[M+H]^+$	Quercetin-3-*O*-glucoside
		301 (25)	$[M-H-\text{Glu}]^-$	303 (15)	$[M+H-\text{Glu}]^+$	
J	16.76	447 (100)	$[M-H]^-$	449 (100)	$[M+H]^+$	Kaempferol-3-*O*-glucoside
		285 (17)	$[M-H-\text{Glu}]^-$	287 (14)	$[M+H-\text{Glu}]^+$	
K	17.9	431 (100)	$[M-H]^-$	433 (100)	$[M+H]^+$	Apigenin-glucoside
		269 (38)	$[M-H-\text{Glu}]^-$	271 (33)	$[M+H-\text{Glu}]^+$	
L	18.44	431 (100)	$[M-H]^-$	433 (100)	$[M+H]^+$	Apigenin-glucoside
		269 (24)	$[M-H-\text{Glu}]^-$	271 (35)	$[M+H-\text{Glu}]^+$	
M	22.46	433 (100)	$[M-H]^-$	435 (100)	$[M+H]^+$	Tetrahydroxycalchone-2′-*O*-glucoside
		271 (15)	$[M-H-\text{Glu}]^-$	273 (21)	$[M+H-\text{Glu}]^+$	

[a] Glu = Glucosyl residue (162 u); Caff = caffeoyl residue (162 u).

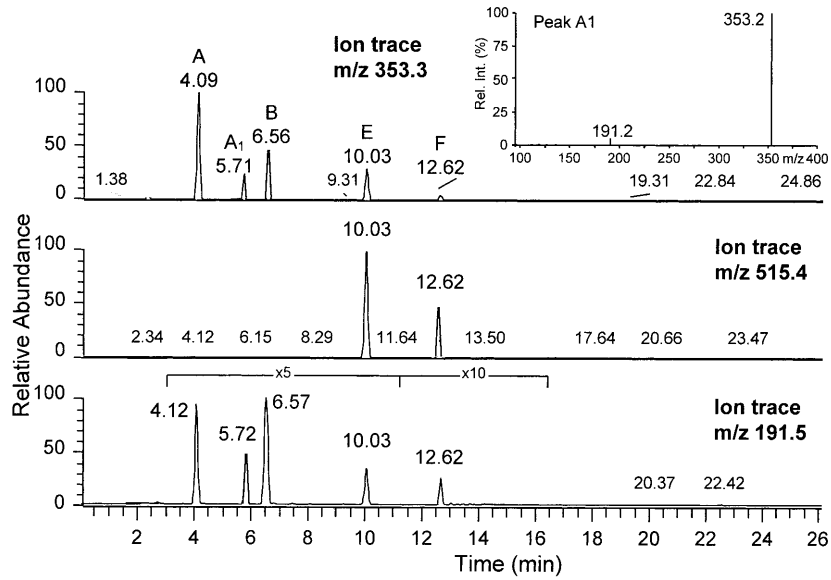

Fig. 3. Reconstituted ion chromatograms traces of $[M-H]^-$ ions and of the daughter ion m/z 191 relative to caffeoyl conjugates in *Helichrysum stoechas* extract. Insert: negative-ion APCI mass spectrum of peak A1.

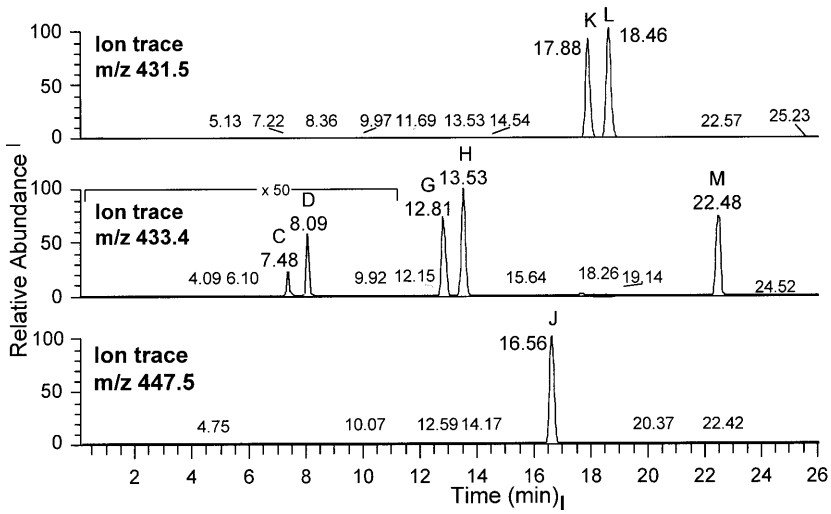

Fig. 4. Reconstituted ion chromatograms traces of $[M-H]^-$ ions of the main peaks in *Helichrysum stoechas* extract.

0.3% (w/w). For HPLC determination of kaempferol-3-*O*-glucoside, the following regression line (peak areas vs concentrations; range 0.5–50 μg ml^{-1}) was obtained: $Y = 28190(\pm 388)X + 14690 \pm 12.130$ ($r^2 = 0.9996$; S.E. of estimate $S_{y,x} = 11490$). The intraday repeatability RSDs ($n = 9$) were 2.21, 1.72 and 1.15% for 5, 25 and 50 μg ml^{-1} respectively. The reproducibility RDSs (3 days, $n = 9$) at the same concentrations were 2.48, 1.93 and 1.55%. For accuracy, recoveries ranged from 97 to 104% and they did not statistically differ from 100%.

In the analyzed *Helichrysum* extracts ($n = 5$) the kaempferol-3-*O*-glucoside concentrations were 20.49 ± 0.69 μg ml^{-1}, corresponding to a 4.098 ± 0.137% (w/w) total content.

3.3. Antioxidant activity

The chain-breaking antioxidant activity of the *H. stoechas* extract was first determined in phosphatidylcholine liposomes, by monitoring the formation of conjugated hydroperoxides during the propagatory phase of the peroxidation process driven by peroxyl radicals. The extract markedly and dose-dependently inhibited the increase in conjugated dienes (6 h after induction), by 17.1% at 1 μg/ml, 54.2% at 5 μg/ml, 76.4% at 10 μg/ml and almost complete inhibition was observed at 20 μg/ml ($IC_{50} = 5.4$ μg/ml). In addition (Fig. 6a), in the samples protected with the extract, the plateau of conjugated dienes was reached later than the control ($t = 6$ h), with a lag time of 8 h at 5 μg/ml ($t = 14$ h) and of 24 h ($t = 30$ h) at 10 μg/ml; at the highest concentration conjugated dienes were far below the plateau even after 30 h.

The antioxidant activity was confirmed in rat erythrocytes (RBC) exposed to cumene hydroperoxide, by measuring the erythrocyte membrane resistance to free radical-induced hemolysis. In control RBC (Fig. 6b), hemolysis started after 60 min incubation and plateaued between 120 and 150 min (71.2 ± 4.8% and 79.2 ± 5.2% hemolysis). The extract from *H. stoechas* dose-dependently delays the onset of the CuOOH-induced hemolysis; at 120 min hemolysis was inhibited by 23.3 ± 1.8% already at 5 μg/ml, by 45.4 ± 3.2% at 10 μg/ml and by 78.2 ± 3.6% at 20 μg/ml ($IC_{50} = 12.1$ μg/ml). The reference compound Trolox at the same concentrations (data not shown) was slightly more effective than the aqueous extract, with an IC_{50} value (120 min) of 5.4 μg/ml.

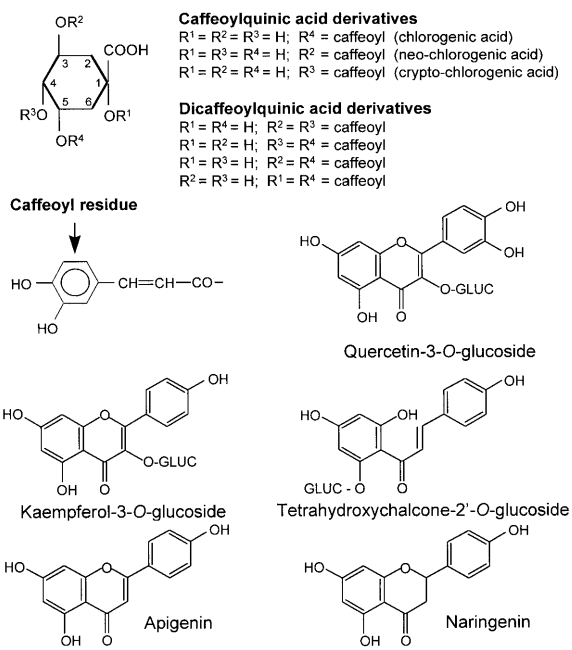

Fig. 5. Polyphenol components of *Helichrysum stoechas*.

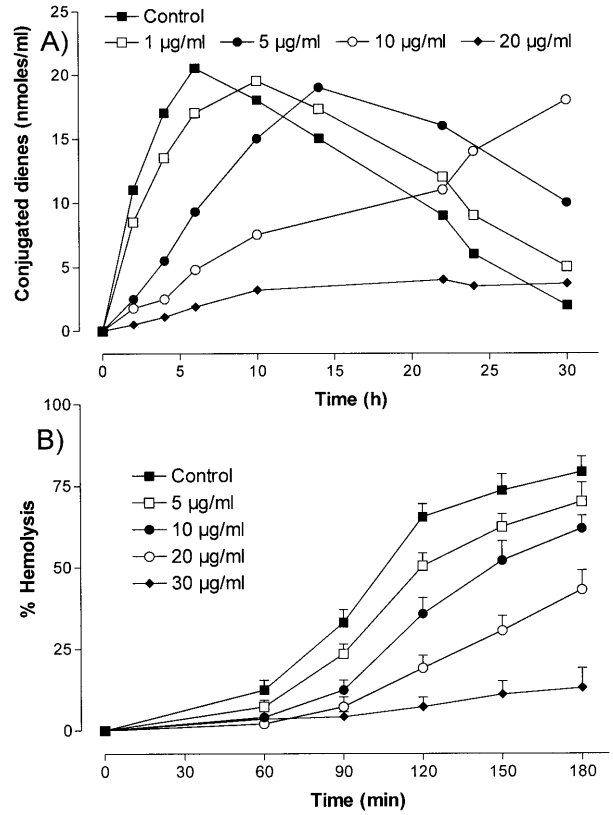

Fig. 6. Antioxidant activity of *Helichrysum stoechas* extract. (a) Chain-breaking antioxidant activity: the extract was incorporated into 2 ml phosphatidylcholine liposomes after ultrasound-induced peroxidation and conjugated dienes spectrophotometrically determined at 233 nm at different incubation times. (b) Protective effect on CuOOH-induced hemolysis. Rat erythrocytes (RBC), suspended in saline to obtain a 1% suspension, were incubated for 180 min in the absence and in presence of increasing concentrations of the extract and the time-course of hemolysis determined turbidimetrically at 710 nm. Results are the mean ± S.D. of 5 independent determinations.

4. Conclusions

The results of this study indicate that the polar fraction isolated from the flowering tops of *Helichrysum stoechas*, a species widely diffused in the Mediterranean area, but until now never investigated for their antioxidant constituents, displays radical scavenging properties, with a potency comparable to that of Trolox, the water-soluble analogue of vitamin E. The biological relevance of these findings is strengthened by the fact that these results have been obtained with a standardized extract, which contains approximately 50% polyphenols and 4% of kaempferol-3-*O*-glucoside, the more prominent component of the extract which is known to be a potent antioxidant.

The coupling of HPLC-UV-DAD with APCI-MS allowed an almost complete on-line identification of the main polyphenol components of the polar fraction of the drug, among which flavonoids (discrimination for flavonoid glycosides between positional isomers is currently under investigation) and caffeoyl conjugates, these last detected in other *H.* species, but never identified in *H. stoechas*, a fact that deserves adequate focus from a chemotaxonomic point of view.

For what concerns the LC conditions, various combination of organic solvents (CH_3OH, CH_3CN) and aqueous modifiers were tested, but the final mobile phase composition gave best results in terms of simultaneous detection of different classes of structurally unrelated polyphenols. In contrast to what recently observed by Pérez-Magariño [14] working with low molecular mass phenols (phenolic acids) and a series of standard flavan-3-ols, APCI source in both positive and negative ion mode supplies high sensitivity for molecular ions of glycosyl derivatives and sufficient fragmentation to obtain information on the aglycon moiety. API-ES (electrospray) ionisation, preliminarily tested in this study, gives predominantly molecular ions: in this case, as recently demonstrated [15] for the identification of flavonol-glycosides and catechin derivatives, an MS/MS approach (in-source collisionally-induced generation of specific daughter ions) is necessary to obtain structural information comparable to those obtained with APCI alone.

Finally, the combination of preliminary testing in vitro for the radical scavenging activity with the analytical screening by LC-MS (supported by UV-DAD detection) represents a valid and rapid approach in the search of new bioactive polyphenols (and of plants containing them) to be used, in the form of standardized fractions, as protective nutrients in food. Diets enriched in glycosyl-flavonoids may be of great benefit to human health contributing to increase the endogenous antioxidant defences, since as recently demonstrated, these compounds circulate unmodified in plasma after oral intake [16,17] and decrease the susceptibility of plasma lipids to peroxidation [18].

Acknowledgements

Financial support from MURST (Cofinanziamento Programma Nazionale 98) is gratefully acknowledged.

References

[1] B. Frei, Natural Antioxidants in Human Health and Disease, Academic Press, San Diego, CA, 1994.
[2] R. Maffei Facino, M. Carini, C. Cipriani, M. Mariani, Acta Ther. 14 (1988) 323–345.
[3] R. Maffei Facino, M. Carini, L. Franzoi, O. Pirola, E. Bosisio, Pharmacol. Res. 22 (1990) 709–721.
[4] P. Pietta, P. Mauri, C. Gardana, R. Maffei Facino, M. Carini, J. Chromatogr. 537 (1991) 449–452.
[5] S.I. El-Dahmy, Zagazig J. Pharm. Sci. 2 (1992) 73–80 C.A. 1994, 120: 265829e.
[6] A.H. Meriçli, K. Ergezen, B. Çubukçu, Fitoterapia 63 (1992) 465.
[7] M.L. Price, L.G. Butler, J. Agric. Food Chem. 25 (1977) 1268–1273.
[8] R. Maffei Facino, M. Carini, G. Aldini, L. De Angelis, Rapid Commun. Mass Spectrom. 11 (1997) 1303–1308.
[9] R. Maffei Facino, M. Carini, G. Aldini, M.T. Calloni, Drugs Exptl. Clin. Res. XXIII (1997) 157–165.
[10] M. Carini, R. Maffei Facino, G. Aldini, M.T. Calloni, L. Colombo, Rapid Commun. Mass Spectrom. 12 (1998) 1813–1819.
[11] K.S. Cholponbaev, V.E. Ruchkin, N.A. Tyukavkina, G.C. Zapesochnaya, V.A. Kurkin, E.S. Matyev, Zdravookhr Kirg. 2 (1990) 29–30 C.A. 113: 129374f — 1990.

[12] R. Gunasegaran, A. Ubeda, M.J. Alcaraz, R. Jayaprakasam, A.G. Nair, Pharmazie 48 (1993) 230–231.
[13] B. Çubukçu, B. Damadyan, Fitoterapia LVII (1986) 124–127.
[14] S. Pérez-Magariño, I. Revilla, M.L. Gonzáles-SanJosé, S. Beltrán, J. Chromatogr. A 847 (1999) 75–81.
[15] E. Gariboldi, D. Mascetti, G. Galli, P. Caballion, E. Bosisio, Pharmac. Res. 15 (1998) 936–943.
[16] P.C.H. Hollman, J.H.M. de Vries, S.D. van Leewen, M.J.B. Mengelers, M.B. Katan, Am. J. Clin. Nutr. 62 (1995) 1276–1282.
[17] G. Paganga, C.A. Rice-Evans, FEBS Lett. 401 (1997) 78–82.
[18] J.W. Lampe, Am. J. Clin. Nutr. 70 (1999) 475S–490S.