Characterization of *Matricaria recutita* L. Flower Extracts by HPLC-MS and HPLC-DAD Analysis

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Key Words

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Summary

Matricaria recutita L. is a spontaneous herbaceous perennial plant and its drug is largely used, as an infusion, for its anti-inflammatory properties, especially for respiratory and gastroenteric tracts. Granular soluble extracts of this drug are also used in children's diets.

The purpose of this work was to investigate the polyphenolic content of different parts of chamomile flowers. Methanolic extracts were compared and directly analysed by HPLC-DAD and HPLC-MS. The comparison between UV-Vis and MS spectra, carried out in positive and negative ionisation mode, allows identification of all the main polyphenolic compounds present in different parts of the flowers.

The findings reported herein both confirm the presence of several flavonoids, described previously, and evidence large amounts of caffeic and ferulic acid derivatives. No other evidence of the presence of these compounds in chamomile flowers has been previously reported.

Quantitative comparison of the flavonoid and phenolic acid derivatives present in receptacles, ligulate, tubular and total flowers was also performed.

Introduction

Matricaria recutita L. or *Chamomilla recutita* L. (Rauschert) is a plant which is widespread in various countries in the world particularly in Eastern Europe, Egypt and Argentina. The drug, composed of fresh and dried flowers, is used as an infusion against several minor diseases such as flogistic processes of mucoses, especially for respiratory and gastroenteric tracts [1, 3].

The spasmolytic activity of chamomile flowers is due both to α-bisabolol and flavonoid glycosides where the apigenin glucosides represent the main components[2].

The consumption of chamomile drug infusion and teas is widely spread in Italy and in other European countries. Granular extracts of this drug, alone or mixed with other herbs, are often present in some baby foods.

So far the main components described as present in chamomile flowers belong only to the classes of volatile derivatives and flavonoid compounds.

Kund and Isaac [4] reported a list of 18 flavonoids extracted from the chamomile plant and successively Exner and co-workers [7] described the presence of an other six highly methylated aglycones, mainly present in lypophilic extracts. Five of these compounds are derivatives of 6-methoxy-quercetin (quercetagetin) and the last is a kaempherol derivative, eupalitin. These authors also described the presence in trace amounts of other free aglycones.

The purpose of this work was to investigate the polyphenolic content of different parts of dried chamomile flowers that constitute the drug. Methanolic extracts were compared and directly analysed by HPLC-DAD and HPLC-MS. The findings reported herein both confirm the presence of several flavonoids described previously, and evidence of large amounts of cinnamic acid derivatives. No other evidence of these compounds in chamomile flowers has been previously reported.

Experimental

Materials

The pure flavonoid compounds (8, 9, 13, 15, and 22) were from Extrasynthese (Sa., Lyon, Nord-Genay, France).

Caffeoylquinic esters (compounds 1, 2, 3, 12) were kindly supplied by Professor Corrado Trogolo, Università la Sapienza , Roma.

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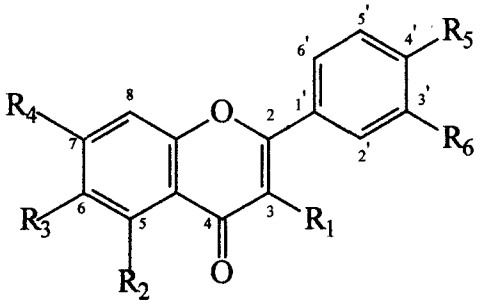


Table I. List of phenolic compounds previously identified in *Chamomilla recutita* L. head flowers. Chemical reference structure and corresponding molecular weights also reported.
glu = Glucose, rut = Rutinose, apio = Apiose, gal = Galactose

Substance	R_1	R_2	R_3	R_4	R_5	R_6	MW	Source ref.
Apigenin	H	OH	H	OH	OH	H	270	[4, 9, 10]
Apigenin-7-O-β-glucoside	H	OH	H	O-glu	OH	H	432	[4, 10]
Apigenin-7-O-apiosyl-glucoside	H	OH	H	O-glu-apio	OH	H	564	[4]
Apigenin-7-O-glucosyl-6"-acetate	H	OH	H	O-glu-6"ac	OH	H	474	[5, 6, 9]
Apigenin-7-O-glucosyl-2"-acetate	H	OH	H	O-glu-2"ac	OH	H	474	[5, 6]
Apigenin-7-O-glucosyl-2",3"-diacetate	H	OH	H	O-glu-2",3"-diac	OH	H	516	[8, 11]
Apigenin-7-O-glucosyl-3",4"-diacetate	H	OH	H	O-glu-3",4"-diac	OH	H	516	[8, 11]
Luteolin	H	OH	H	OH	OH	OH	286	[4, 10]
Luteolin-7-O-β-glucoside	H	OH	H	Oglu	OH	OH	448	[4]
Luteolin-4'-O-β-glucoside	H	OH	H	OH	Oglu	OH	448	[4]
Luteolin-7-O-β-rutinoside	H	OH	H	O-rut	OH	OH	594	[4]
6-Hydroxy-luteolin-7-glucoside	H	OH	OH	O-glu	OH	OH	464	[4]
Quercetin	OH	OH	H	OH	OH	OH	302	[4]
Quercetin-7-O-β-glucoside	OH	OH	H	O-glu	OH	OH	464	[4]
Quercetin-3-O-β-rutinoside	O-rut	OH	H	OH	OH	OH	610	[4]
Quercetin-3-O-β-galactoside	O-gal	OH	H	OH	OH	OH	464	[4]
Patuletin	OH	OH	OCH_3	OH	OH	OH	332	[4]
Patuletin-7-O-β-glucoside	OH	OH	OCH_3	O-glu	OH	OH	494	[4]
Isorhamnetin	OH	OH	H	OH	OH	OCH_3	316	[4]
Isorhamnetin -7-O-β-glucoside	OH	OH	H	O-glu	OH	OCH_3	478	[4]
Chrysoeriol-7-O-β-glucoside	H	OH	H	O-glu	OH	OCH_3	462	[4]
Jaceidin	OCH_3	OH	OCH_3	OH	OH	OCH_3	360	[7]
Chrysosplenol	OCH_3	OH	OCH_3	OCH_3	OH	OH	360	[7]
Eupatoletin	OH	OH	OCH_3	OCH_3	OH	OH	360	[7]
Spinacetin	OH	OH	OCH_3	OH	OH	OCH_3	346	[7]
Axillarin	OCH_3	OH	OCH_3	OH	OH	OH	346	[7]
Eupalitin	OH	OH	OCH_3	OCH_3	OH	H	330	[7]
Chrysosplenetin	OCH_3	OH	OCH_3	OCH_3	OH	OCH_3	374	[7,10]
Astragalin	O-glu	OH	H	OH	OH	OH	286	[10]

All solvents used were HPLC grade; CH_3CN and MeOH for HPLC were from E. Merck, (Darmstadt, Germany) while water was from Baker (J.T.Baker, Italy).

The trifluoroacetic acid was from Fluka (Fluka, Sigma Aldrich Company). The dried head flowers of *Chamomilla recutita* L. (Rhauschert) or *Matricaria recutita* L. were purchased on the Italian market using the same commercial batch.

Sample Preparation

About 100 mg dried head flowers of *Matricaria recutita* L. were used to prepare each of the following samples: total flowers (A), ligulate flowers (B), tubular flowers (C) and receptacles (D). These samples were extracted by refluxing with MeOH (80 mL) for 1 h, were then filtered and the volumes adjusted to obtain a final concentration of 1g dried material 100 mL^{-1}. The methanolic solutions were filtered (0.45μm) and directly analysed by HPLC-DAD and or HPLC-MS.

HPLC-DAD

Apparatus

HPLC-DAD analysis was performed on a Solvent Delivery System P4000 equipped with a UV6000LP DAD detector and autosampler AS3000, managed by a ChromQuest Chromatography data system (all from ThermoQuest, San Jose, CA, USA).

Chromatographic Procedure

The column was a 3.0 × 150 mm (3 μm) Inertsil ODS-3 (Chrompack, Netherland) equipped with a pre-column (2.0 × 10 mm, 5 μm) of the same phase; the oven temperature was 30 °C. The eluents were A: H_2O adjusted to pH 2.5 by CF_3COOH and B: CH_3CN. The following concave (type 2) solvent gradient was applied: from 92 % A and 8 % B to 30 % A and 70 % B within 50 min. Flow elution was 0.4 mL min^{-1} and 2 μL were injected for all samples. Chromatograms were acquired at 335 nm and UV-Vis spectra were recorded in the range 200–450 nm.

HPLC-DAD quantitative analyses were expressed in area percent at 335 nm for both caffeoylquinic acids and flavonoids derivatives.

HPLC-MS

Spectra were registered in negative and positive ion mode, using two different instruments. The positive MS spectra were performed on an LCQ electrospray (ThermoQuest, San Jose, CA, USA) directly coupled to the HPLC-DAD (Hewlett & Packard, Palo Alto, CA, USA) applying the same chromatographic conditions previously described but with a linear gradient. Capillary temperature was 220 °C, capillary voltage 3.0 V, source voltage 4.2 kV, tube lens voltage 30 V and collision energy 35 %.

The negative MS spectra were performed on an API-Electrospray (Hewlett & Packard, Palo Alto, CA, USA) with capillary temperature 350 °C and capillary voltage 3500 V; fragmentors applied were in the range 80–180 V.

Result and Discussion

The aim of this work was to optimise the HPLC analytical conditions and to carry out a quali-quantitative analysis in order to evaluate the polyphenolic content in different parts of the chamomile flowers.

The choice of an Inertsil ODS-3 column, with particle size 3 μm made it possible both to carry out the best separation and to detect compounds present in traces.

Several flavonoid derivatives have been previously found in this drug, many glycosides (4, 7, 9, 10) and some free aglycones (4, 5, 6, 8, 9, 10, and 11). Apigenin derivatives have been described as the main components of the flowers and luteolin glycosides have been found in appreciable amounts. By extraction with lipophylic solvents such as petrol ether and $CHCl_3$, some minor components such chrysosplenetin, astragalin and eupalitin have also been detected [7, 10].

All the flavonoids identified in the chamomile flowers to date are listed in Table I and their chemical reference structure is also reported.

Table II. Polyphenolic compounds identified in methanolic extract of *Matricaria recutita* L. head flowers.

N°	Component	t_R
1	5-Caffeoylquinic acid	8.64
2	3-Caffeoylquinic acid	15.71
3	4-Caffeoylquinic acid	16.38
4	Quinic acid	18.4
5	Ferulic acid-1-O-glycoside	18.63
6	Caffeoylquinic acid derivative	24.59
7	Ferulic acid -7-O-glycoside	25.50
8	Quercetin-3-O-glalactoside	27.73
9	Quercetin-7-O-glucoside	28.39
10	Patuletin-7-O-glucoside	28.74
11	Dicaffeoylquinic acid derivative	29.73
12	1,3-Dicaffeoylquinic acid	30.77
13	Apigenin-7-O-glucoside	30.89
14	Quercetin derivative	31.28
15	Luteolin-4'-O-glucoside	31.40
16	Dicaffeoylquinic acid derivative	31.79
17	Quinic acid derivative	32.86
18	Apigenin-7-O-glucosyl-2"-acetate	33.84
19	Apigenin-7-O-glucosyl-6"-acetate	36.31
20	Caffeoylquinic acid derivative	36.80
21	Apigenin-7-O-glucosyl-diacetate	37.51
22	Apigenin	40.25
23	Luteolin-7-O-rutinose	46.00
24	Ferulic acid derivative	48.5

Extraction with refluxing methanol was applied because previous tests performed with cold methanol or ethanol evidenced the same qualitative profiles but lower amounts of the polyphenolic substances.

HPLC-DAD and MS analysis were applied to identify and quantify all the polyphenolic compounds present in the methanolic extracts of total flowers (A), ligulate flowers (B), tubular flowers (C) and receptacles (D). The analyses were directly performed on the total extracts without any manipulation of the sample and the resulted chromatographic profiles at 335 nm are shown and compared in Figure 1. Our findings confirmed the presence of the main flavonoid compounds quoted in the literature and highlighted the co-presence of several cinnamic acid derivatives which had not been previously cited.

The identification of the acid derivatives 1, 2, 3, 12, and of flavonoid compounds, 12, 13, 15 and 22 was performed by comparing their t_r values, UV-Vis and MS spectra with those obtained with the corresponding pure standards.

To collect useful information on the chemical structure of the other compounds listed in Table II, HPLC-MS investigations were performed. Both different fragmentor values and positive and/or negative ionization were applied to modulate the fragmentation pattern.

The positive MS spectra of the two intense peaks at t_r 18.63 (5) and t_r 25.5 (7) are reported respectively in Figures 2A and 2B, where the main species are at *m/z* 735, 713, 195 and 177. These results suggest, both for compound 5 and 7, the structure of two ferulic acid

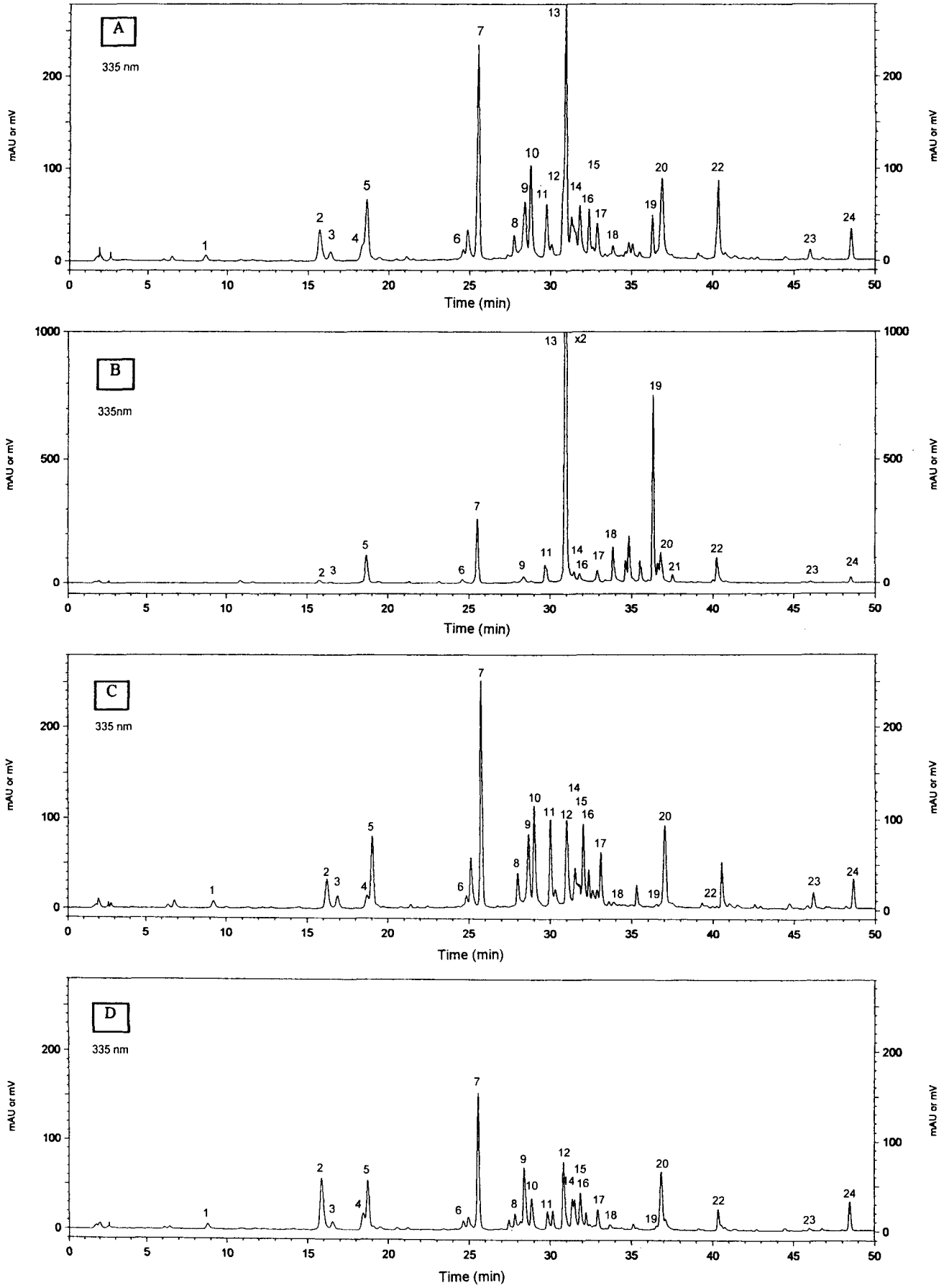
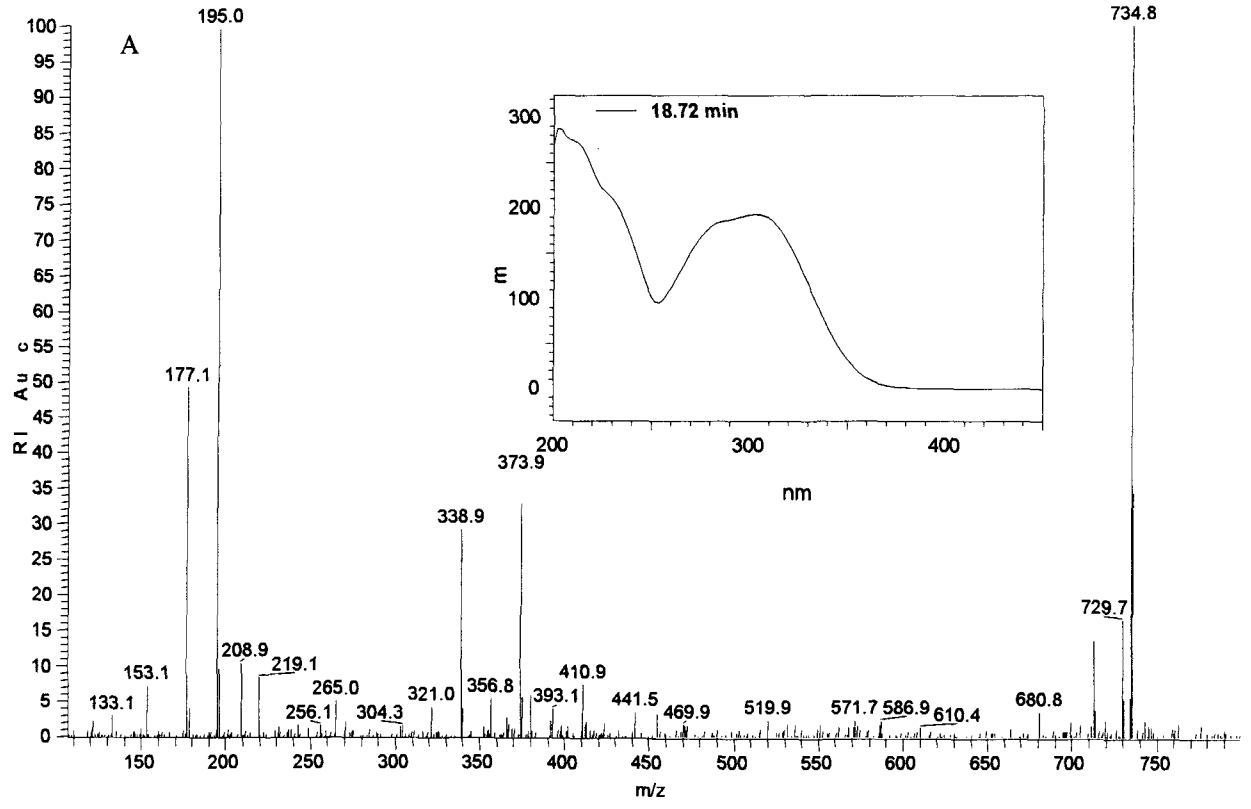


Figure 1

HPLC-DAD profiles of sample A (total flowers), B (ligulate flowers), C (tubular flowers), D (receptacles) all at 335 nm. List of identified compounds, with corresponding retention times, in Table II.



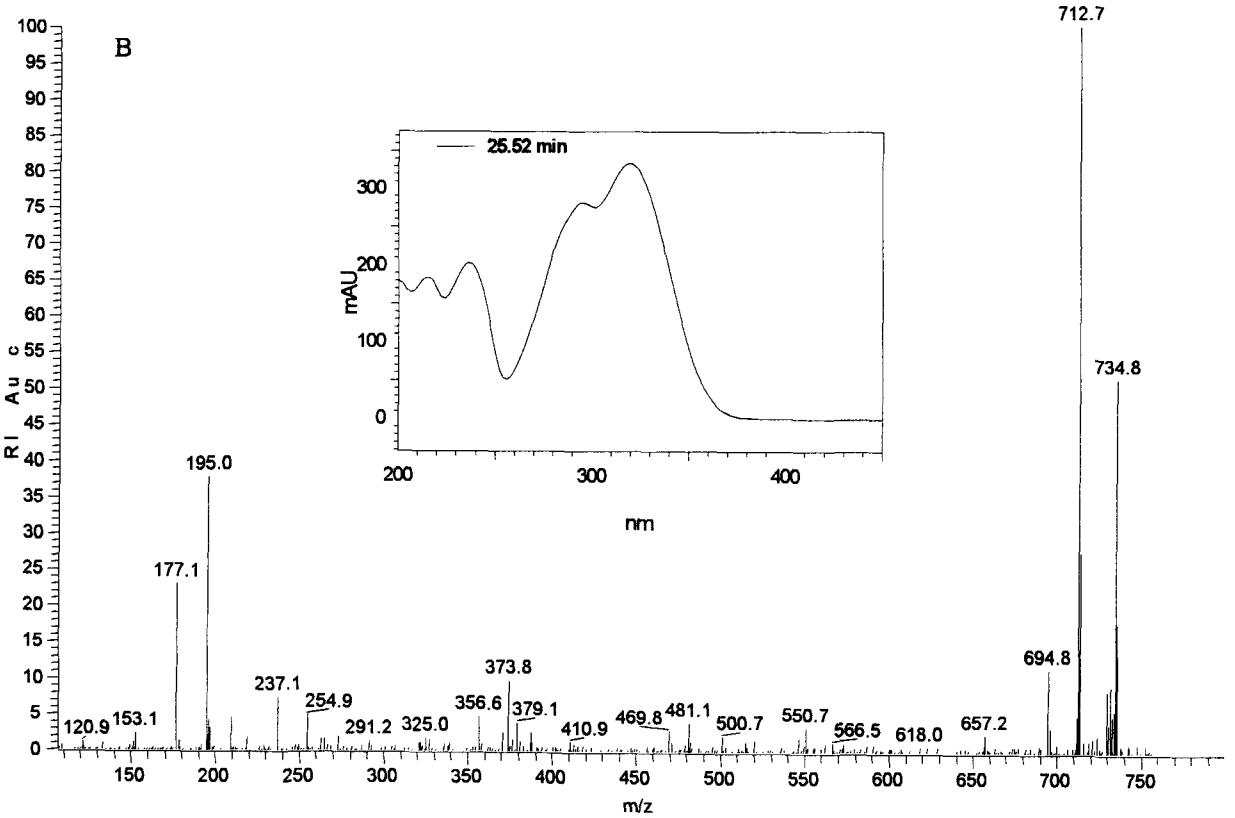


Figure 2
UV-Vis and positive MS spectra of compounds **5** (2A) and **7** (2B).

Table III. Percent area at 335 nm for methanolic extracts (1 % *w/v*) from total, ligulate, tubular flowers and receptacles.
API = flavonoids containing apigenin, QUE = flavonoids containing quercetin, LUT = flavonoids containing luteolin, PAT = flavonoids containing patuletin, AC = caffeic and ferulic acid derivatives, OpuD = other phenolic unidentified derivatives

	API	QUE	LUT	PAT	AC	OpuD
ligulate flower	68.6	2.1	0.1	0.2	17.7	11.3
tubular	0.9	12.4	2.1	7.6	61.4	15.6
receptacle	0.8	10.0	3.0	4.0	64.8	17.4
total flower	16.8	9.9	1.9	6.5	39.1	25.8

glycosides. In fact, the signals at *m/z* 195 together with the ion *m/z* 177 can be related to the molecular ion of ferulic acid and to the corresponding $[M\text{-}H_2O]^+$ fragment, while the signal at *m/z* 713 represents the free dimer form $[2M+H]^+$. In addition ions at *m/z* 735 correspond to the adduct with sodium $[2M+Na]^+$, the molecular ions $[M+H]^+$, at *m/z* 357, appear at very low intensity.

The fragmentation pattern obtained in negative ionisation mode, fragmentor 120 V, evidenced the presence of dimer forms at *m/z* 711, $[2M\text{-}H]^-$, both for 5 (18 %) and 7 (67 %) while the main ions are at *m/z* 355 (100 %) which corresponds to the $[M\text{-}H]^-$ moiety. In addition ions at *m/z* 193 (70–80 %) related to the ferulic acid molecular ion $[M\text{-}162]^-$, and at *m/z* 149 (35–55 %), which evidences loss of CO_2 from the acidic moiety, are also present.

Observing the findings obtained by negative and positive MS spectra it was possible to hypothesize compounds 5 and 7 as the two monoglucosides of the ferulic acid with glucose linked at the carboxylic group or at the para-hydroxyl group. Compound 7 evidences a UV-Vis spectrum perfectly overlapped with that of free ferulic acid as happens when comparing UV-Vis spectra of chlorogenic and caffeic acid, confirming the presence of an ester linkage. In fact, the ester linkage does not modify the typical chromophore of these cinnamic acid derivatives. In the light of these findings, component 7 corresponds to the 4-hydroxy-3-methoxycinnamic acid glucosyl ester. On the other hand component 5 necessarily corresponds to the ferulic acid ether glucoside (4-hydroxyglucosyl-3-methoxycinnamic acid).

The structure of the caffeoylquinic esters, compounds 6, 11 and 16, were likewise attributed after comparison both of their positive and negative MS spectra, and their related UV-Vis spectra. In addition the behaviour of the available corresponding standards showing a similar structure (cynarine, clorogenic acid) was taken into account.

The UV-Vis spectra of compounds 18 and 19 suggested the presence of the apigenin moiety and their negative MS spectra (fragmentor 80 and 120 V) showed only an intense $[M\text{-}H]^-$ ion at *m/z* 473 without fragmentation attributed to the apigenin glucosyl monoacetates. Increasing the fragmentation energy (fragmentor 180 V) the ion at *m/z* 269, typical of the apigenin moiety, appeared at 40–50 % intensity. The data collected confirmed the structure of these derivatives, previously described [5, 6, 9], as apigenin glucosyl monoacetates. The apigenin monoglucoside di-acetate (21), present in trace amounts only in sample B, was determined in the same way. Applying the selected ion monitoring technique the signals at *m/z* 515 $[M\text{-}H]^-$ and 269 were extrapolated from the total ion current in order to obtain a selective MS profile able to detect also small amounts of this glycoside.

The MS spectra of patuletin 7-O-glucoside (10) was obtained from the HPLC-MS analysis of samples A and C in which this glucoside is present in higher concentration. The UV-Vis spectrum showed the typical shape of a very hydroxylated flavonol (λ_{max} 356 nm) and its positive MS spectrum showed both $[M+H]^+$ at *m/z* 495 (100 %) and at *m/z* 333 (25 %) corresponding to the patuletin moiety. The structure of the patuletin 7-O-glucoside previously identified by Kunde and Isaac [4] was also confirmed by applying different fragmentation energy. It is interesting to note that the positive mode showed good response for this compound in terms of ionisation as with the negative mode.

A quantitative comparison of samples A, B, C and D, expressed as area percent values at 335 nm, is reported in Table III. The amount of the flavonoid fraction is expressed in relation to the different aglycones and the caffeic and ferulic acid derivatives are summarised as acids. This wavelength was applied because the main polyphenolic compounds present in the chamomile flowers show a maximum absorption near 335 nm.

The ligulate flower extract contains the highest concentration of apigenin glucosides (13, 18, 19) and low percentages of caffeoyl esters (1, 2, 3, 4), quercetin (8, 9, 14), luteolin (15, 23), and patuletin (10) glycosides. On the other hand the sum of the two ferulic acid monoglucosides (5, 7) is about the same for ligulate (B) and tubular extract (C). As expected, the receptacle extract (sample D) shows the lowest content for both flavonoid and cinnamic acid derivatives.

The findings of our investigation confirmed the presence of the main flavonoid compounds previously de-

scribed, and evidenced at the same time a medium-high content, up to 64.8 %, of caffeic and ferulic acid derivatives. The presence of this latter group of compounds, which had not been reported before, certainly modulates the biological activity of the chamomile flower preparations. Recently some investigations have been carried out in order to elucidate the bioavailability of these phenolic acids in humans [13]. Infusions and teas of this drug are frequently consumed and certainly in a polar medium, such as hot water, the amount of these derivatives could be not negligible. In conclusion, it is interesting to underline that the weight of the tubular flowers ranges between 50 and 70 % of total flowers [12] represent a very rich source of caffeic and ferulic acid derivatives.

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