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Evaluation of the content and stability of the constituents of mother tinctures and tinctures: The case of *Crataegus oxyacantha* L. and *Hieracium pilosella* L.

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Abstract

As a part of our investigations on the content and stability of herbal drug preparations, we evaluated the content and stability of tinctures and mother tinctures of Hawthorn leaves and flowers and Hawkweed. Hawthorn preparations are mainly used by patients with cardiac diseases; Hawkweed is employed for the treatment of cellulitis and obesity due to its diuretic properties. Both tinctures (DER 1:5) and mother tinctures (DER 1:10) are herbal preparations reported in the European Pharmacopoeia. The first preparation is obtained using dried herbal drugs; the latter is a homoeopathic preparation obtained with fresh plant material, often used in substitution of tinctures. The aim of this work was to assess the qualitative and quantitative profile of the constituents of the investigated preparations and the chemical stability of their marker constituents from long-term testing using HPLC assays.

Characteristic constituents of Hawthorn leaves and flowers are flavonoids such as vitexin-2″-*O*-rhamnoside and hyperoside and oligomeric procyanidins. Charcteristic constituents of Hawkweed are caffeoyl-quinic acid derivatives, flavonoids and a coumarin:umbelliferone.

Our investigation showed that Hawthorn mother tincture had a higher concentration of procyanidins with respect to the tincture but the stability of these constituents were very low in both preparations. Total flavonoidic content was 3.33 mg/ml, about 1.5 times more than the content of mother tincture and the shelf-life t_{90} was about 7 months for both preparations.

For Hawkweed preparations a content of caffeoyl-quinic acid derivatives (ca. 4 mg/ml) was found, but their stability was good only in the tincture. The concentrations of flavonoids and umbelliferone were two times as much in the tincture with respect to the mother tincture. Stability of these two classes of constituents was good for both preparations over a 9-month period.

Keywords: Hawthorn leaves and flowers; Hawkweed; Tinctures and mother tinctures; Flavonoids and procyanidins; Caffeoyl-quinic acid derivatives; Flavonoids and umbelliferone; Qualitative and quantitative profile; Stability

1. Introduction

In continuing our investigations on tinctures of herbal drugs [1,2], we now report the analysis and stability of tinctures and mother tinctures of two widely used herbal drugs: Hawthorn leaves and flowers and Hawkweed. Hawthorn (*Crataegus monogina* Jacq. (Lindm.), Rosaceae) preparations are mainly employed for their positive effects in patients with cardiac diseases [3]. Characteristic constituents are flavonoids with a minimum content of 1.5% expressed as hyperoside and include vitexin-2″-*O*-rhamnoside, acetyl-vitexin-2″-*O*-rhamnoside and hyperoside [4]. Studies concerning the HPLC analysis of Hawthorn leaves are recently reported, but only concerning a few constituents (free flavones and flavonols) [5]. Hawkweed (*Hieracium pilosella* L., Asteraceae) is employed for the treatment of cellulitis and obesity for its strong diuretic properties [6]. Hawkweed is not reported in the European Pharmacopoeia but there is a monograph in the French Pharmacopoeia which states that it should contain at least the 2.5% of caffeoyl-quinic acid derivatives, expressed as chlorogenic acid [7]. Another characteristic substance of Hawkweed is umbelliferone, a coumarin [6]. Only a recent study is concerning the content of phenolic constituents of Hawkweed [8], including caffeic acid derivates and flavonoids (luteolin and glycosides; quercetin).

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In the present investigation, the same herbal drug material was used for the two preparations obtained according to the European Pharmacopoeia. Tinctures [9] were obtained by macerating the dried herbal drug in the hydroalcoholic solvent (1:5) and mother tinctures using fresh herbal material in the hydroalcoholic solvent in the portion of 1:10 (calculated on the dried weight of the herbal drug), according to the homoeopathic preparations [10]. Mother tinctures are often used instead of tinctures, even if to our knowledge there are no data concerning the equivalence of such preparations.

2. Experimental

2.1. Chemicals

Acetonitrile and methanol HPLC grade were provided by Sigma–Aldrich (Steinheim, Germany); 85% formic acid was provided by Carlo Erba (Milan, Italy). Water was purified by a Milli-Q_{plus} system from Millipore (Milford, MA, USA).

2.2. Standards

Chlorogenic acid, vitexin, hyperoside, vitexin-2″-*O*-rhamnoside, isoquercitrin, apigenin, luteolin, luteolin-7-*O*-glucoside and catechin were purchased from Extrasynthese (Genay, France).

Umbelliferone (purity 99%) was obtained from Sigma–Aldrich Chemie (Steinheim Germany). Quercetin was obtained from Fluka (Milano, Italy).

2.3. Herbal preparations

Mother tinctures were commercial samples (Hawthorn leaves and flowers mother tincture B035/05, Hawkweed mother tincture B033/05) kindly offered by "Principio attivo"soc.coop.a.r.l. (Carrara, Italy). Tinctures were prepared by one of the authors (F.E.) using the same vegetal material employed for the preparation of the mother tinctures. The alcoholic solutions used for Hawthorn preparations were 55% (v/v) and for Hawkweed preparations 60% (v/v).

Batches (about 200 ml) of each preparation were stored in brown glass in an oven at a temperature of 25 ± 2 °C. Analyses were conducted on samples on the day of preparation and after 3, 5, 7 and 9 months of storage. Triplicate HPLC determinations were performed on each sample, after dilution of 1 ml of the preparation in 4 ml of methanol and centrifugation.

2.4. Stability studies

Thermal stability testing was carried out at 25 ± 2 °C (60 ± 5% RH). The climatic chambers employed were previously reported [2].

2.5. HPLC apparatus

2.5.1. HPLC–DAD analysis instrumentation

The HPLC system consisted of a HP 1090L instrument with a Diode Array Detector and managed by a HP 9000 workstation (Hewlett and Packard, Palo Alto, CA, USA). The column was a Jupiter C_{18} (5 μm, 250 mm × 4.6 mm, 300 Å, Phenomenex Torrance, CA, USA) maintained at 26 °C. The mobile phases were linear solvent gradients CH_3CN/MeOH/H_2O with HCOOH (pH 3.2), with a flow rate of 1 ml/min for Hawthorn samples and 0.9 ml/min for Hawkweed samples. Timetables are reported in Tables 1 and 2. Injected volume of the sample was 10 μl solution. UV–vis spectra were recorded in the range 190–450 nm, and chromatograms were acquired at 225, 270, and 350 nm. Peaks were detected at 350 nm for flavonoids, umbelliferrone and caffeoyl-quinic acid derivates; and at 270 nm for procyanidins. Typical chromatograms of mother tinctures are reported in Figs. 1 and 3. Similar HPLC profiles have been obtained for tinctures. Structures of the characteristic constituents are reported in Figs. 2 and 4.

Table 1
Mobile-phase composition used for the HPLC–DAD and HPLC–MS analysis of Hawthorn

Min	H_2O (%)	MeOH (%)	CH_3CN (%)
0	100	0	0
10	85	0	15
30	70	10	20
40	10	15	75
55	10	10	80
60	100	0	0
70	100	0	0

2.5.2. HPLC–MS analysis instrumentation

The HPLC system described above was interfaced with a HP 1100 MSD API-electrospray (Hewlett and Packard, Palo Alto, CA, USA). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed use of analytical conditions similar to those used for HPLC–DAD analysis in order to achieve the maximum sensitivity of ESI values.

The same column, time period and flow rate were used during the HPLC–MS analyses. Since phosphoric acid was not suitable for HPLC–MS operations, separation was performed using aqueous formic acid (pH 3.2), without appreciable variations in the chromatographic profile.

Mass spectrometry operating conditions were: positive polarity, fragmentor 120, gas temperature 350 °C at a flow rate

Table 2
Mobile-phase composition used for the HPLC–DAD and HPLC–MS analysis of Hawkweed

Min	H_2O (%)	MeOH (%)	CH_3CN (%)
0	100	0	0
10	85	0	15
25	85	0	15
30	70	10	20
45	40	30	30
50	0	0	100
55	0	0	100
60	100	0	0
70	100	0	0

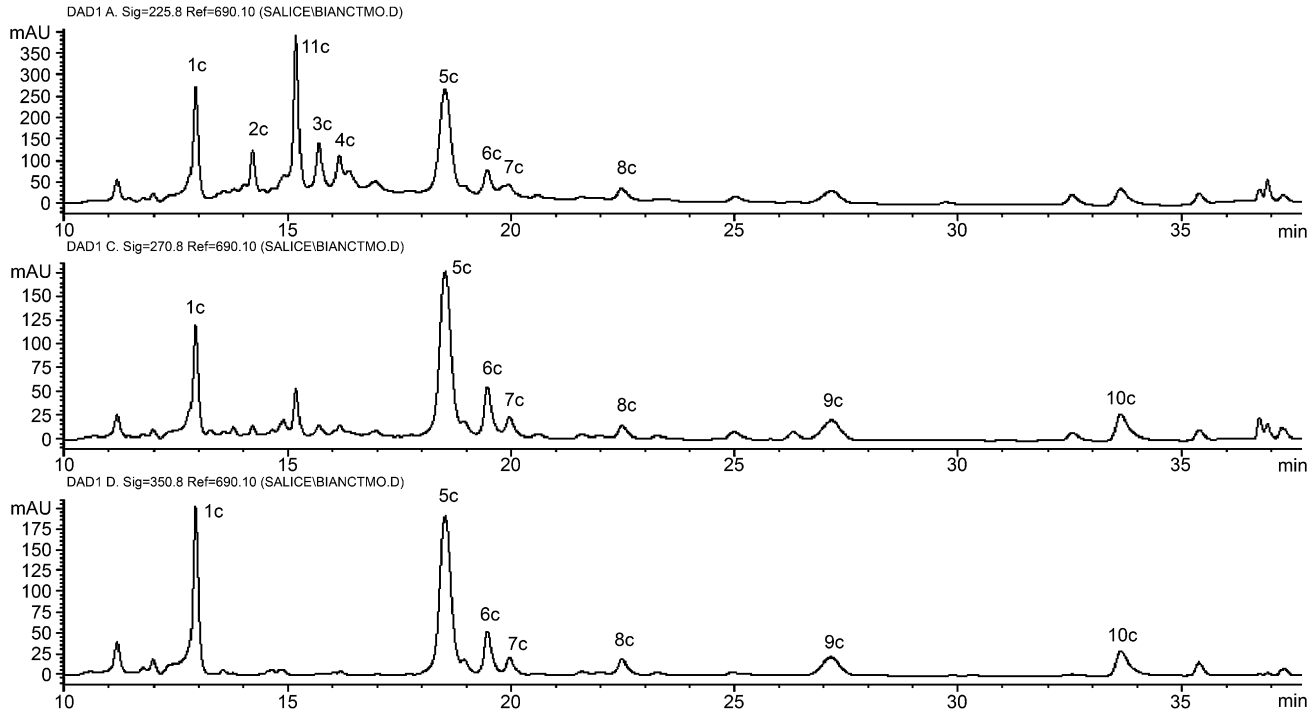

Fig. 1. Profiles at 225, 270 and 350 nm of the chromatogram of Hawthorn mother tincture with the HPLC–DAD–MS attributions of the components.

of 10 ml/min (Hawthorn samples) or 11.5 ml/min (Hawkweed samples), nebulizer pressure 30–60 psi, quadrupole temperature 35 °C and capillary voltage 3500 V. Full scan spectra from 100 to 1000 *m/z* were obtained. Injected volume was 10 μl.

2.6. Identification of peaks and peak purity

Identification of all constituents was performed by HPLC–DAD and MS analysis and/or by comparing the retention time and the UV spectrum of the peaks in the samples with those of authentic reference samples. The purity of peaks was checked by a Diode Array Detector coupled to the HPLC system, comparing the UV spectra of each peak with those of authentic references samples and/or by examination of the MS spectra.

2.7. Linearity, repeatability and reproducibility

The linearity range of responses was determined on five concentration levels with three injections for each level. Calibration graphs for HPLC were recorded with sample amounts ranging from 0.10 to 2.5 μg ($r > 0.99$).

To evaluate the repeatability, six samples of each tincture from the same batch were analyzed by HPLC. The contents of each constituent were evaluated to calculate the relative standard deviation.

The following data were obtained: chlorogenic acid 2.00%, hyperoside 2.40%, isoquercitrin 2.50%, vitexin 1.98%, quercetin 2.00%, apigenin 1.90%, luteolin 2.00%, luteolin 1.56%, luteolin-7-*O*-glucoside 2.80%, catechin 2.33% and umbelliferone 2.15%.

To evaluate the reproducibility of the injection integration, the standard solutions (2.5–10.0 μg/25 μl) and of each tincture sample were injected six times and the relative standard deviation values were calculated. The following data were obtained: chlorogenic acid 1.00%, hyperoside 1.60%, isoquercitrin 0.99%, vitexin 0.85%, quercetin 1.25%, apigenin 0.78%, luteolin 1.12%, luteolin 0.98%, luteolin-7-*O*-glucoside 1.75%, catechin 1.06% and umbelliferone 1.54%.

2.8. Quantitation of constituents

All the tinctures were analyzed in triplicate and quantitation of the constituents was obtained using calibration graphs with six data points. Chlorogenic acid was used to quantify caffeoyl quinc acid derivatives in both plant material preparations. Acetyl-vitexin-rhamnoside and vitexin-2″-*O*-rhamnoside content in Hawthorn were quantified as vitexin while proantocyanidins were expressed as catechin. All the other flavonoids in both Hawthorn and Hawkweed preparations were quantified as hyperoside.

3. Results and discussion

In continuing our studies on the constituents and stability of herbal drug preparations, the present work reports the qualitative and quantitative profiles of the constituents of tinctures and mother tinctures of Hawthorn leaves and flowers and Hawkweed and their chemical stability. All the constituents were evaluated because the active ones are not known and the biological effects of these preparations are generally considered to arise from the whole phytocomplex.

Two simple RP-HPLC methods that optimised the separation of all the classes of constituents were obtained for the preparations of Hawthorn and Hawkweed. The HPLC methods are

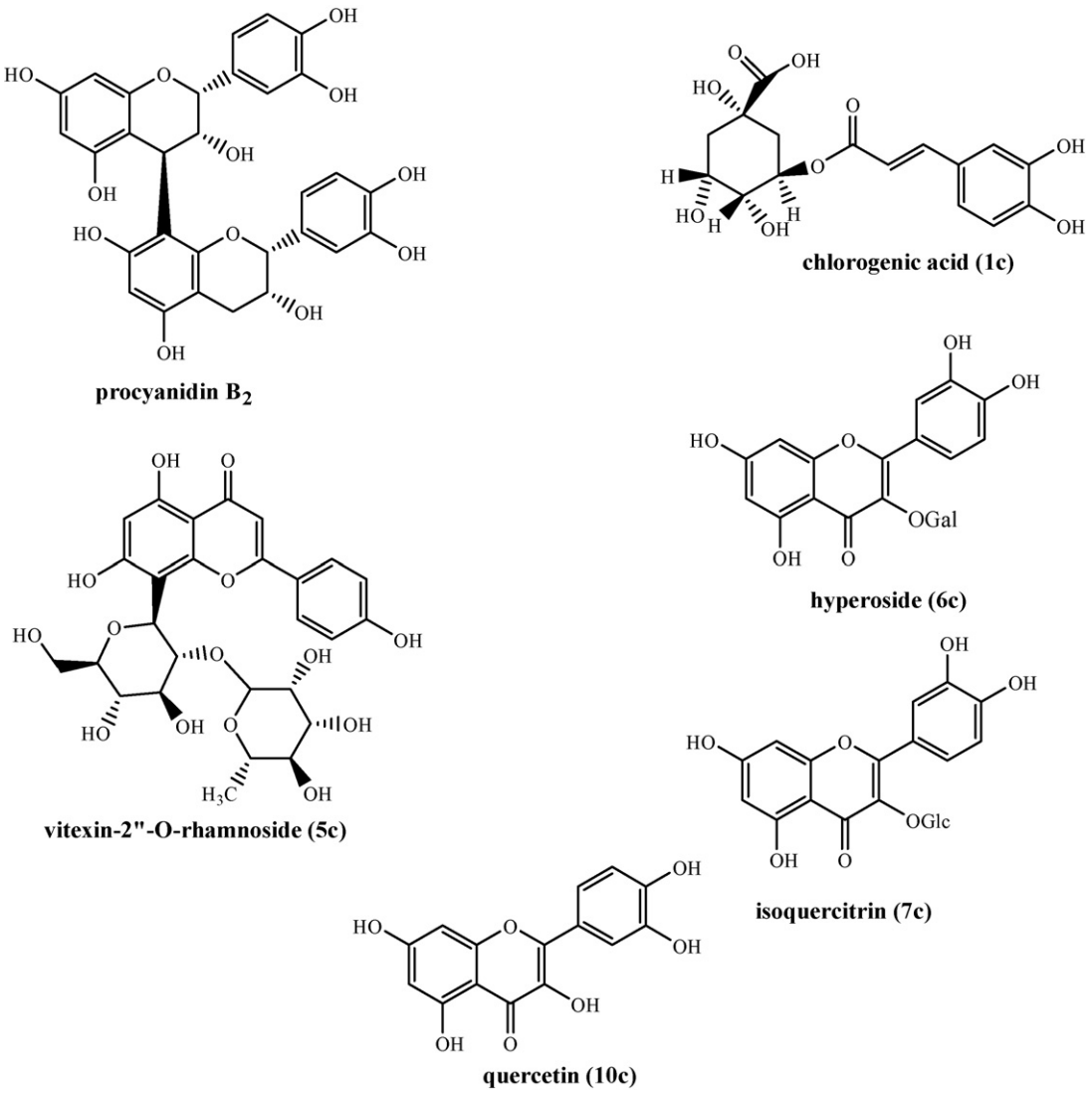

Fig. 2. Chemical structures of Hawthorn compounds: chlorogenic acid (1c), procyanidin B2, vitexin-2″-*O*-rhamnoside (5c), hyperoside (6c), isoquercitrin (7c) and quercetin (10c).

reported in Tables 1 and 2. Identification of constituents was obtained by the combination of DAD and MS data. The same HPLC methods were also used for the samples submitted to the thermal stability testings because no interferences with eventual degradation products were observed. All the analyses were performed in triplicate.

In the chromatogram of mother tincture from Hawthorn leaves and flowers (Fig. 1) 11 peaks were identified (1c–11c).

Peak 1c displayed the typical UV absorption of caffeic acid derivates with maxima at about 236 and 327 nm. Peak 1c was identified as chlorogenic acid by comparison of t_R, UV and mass spectrum with those of an authentic sample. Peak 1c showed $[M+Na]^+$ ion at *m/z* 377 and a characteristic fragment ion at *m/z* 163 due to the loss of quinic acid. These data were confirmed by comparing the t_R, UV and MS spectra with those of an authentic sample.

Peaks 2c, 3c and 4c displayed the typical UV absorption of procyanidins with maxima at about 270 nm. A detailed analysis of their mass spectra led to their identification. Peak 2c was tentatively identified as a dimeric procyanidin: it showed $[M+H]^+$ and $[M+Na]^+$ ions at *m/z* 579 and 601 and a characteristic fragment at *m/z* 291 due to the loss of an epicathechin moiety. Peaks 3c and 4c had identical mass spectra and were tentatively identified as trimeric procyanidins. They showed $[M+H]^+$ and $[M+Na]^+$ ions at *m/z* 867 and 890 and some characteristic fragments at *m/z* 577 and 291 due to the loss of one and two units, respectively, of epicatechin.

Peaks 5c and 9c displayed identical UV spectra with maxima at 271 and 334 nm, typical of isovitexin derivatives.

Peak 5c was identified as vitexin-2″-*O*-rhamnoside because it showed $[M+H]^+$ ion at *m/z* 579 and a fragment ion at *m/z* 433 due to the loss of a rhamnose unit. These data were confirmed by comparing the t_R, UV and MS spectra with those of an authentic sample. Peak 9c was tentatively identified as acetyl-vitexin-rhamnoside because it showed $[M+H]^+$ ion at *m/z* 621 and another characteristic ion at *m/z* 433 due to the loss of a rhamnose unit with an acetyl group.

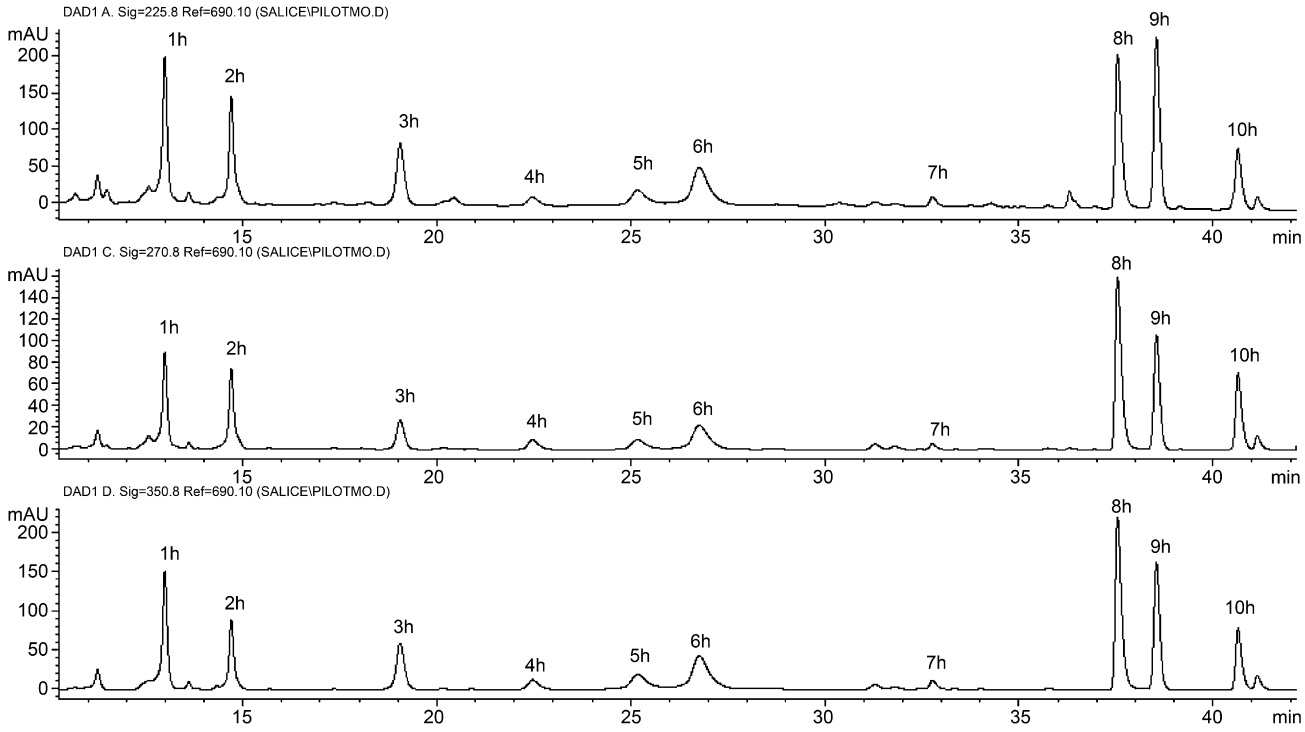

Fig. 3. Profiles at 225, 270 and 350 nm of the chromatogram of Hawkweed mother tincture with the HPLC–DAD–MS attributions of the components chromatogram.

Peaks 6c, 7c, 8c, and 10c displayed identical UV absorptions with maxima at about 256 and 355 nm, typical of flavonols.

Peak 6c was identified as hyperoside. The peak showed $[M+Na]^+$ ion at *m/z* 487 and a characteristic ion fragment at *m/z* 303 due to the loss of a hexose unit. These data were confirmed by comparing the t_R, UV and MS spectra with those of an authentic sample. Peak 7c was identified as isoquercitrin. It showed $[M+Na]^+$ ion at *m/z* 487 and a characteristic ion fragment at *m/z* 303 due to the loss of a hexose unit. These data were confirmed by comparing the t_R, UV and MS spectra with

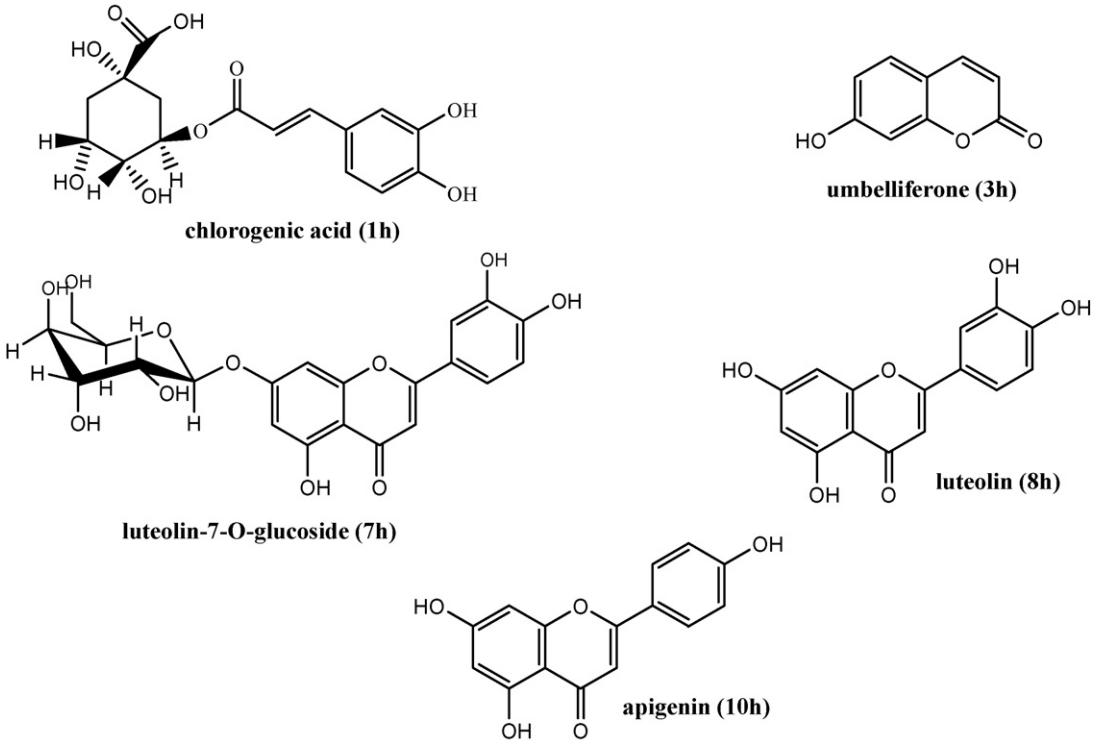

Fig. 4. Chemical structures of Hawkweed compounds: chlorogenic acid (1h), umbelliferone (3h), luteolin-7-*O*-glucoside (4h), luteolin (8h) and apigenin (10h).

Table 3
Peak identification and concentration of the constituents in Hawthorn preparations

	Retention time (min)	Constituent	Concentration in mother tincture DER 1:10 (mg/ml)	Concentration in tincture DER 1:5 (mg/ml)
1c	12.9	Chlorogenic acid	1.12	0.73
2c	14.2	Dimeric procyanidin	0.25	0.11
3c	15.7	Trimeric procyanidin	[a]	[a]
4c	16.2	Trimeric procyanidin	1.49[a]	0.81[a]
5c	18.5	Vitexina-2″-*O*-rhamnoside	1.38	0.94
6c	19.4	Hyperoside	0.20	0.36
7c	19.9	isoquercitrin	0.08	0.11
8c	22.5	Methoxy-quercetin-glycoside	0.07	0.06
9c	27.2	Acetyl-vitexin-2″-*O*-rhamnoside	0.19	1.84
10c	33.6	Quercetin	0.09	0.02
		Total flavonoids	2.02	3.33
		Total procyanidins	1.74	0.92

[a] Trimeric procyanidins are expressed as the sum of the two isomers.

those of an authentic sample. Peak 8c was tentatively identified as metoxy-quercetin-glycoside. It showed $[M+Na]^+$ ion at *m/z* 501, and two characteristic ions at *m/z* 317 and 163, the first due to the loss of a hexose while the second is a hexose. Peak 10c was identified as quercetin because of the same t_R, UV and MS spectra with those of an authentic sample of quercetin.

Peak 11c was not assigned based on UV and MS data, probably a terpenoidic derivative, due to the absence of chromophore moieties, typical of polyphenols.

Structures of Hawthorn constituents chlorogenic acid (1c), procyanidin B2, vitexin-2″-*O*-rhamnoside (5c), hyperoside (6c), isoquercitrin (7c) and quercetin (10c) are reported in Fig. 2.

The chromatogram obtained from Hawthorn tincture samples presented the same chromatographic profile.

In the chromatogram obtained from Hawkweed mother tincture (Fig. 3) 10 peaks (1h–10h) were identified.

Peaks 1h, 2h, 5h, 6h, 7h, and 9h displayed the typical UV absorption of caffeic acid derivates with maxima at about 236 and 327 nm. Peaks 4h, 8h and 10h displayed identical UV absorptions with maxima at about 256 and 348 nm, typical of flavones.

Peak 1h displayed the typical UV absorption of caffeic acid derivates with maxima at about 236 and 327 nm. Peak 1h was identified as chlorogenic acid by comparison of t_R, UV and mass spectrum with those of an authentic sample. Peak 1c showed $[M+Na]^+$ ion at *m/z* 377 and a characteristic fragment ion at *m/z* 163 due to the loss of quinic acid. These data were confirmed by comparing the t_R, UV and MS spectra with those of an authentic sample. Peak 2h presented an identical mass spectrum and was tentatively identified as an isomer of chlorogenic acid.

Peak 5h and 6h were tentatively identified as *p*-coumaroil derivates of caffeic acid. Their mass spectra were identical, showing $[M+H]^+$ and $[M+K]^+$ ions at *m/z* 500 and 539. Other characteristic ions at *m/z* 320 and 163 were due to the loss of a unit of quinic acid, and the loss of this moiety plus a coumaric moiety.

Peak 7h was tentatively identified as a di-caffeoyl-quinic acid. It showed a caracteristic $[M+Na]^+$ ion at *m/z* 539. Finally, peak 9h was tentatively identified as a tri-caffeoyl-quinic-acid; it showed $[M+H]^+$ ion at *m/z* 677.

Peak 4h was identified as luteolin-7-*O*-glucoside. It showed $[M+H]^+$ ion at *m/z* 447 and the aglicone fragment at *m/z* 287. These data were confirmed by comparing the t_R, UV and MS spectra with those of an authentic sample. Peak 8h was identified as luteolin: it showed $[M+H]^+$ ion at *m/z* 287. These data were

Table 4
Peak identification and concentration of the constituents in Hawkweed preparations

	Retention time (min)	Constuituent	Concentration in mother tincture DER 1:10 (mg/ml)	Concentration in tincture DER 1:5 (mg/ml)
1h	13.0	Chlorogenic acid	0.73	0.95
2h	14.7	Iso-chlorogenic acid	0.37	0.11
3h	19.0	Umbelliferon	0.27	0.50
4h	22.4	Luteolin-7-*O*-glucoside	0.07	0.13
5h	25.2	*p*-Coumaroil-chlorogenic acid derivate	1.20	1.35
6h	26.7	*p*-Coumaroil-chlorogenic acid derivate	0.10	0.19
7h	32.6	Di-caffeoyl quinic acid	0.41	0.64
8h	37.5	Luteolin	1.44	1.38
9h	38.5	Tri-caffeoyl quinic acid	0.10	0.19
10h	40.6	apigenin	3.84	4.00
		Total caffeoyl-quinic deriVATES	0.58	0.95
		Total flavonoids	0.73	0.95

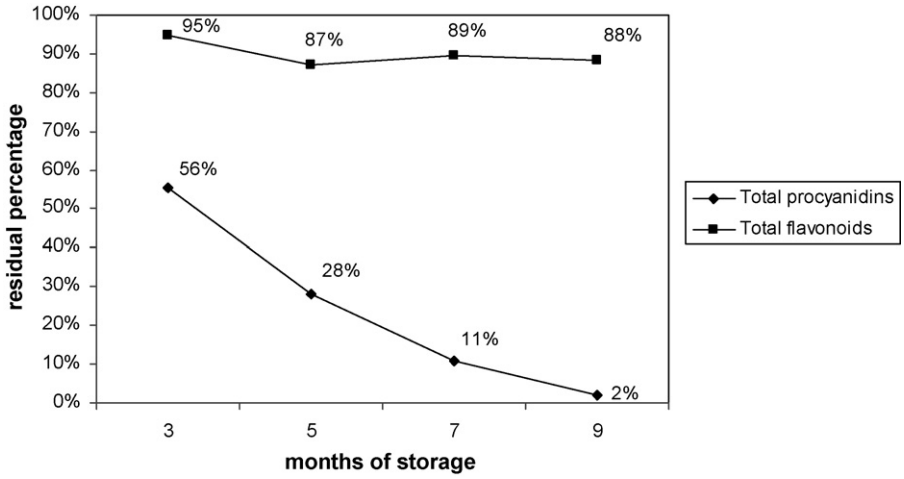

Fig. 5. Stability profiles of Hawthorn mother tincture constituents during 9-month storage period.

confirmed by comparing the t_R, UV and MS spectra with those of an authentic sample. Peak 10h was identified as apigenin: it showed $[M+H]^+$ ion at *m/z* 271. These data were confirmed by comparing the t_R, UV and MS spectra with those of an authentic sample.

Peak 3h was identified as umbelliferone by comparison of t_R, UV and mass spectrum with those of an authentic sample. Peak 3h showed $[M+NaH]^+$ ion at *m/z* 163.

Structures of Hawkweed constituents chlorogenic acid (1h), umbelliferone (3h), luteolin-7-*O*-glucoside (4h), luteolin (8h) and apigenin (10h) are reported in Fig. 4.

Hawkweed tincture samples presented the same chromatographic profiles.

Chlorogenic acid was used as reference constituent for the quantitative analysis of all caffeoyl-quinic acids; vitexin was used for all vitexin derivatives; hyperoside was used for the quantification of all flavonols and flavones. Catechin was used as reference constituent for the quantitative analysis of all procyanidins and umbelliferone standard reference for quantification of umbelliferone.

Quantitative analyses of the constituents of tinctures and mother tinctures of the two herbal drugs are reported in Tables 3 and 4.

Tinctures and mother tinctures were submitted to long-term testing at $25 \pm 2\,^{\circ}C$ for 9 months.

The analyses were carried out in triplicate after 3, 5, 7, and 9 months of storage. Results are reported in Figs. 5–8 and are expressed as residual percentage of flavonoids and of procyanidins for Hawthorn preparations and as residual percentage of caffeoyl-quinic acid derivatives and flavonoids in Hawkweed preparations.

Data are concerning the total amount of a class of constituent (flavonoids, procyanidins, caffeoyl-quinic acid derivatives) rather than each single constituent because for these preparations the active constituents are not known and as a consequence the biological effects are generally considered to arise from the whole phytocomplex.

Flavonoids and caffeoyl-quinic acid derivatives have a good stability in all the tested preparations during the investigated storage period. On the contrary, the content of procyanidins

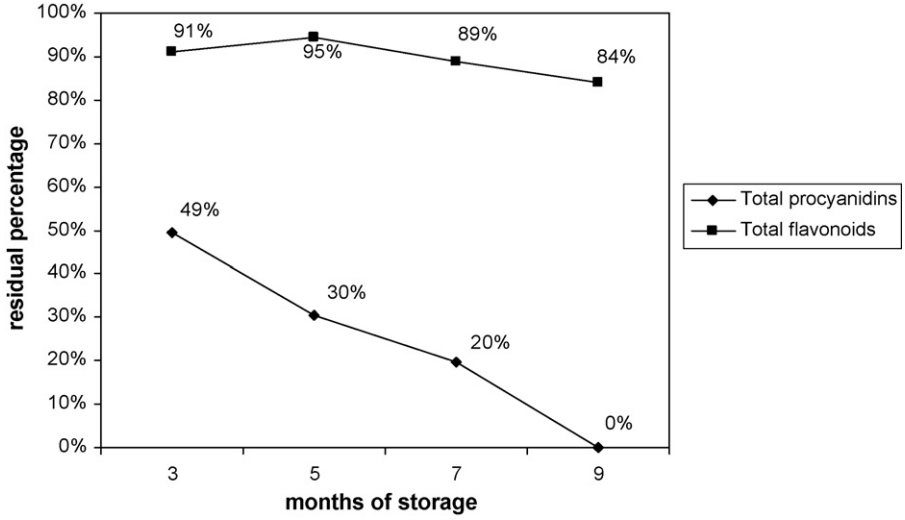

Fig. 6. Stability profiles of Hawthorn tincture constituents during 9-month storage period.

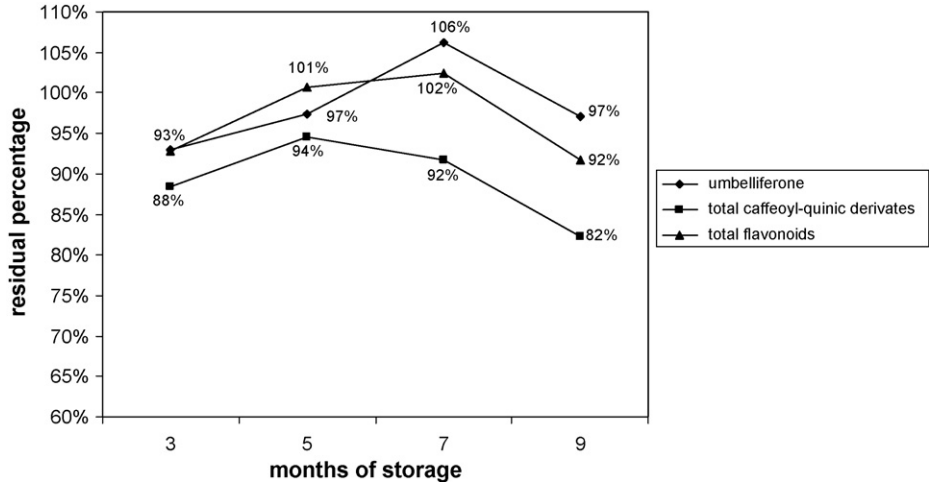

Fig. 7. Stability profiles of of Hawkweed mother tincture constituents during 9-month storage period.

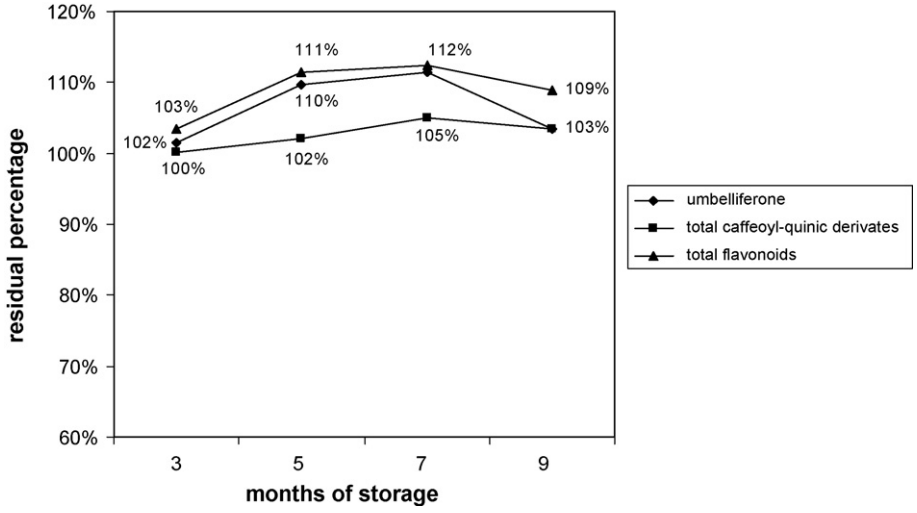

Fig. 8. Stability profiles of of Hawkweed tincture constituents during 9-month storage period.

decrease dramatically and after 3-month storage period the residual amount was about 50% and it was completely degraded after 9-month storage period.

4. Conclusions

In our investigation, tinctures and mother tinctures were prepared from the same plant material using two herbal drugs: Hawthorn leaves and flowers and Hawkweed. These preparations have different modes of preparation [9,10] but often they are indicated by physicians and herbalists as equivalent and interchangeble, using the same dosages.

A rapid and simple HPLC assays was developed and validated for the preparations of each herbal material. These analytical methods provided a satisfactory accuracy, specificity and reproducibility, together with good separations of different classes of constituents such as flavonoids, procyanidins, caffeoyl-quinic derivatives and coumarins. Good linearity of the calibration curves was achieved between 0.1 and 2.5 μg ($r > 0.99$); the repeatability and reproducibility of the methods were satisfactory.

Concerning the qualitative HPLC profiles, tinctures and mother tinctures were very similar, while quantitative analyses revealed some significant differences. In the case of Hawthorn, the concentration of flavonoids was, as expected, higher in tincture but the composition of single flavonoids was quite different: tincture showed a low concentration of vitexin-2″-*O*-rhamnoside and a very high concentration of acetyl-vitexin-2″-*O*-rhamnoside. Total procyanidins content in mother tincture was double respect to tincture (while a half content was expected according to the preparation method). Concerning stability, flavonoids showed good shelf-lives in both preparations while procyanidins showed very low stability at these conditions.

Hawkweed preparations showed similar content of total caffeoyl-quinic derivatives while, as expected, total flavonone and umbelliferone content was half in mother tincture with respect to tincture. The highest stability of caffeoyl-quinic

derivatives and flavonoids, during storage, was found for tincture.

Differences, mainly in the quantitative profile and stability of the constituents between tinctures and mother tinctures, have been found in the present investigation. These two preparations can represent acceptable herbal drug preparations because of their low cost, versatility and ease of preparation, but they cannot always be considered interchangeable but should be judged case by case.

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