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Stability of the constituents of Calendula, Milk-thistle and Passionflower tinctures by LC-DAD and LC-MS

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Abstract

As a part of our investigations on the stability of tinctures, we evaluated 40 and 60% v/v tinctures of Calendula flower, Milk-thistle fruit and Passionflower. These preparations are widely employed in phytotherapy, thus Calendula is used externally for anti-inflammatory properties, Milk-thistle and Passionflower are employed for hepatic injuries and in tenseness with difficulty in falling asleep, respectively. Aim of this work was to assess the chemical stability of their active or marker constituents from accelerated and long-term testing by using HPLC. For Calendula flower and Passionflower active constituents are not known, however, flavonoids seem to have a crucial importance for the activity, and thus are considered the markers of Calendula and of Passionflower. Active constituents of Milk-thistle are represented by silymarin that is a phytocomplex mainly constituted by three flavolignans: silybin, silychristin and silydianin. Our investigation showed a very low thermal stability of the constituents from accelerated and long-term testing and determined by HPLC-DAD and -MS analyses and was related both to the class of flavonoids and water content of the investigated tinctures. Thus, shelf-lives at 25 °C of the most stable tincture (Passionflower 60% v/v) was about 6 months and only about 3 months the stability of Milk-thistle tinctures.

Keywords: Calendula flower tinctures; Milk-thistle fruit tinctures; Passionflower tinctures; Accelerated and long-term stability testing; High-performance liquid chromatography; Active and marker constituents

1. Introduction

In continuing of our investigations on stability of herbal drug preparations (HDPs) and herbal medicinal products (HMPs) [1,2], the constituents' content of Calendula flower, Milk-thistle and Passionflower tinctures after thermal stability testing was evaluated as contribution in this field. Thus, if quality control and stability testing are mandatory and well-described for pharmaceutical synthetic active substances and related finished products [3], in the field of HDPs and HMPs there are only a few reports [1,4,5]. To our best knowledge, this work represent the second investigation on thermal stability of tinctures, recently

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we have reported the very low stability of the constituents of artichoke and St. John's worth tinctures [2].

These tinctures as such or as components of other liquid preparations are marketed mainly as galenicals [6–11]. Preparations based on Calendula flower are used for inflammations of the skin and mucosa, as an aid to wound healing [8,10,12–15]. Those based on Milk-thistle fruit for dyspeptic complaints, for hepatic injuries caused by toxic substances and as adjuvant in the treatment of chronic inflammatory hepatopathies and cirrhosis of the liver [6,16]. Passionflower preparations for tenseness, restlessness and irritability with difficulty in falling asleep [6,9,17].

A general monograph on tinctures is available in the European Pharmacopoeia reporting the ratio between herbal drug and tincture. No information concerning the quality control and stability studies are indicated besides the general advice to store them protected from light [18].

Although this, tinctures still represent the most used pharmaceutical form of plant origin and represent both HDP and HMP: they are administered both as such and as an ingredient of many liquid formulations. In addition, after evaporation to dryness, they are used in solid preparations such as capsules and tablets.

2. Experimental

2.1. Chemicals

Acetonitrile and methanol, absolute ethanol and 85% *o*-phosphoric acid were HPLC grade from Merck (Darmstadt, 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

Indena Research Laboratories (Settala, Milan, Italy) kindly provided rutin (international standard, batch no. K12408717, standard purity 88.17% considering the content of residual solvents, moisture and amount of impurities). Homoorientin, isoquercitrin, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, isovitexin, silybin, taxifolin and vitexin were purchased from Extrasynthese (Genay, France).

2.3. Plant materials and sample preparation

Calendula officinalis L. (dried flowers, lot no. 61174), *Passiflora incarnata* L. (dried flowering tops, lot no. 60698), *Silybum marianum* (L.) Gaertn. (dried fruits, lot no. 56313) were kindly offered by Aboca S.p.A., Sansepolcro, Arezzo, Italy.

Tinctures were obtained according to European Pharmacopoeia [3]. Two-hundred grams of dried plant material were macerated using 40 and 60% v/v hydro-alcoholic solutions to obtain 1000 ml of tincture. Batches (about 200 ml) of each tincture were stored in brown glass in an oven. They were analyzed, as such, monthly during a 6-month period for long-term testing (25 ± 2 °C), and fortnightly during a 3-month period for accelerated testing at 40, 50 and 60 ± 2 °C. Triplicate HPLC determinations were performed on each sample, after filtration.

2.4. Stability study

The thermal stability testing was carried out in triplicate at different temperature conditions: $+25 \pm 2$ °C ($60 \pm 5\%$ RH) for the long-term testing and at $+40 \pm 2$ °C ($75 \pm 5\%$ RH), $+50$ and $+60$ °C for accelerated testing. 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 (Helwett & Packard, Palo Alto, CA, USA). The column was a LiChrosorb RP18 (5 μm, 250×4 mm i.d.) (Merck, Darmastadt, Germany) maintained at 26 °C and equipped with a precolumn LiChrosorb RP18 (5 μm, 10×4 mm i.d.) (Merck, Darmastadt, Germany). The mobile phase was a

four-step linear solvent gradient CH_3CN/H_2O with H_3PO_4 (pH 3.0) during a 40-min period at a flow rate of 1.3 ml/min and was previously reported [19]. Tinctures submitted to thermal stability were filtered through a cartridge-type sample filtration unit with a polytetrafluoroethylene (PTFE) membrane before HPLC analysis. Injected volume of sample was 25 μl solution. UV–vis spectra were recorded in the range 190–450 nm, and chromatograms were acquired at 254, 280, 330 and 350 nm. Peaks were detected at 350 nm for Calendula and Passionflower tinctures; and were detected at 280 nm for Milk-thistle tinctures. Typical chromatograms of tinctures are reported in Figs. 1, 3 and 5.

2.5.2. HPLC-MS analysis instrumentation

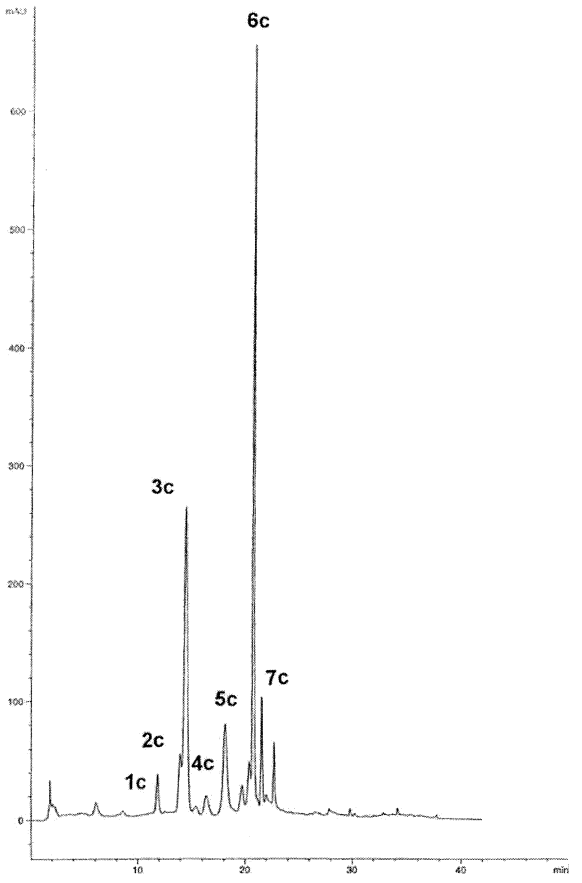
The HPLC system described above was interfaced with a HP 1100 MSD API-electrospray (Hewlett & Packard, Palo Alto, CA, USA). The interface geometry, with an orthogonal position of the nebulizer with respect to the capillary inlet, allowed the use of analytical conditions similar to those used for the HPLC-DAD analysis. Mass spectrometer conditions were optimized in order to achieve the maximum sensitivity of ESI values. The same column, time period and flow rate were used. Since phosphoric acid was not suitable for HPLC-MS operations, separation was performed using aqueous formic acid (pH 3.0), without appreciable variations in the chromatographic profile. Mass spectrometry operating conditions were: gas temperature 350 °C at a flow rate of 10 l/min, nebulizer pressure 30 p.s.i., quadrupole temperature 30 °C, and capillary voltage 3500 V. Full scan spectra from m/z 100 to 800 in the positive ion mode were obtained (scan time 1 s). Injected volume of samples was 25 μl solution.

2.6. Identification of constituents and peak purity

Identification of all constituents was performed by HPLC-DAD and -MS analysis and/or by comparing the retention time of the peaks in the extracts with those of the 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 reference samples and/or by examination the MS spectra.

2.7. Linearity, repeatability and reproducibility

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 ($r > 0.99$).



1c quercetin-3-O-rutinosylrhamnoside
2c rutin
3c isorhamnetin-3-O-rutinosylrhamnoside
4c isoquercitrin
5c isorhamnetin-3-O-glucosylglucoside
6c narcissin
7c isorhamnetin-3-O-glucoside

Fig. 1. Profile of the chromatogram of Calendula tincture (60% v/v) with the HPLC-DAD–MS attributions of the components detected.

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: homoorientin 2.55%, hyperoside 2.60%, isoquercitrin 2.40%, isorhamnetin 2.80%, isorhamnetin-3-*O*-glucoside 2.10%, isorhamnetin-3-*O*-rutinoside 2.22%, isovitexin 1.90%, quercetin 2.00%, rutin 1.90%, silybin 2.00%, taxifolin 2.80% and vitexin 2.06%.

To evaluate the reproducibility of the injection integration, the standard solutions of rutin (2.5 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: homoorientin 1.09%, hyperoside 1.30%, isoquercitrin 1.22%, isorhamnetin 1.29%, isorhamnetin-3-*O*-glucoside 1.00%, isorhamnetin-3-*O*-rutinoside 1.12%, isovitexin 0.90%, quercetin 1.10%, rutin 0.90%, silybin 1.00%, taxifolin 1.56% and vitexin 1.12%.

2.8. Quantitation of flavonoids

All the tinctures were analyzed in triplicate and a calibration graph with six datapoints of external standard was used. The contents of constituents were calculated taking into account the mean of the response factor of rutin in the reference solutions, i.e. $\text{area/concentration}_{(\text{mg/ml})} \times \text{purity}/100$, and the response factor of the considered constituent relative to rutin (RRF). This value was determined by calculating the ratio between the average response factor of each compound and the average response factor of rutin at 350 or 280 nm. All flavanolignans were evaluated as silybin, isorhamnetindiglycosides and isorhamnetintriglycosides as narcissin, apigenin-6,8-diC-glycosides as isovitexin, and quercetintriglycoside as rutin.

2.9. Evaluation of the results obtained from tinctures subjected to accelerate storage

Data obtained from the accelerated testing (40, 50, 60 ± 2 °C) were used to predict the shelf life at 25 °C, by the Arrhenius law, as previously reported [2].

3. Results and discussion

In continuing our investigation on the stability of HDPs [1,2], we now report on the chemical stability of tinctures of Calendula flower, Milk-thistle fruit and Passionflower through the determination of the stability of their flavonoidic content, which represent the active constituents or the markers of these herbal drugs. A simple RP-HPLC method [19] that optimized the separation of all classes of flavonoids (flavones, flavonols, flavanonols and flavanolignans) was used. Identification of constituents was obtained by the combination of DAD and MS data.

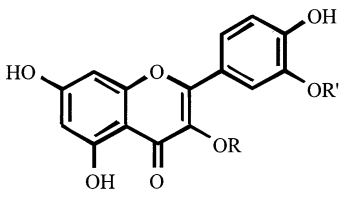
All the tinctures, after their preparation according to European Pharmacopoeia were subjected to long-term testing at 25 ± 2 °C for 6 months, and also to accelerated testing at three different temperatures (40, 50 and 60 ± 2 °C) for 3 months.

The analyses of tinctures subjected to long-term testing were carried out in triplicate every 30 days. Limits of acceptance for the stability evaluation were considered 90%, i.e. 10% potency loss from the initial value of the sample for flavonoids considered as markers (i.e. flavonols of Calendula and *C*-glycosylflavones of Passionflower). The limit for known active constituents (i.e. flavanolignans of Milk-thistle) was considered 95%, i.e. 5% potency loss from the initial assay value of the sample [20].

In these studies the total content of flavonoids was also considered because the biological effects of herbal drugs and their preparations are generally considered to arise from the whole phytocomplex rather than from a single constituent.

In the chromatograms of 60% v/v tinctures from Calendula flowers (Fig. 1) seven peaks are presents (1c–7c). All the constituent 1c–7c (Fig. 2) displayed identical UV absorptions with maxima at about 256 and 355 nm, typical of flavonols. Moreover, the presence in all mass spectra of an ion at m/z 303 or 371 indicated that constituents 1c–7c were quercetin or isorhamnetin derivatives.

Thus, peaks 2c, 4c, 6c, 7c were identified as rutin, isoquercitrin, narcissin, isorhamnetin-3-*O*-glucoside, respectively, by comparison of t_R, UV and MS spectra with those of an authentic sample. Peak 1c showed $[M + H]^+$ and $[M + Na]^+$



1c: R=rut-glc R'=H quercetin-3-O-rutglc
2c: R=rut R'=H rutina
3c: R=rut-glc R'=Me isorhamnetin-3-O-rutglc
4c: R=glc n R'=H isoquercetin
5c: R=glc-glc R'=Me quercetin-3-O-glcglc
6c: R=rut R'=Me narcissin
7c: R=glc R'=Me isorhamnetin-3-O

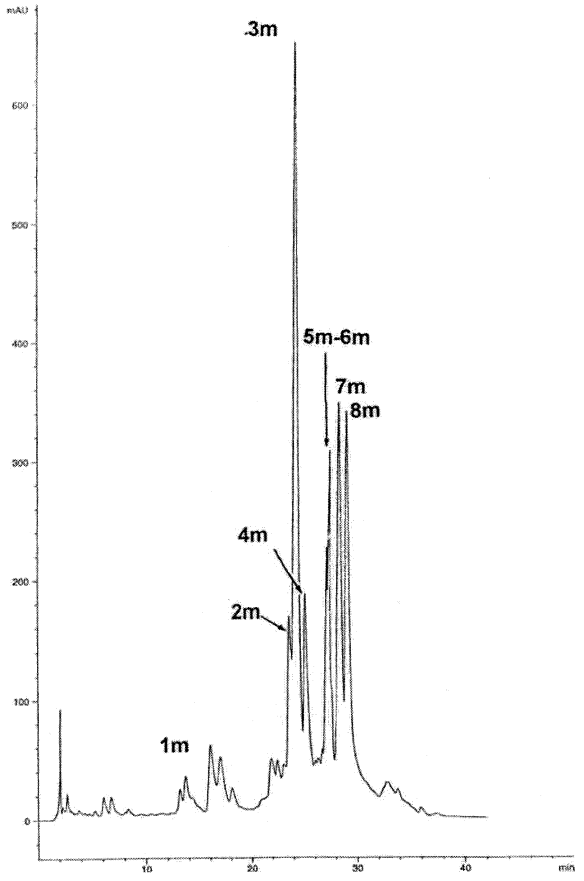
Fig. 2. Structures of the constituents of Calendula tinctures.

ions at m/z 757 and 779, respectively, and three fragment ions at m/z 611, 465 and 303 due to the loss of a deoxy-hexose unit, another deoxy-hexose unit and a hexose unit, respectively and was identified as quercetin-3-*O*-rutinosylrhamnoside. Peak 3c showed $[M + H]^+$ and $[M + Na]^+$ ions at m/z 769 and 791, respectively, and three fragment ions at m/z 625, 479 and 317 due to the loss of a deoxy-hexose unit, another deoxy-hexose unit and a hexose unit, respectively and was identified as isorhamnetin-3-*O*-rutinosylrhamnoside. Peak 5c displayed UV spectra with maxima at 259 and 356 nm and two shoulders at 270 and 300 nm. It showed $[M + H]^+$ and $[M + Na]^+$ ions at m/z 641 and 663, respectively, and two fragment ions at m/z 479 and 317 due to the loss of a hexose unit and another hexose unit, respectively. It was tentatively identified as isorhamnetin-3-*O*-glucosylglucoside.

The chromatogram of 40% v/v Calendula tincture was qualitatively similar and the presence of all the constituents 1c–7c was confirmed by comparison of the spectroscopic and the chromatographic data.

In addition, both Calendula tinctures showed a similar total content of flavonols that was mainly represented by isorhamnetin derivatives, while quercetin derivatives represented only 15% of the total flavonols (Table 1). Therefore, in the long-term testing quercetin derivatives were considered together. Over time the flavonol content of the two tinctures gave rise to different decomposition percentages as reported in Table 1.

In the chromatogram of 60% v/v tinctures from Milk-thistle (Fig. 3) eight peaks are present (1m–8m). All the constituents 1m–8m (Fig. 4) displayed identical UV absorptions with maxima at about 290 nm, typical of dihydroflavonols. Peaks 1m and 7m were identified as taxifolin and silybin by comparing their t_R UV and MS spectra with those of an authentic sample. Peaks 2m, 3m and 8m showed $[M + H]^+$ and $[M + Na]^+$ ions at m/z



1m taxifolin
2m silydianin
3m silychristin
4m oxyderivative of silybin/isosilybin isomers
5m-6m 2,3-dehydroderivatives of silybin/isosilybin isomers
7m silybin
8m isosilybin

Fig. 3. Profile of the chromatogram of Milk-thistle tincture (60% v/v) with the HPLC-DAD–MS attributions of the components detected.

Table 1
Content of constituents in Calendula tinctures determined by HPLC, after long-term testing at 25 ± 2 °C

Storage period (days)	Isorhamnetin-3-rutglc		Isorhamnetin-3-glcglc		Narcissin		Isorhamnetin-3-glc		Quercetin derivatives		Total flavonols	
	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%
Tincture 60% v/v												
Initial	16.6 ± 0.52		7.1 ± 0.60		21.4 ± 0.72		3.2 ± 0.11		8.8 ± 0.50		57.1 ± 1.18	
30	15.6 ± 0.98	94.0	6.4 ± 0.72	90.1	21.0 ± 1.34	98.1	5.0 ± 0.89	156.3	8.5 ± 1.00	96.6	56.5 ± 1.32	98.9
90	14.2 ± 0.82	85.3	5.1 ± 0.57	71.8	20.1 ± 1.32	94.0	8.0 ± 0.72	284.4	8.3 ± 1.23	94.0	5.7 ± 1.00	97.5
120	13.4 ± 0.88	80.7	4.9 ± 0.80	69.1	19.6 ± 1.50	91.5	8.3 ± 0.69	259.3	8.0 ± 0.99	91.0	54.2 ± 1.21	95.0
150	12.8 ± 1.12	77.1	4.7 ± 0.67	66.2	18.9 ± 1.34	88.3	8.5 ± 0.99	265.6	7.9 ± 1.13	90.0	52.8 ± 1.43	92.4
Tincture 40% v/v												
Initial	18.6 ± 0.80		9.4 ± 0.64		20.4 ± 0.86		2.6 ± 0.13		7.4 ± 0.80		58.4 ± 1.19	
30	16.9 ± 1.05	90.9	8.1 ± 0.92	86.2	19.8 ± 1.43	97.1	2.6 ± 0.78	153.8	7.0 ± 0.99	94.6	55.8 ± 1.26	95.5
90	15.9 ± 1.32	85.5	7.7 ± 1.09	81.9	18.6 ± 1.56	91.2	4.0 ± 0.67	203.8	6.8 ± 0.88	91.9	54.3 ± 1.56	93.0
120	14.8 ± 1.02	79.6	7.4 ± 0.89	78.7	17.9 ± 1.45	87.7	5.3 ± 0.99	223.1	6.5 ± 0.95	87.8	52.4 ± 1.38	89.7
150	14.0 ± 1.42	75.3	7.0 ± 0.99	74.5	18.0 ± 1.76	88.2	5.8 ± 0.67	211.5	6.0 ± 0.98	81.1	50.5 ± 1.25	86.5

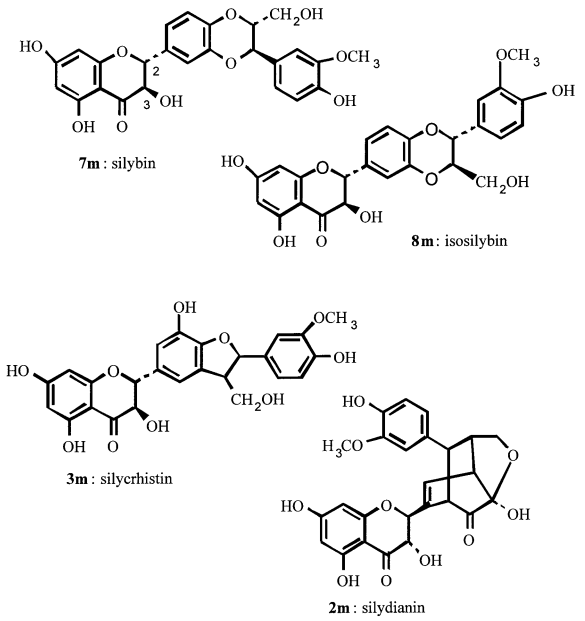


Fig. 4. Structures of the constituents of Milk-thistle tinctures.

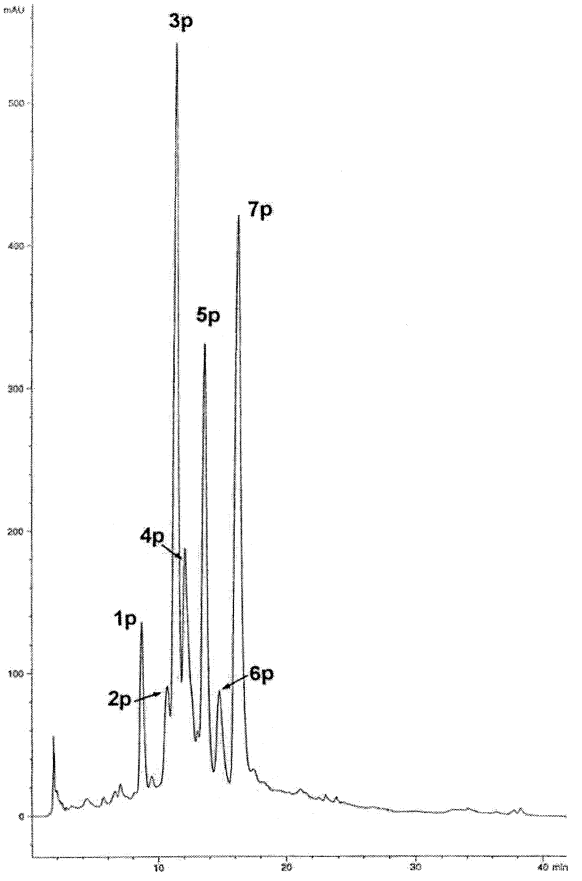
483 and 505, respectively and other characteristic fragment ions. According to these data peak 8m was tentatively identified as isosilybin, peak 3m as silychristin and peak 2m as silydianin [19].

Peak 4m showed $[M+H]^+$ and $[M+Na]^+$ ions at m/z 499 and 521, respectively. These data indicated that peak 4m contained an additional oxygen compared to the other flavanolignan. Peaks 5m and 6m showed $[M+H]^+$ and $[M+Na]^+$ ions at m/z 481 and 503, respectively. These data were in accordance with the presence of two 2,3-dehydroderivatives of silybin/isosilybin isomers.

Milk-thistle 40% v/v tincture showed a similar chromatogram to that of 60% v/v but constituent content was very different (Table 2).

Both Milk-thistle 40 and 60% v/v tinctures were less stable than Calendula tinctures (Table 2). Taxifolin, a dihydroflavonol, represented only 0.03% of the total flavonoids, therefore it was not considered in the long-term testing (Table 2). The two tinctures showed a very different content of the phytocomplex and over time the flavanolignan content of the two tinctures gave rise to different decomposition percentages (Table 2).

In the chromatogram of 69% v/v Passionflower tincture (Fig. 5) seven peaks (1p–7p) were evidenced. Structures of compounds 1p–7p are reported in Fig. 6. Peak 4p was identified as homoorientin, peak 6p as vitexin, peak 7p as isovitexin by comparison their chromatographic and spectroscopic data with authentic samples. Peaks 5p displayed identical UV spectra with maxima at 271 and 334 nm, typical of isovitexin

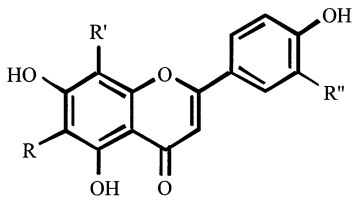


1p 6,8-di-C-glucosylapigenin
2p isoschaftoside
3p schaftoside
4p homoorientin
5p isovitexin-2"-O-glucoside
6p vitexin
7p isovitexin

Fig. 5. Profile of the chromatogram of Passionflower tincture (60% v/v) with the HPLC-DAD–MS attributions of the components detected.

Table 2
Content of constituents (means ± SD of three replicates) in Milk-thistle tinctures determined by HPLC, after long-term testing at 25 ± 2 °C

Storage period (days)	Silydianin		Silychristin		Oxyderivative		2,3-Dehydro derivatives		Silybin		Isosilybin		Total flavanolignans	
	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%
Tincture 60% v/v														
Initial	10.3 ± 0.33		52.5 ± 1.06		18.8 ± 0.84		19.9 ± 0.65		39.4 ± 0.90		29.4 ± 0.79		170.3 ± 1.09	
30	10.2 ± 0.99	99.0	52.3 ± 1.89	99.6	18.6 ± 1.45	98.9	22.6 ± 1.76	113.6	38.8 ± 2.00	98.5	28.7 ± 1.37	97.6	171.2 ± 1.9	97.4
60	9.9 ± 0.98	96.1	51.8 ± 1.97	98.7	18.2 ± 1.32	96.8	20.5 ± 1.68	103.0	38.4 ± 2.22	97.5	28.2 ± 01.72	95.9	167.0 ± 1.1	95.0
90	9.6 ± 0.70	93.2	51.3 ± 2.08	97.7	18.0 ± 1.34	95.7	19.0 ± 1.46	95.5	37.6 ± 1.82	95.4	26.1 ± 1.56	88.8	161.6 ± 1.0	91.9
Tincture 40% v/v														
Initial	4.8 ± 0.28		31.5 ± 0.99		9.6 ± 0.60		13.8 ± 0.55		13.5 ± 0.46		15.4 ± 0.70		88.6 ± 1.10	
30	4.6 ± 0.92	95.8	30.1 ± 1.68	95.6	9.2 ± 0.99	95.8	14.7 ± 1.05	106.5	12.4 ± 1.02	89.8	13.9 ± 0.99	90.3	84.9 ± 1.40	95.8
60	4.5 ± 0.89	93.8	29.4 ± 1.39	93.3	8.8 ± 1.34	91.7	14.2 ± 1.39	102.9	11.6 ± 1.15	84.1	13.1 ± 1.37	85.1	81.6 ± 1.52	92.1
90	4.3 ± 0.69	89.6	28.3 ± 1.46	89.8	8.2 ± 0.76	85.4	13.9 ± 1.22	100.7	10.8 ± 0.82	80.0	12.6 ± 1.19	81.8	78.1 ± 1.32	88.2



1p: R=R'=glc R"=H vicin-2
2p: R=glc R'= ara R"=H isoschaftoside
3p: R= ara R'=glc R"=H schaftoside
4p: R=glc R'= H R"=OH homorientin
5p: R=glc-2"-glc R'=R"=H isovitexin-2"-O-glc
6p: R= R"=H R'=glc vitexin
7p: R= glc R'= R"=H isovitexin

Fig. 6. Structures of the constituents of Passionflower tinctures.

derivatives. Peak 5p exhibited $[M + H]^+$ and $[M + Na]^+$ ions at m/z 595 and 617 and a characteristic $[M + H - 162]^+$ ion at m/z 433 due to the loss of a terminal hexose moiety. It was identified as 2″-glucosylisovitexin. Peaks 1p, 2p and 3p displayed identical UV spectra with maxima at 274 and 334 nm. Peaks 2p and 3p exhibited the same $[M + H]^+$ and $[M + Na]^+$ ions at m/z 565 and 587. These data suggested the presence of an apigenin nucleus plus a pentose and glucose. However, the absence for both peaks 2p and 3p spectra of the fragment ions $[M + H - 162]^+$, $[M + H - 146]^+$ or $[M + H - (162 + 146)]^+$ at m/z 403, 419 and 257 (corresponding to the aglycone apigenin) suggested that peaks 2p and 3p were 6,8-*C*-glycosides of apigenin. These suggestions were confirmed by the typical fragment ions $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$ and $[M + H - 3H_2O]^+$ that were evidenced at m/z 547, 529 and 511. Other characteristic fragment ions $[M + H - 2H_2O - CH_2O]^+$, $[M + H - 2H_2O - 2CH_2O]^+$, $[M + H - 5H_2O - CH_2O]^+$ and $[M + H - 6H_2O - CH_2O]^+$ were also evidenced at m/z 499, 469, 445 and 427. From these data, peak 3p was tentatively identified as schaftoside and the minor constituent peak 2p was tentatively identified as 6-*C*-arabinosyl-8-*C*-glucosylapigenin (isoschaftoside). Peak 1p exhibited the same $[M + H]^+$ and $[M + Na]^+$ ions at m/z 595 and 617 of peak 5p. These data suggested the presence of a apigenin nucleus plus two glucose moieties, and thus it was tentatively identified as 6,8-diC-glucosylapigenin (vicenin-2).

Passionflower 40 and 60% v/v tinctures showed similar chromatograms but a different total content of *C*-glycosylflavones, mainly represented by isovitexin and schaftoside/isoschaftoside (Table 3). During time the *C*-glycosylflavone content of the two tinctures showed different decomposition percentages as reported in Table 3.

Rutin was used as reference substance for the quantitative analysis of flavonoids. It was found to be stable in methanol solution at room temperature for at least 48 h. Flavonoids in the tinctures were stable at room temperature for at least 48 h. The content of each flavonoids was calculated using the response factor, RRF, calculated as described in Section 2.7.

Tinctures submitted to accelerated degradation methods were analyzed by the Arrhenius law to predict the shelf life of single constituents at 25 °C. Mean concentration–time data for metabolite content were analyzed by linear and exponential regression analysis and decomposition was assumed if the slope of regression line was significant. Only a few constituents showed a degradation rate fitting with a 1st order kinetic and the data obtained after storage at 25 ± 2 °C confirmed a very short shelf life (Table 4). These results were in approximate agreement with the shelf life found by long-term testing. The studies yielded good correlation coefficients ($R > 0.9000$) for the constituents reported in Table 4 that best fit 1st order kinetic leading to the possibility of predicting shelf life at 25 °C by the Arrhenius law. The data obtained from accelerated degradation methods expired a decomposition of the phytocomplex but the rate of degradation did not fit the law. So, only long-term testing was used to establish the t_{90} shelf life of each tincture.

4. Conclusions

In our investigation, two different alcoholic degrees, i.e. 40 and 60% v/v, were used to prepare the tinctures of Calendula flower, Milk-thistle fruit and Passionflower. The analysis was performed on tinctures as such, after filtration and a rapid and simple RP-HPLC assay was developed

Table 3
Content of constituents in Passionflower tinctures determined by HPLC, after long-term testing at 25 ± 2 °C

Storage period (days)	Vicinin-2		Schaftoside/isoschaftoside		Homoorientin		Isovitexin-2″-glc		Vitexin		Isovitexin		Total *C*-glycosilflavones	
	Mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%	mg/100 ml ± SD	%
Tincture 60% v/v														
Initial	17.0 ± 0.52		59.6 ± 1.48		25.6 ± 0.89		36.1 ± 1.14		18.3 ± 0.89		58.9 ± 1.45		215.5 ± 1.78	
30	17.0 ± 1.19	100	59.5 ± 2.49	99.8	25.6 ± 1.10	100	33.8 ± 2.26	93.6	18.4 ± 1.00	100.5	59.0 ± 2.58	100.2	215.3 ± 3.18	99.0
90	16.9 ± 1.32	99.4	59.5 ± 2.32	99.8	25.4 ± 1.35	99.2	32.6 ± 2.86	90.3	18.2 ± 1.18	99.5	59.5 ± 2.23	101.0	212.1 ± 2.04	98.4
120	16.6 ± 0.98	97.6	59.2 ± 2.63	99.3	25.2 ± 1.68	98.4	29.8 ± 1.99	82.5	17.9 ± 0.98	97.8	58.0 ± 2.32	98.5	206.7 ± 1.98	95.9
180	16.0 ± 0.82	94.1	58.3 ± 2.54	97.8	24.4 ± 1.32	95.3	28.0 ± 2.06	77.6	17.0 ± 1.58	92.9	54.7 ± 1.45	92.8	198.4 ± 2.09	92.1
Tincture 40% v/v														
Initial	14.2 ± 1.01		47.5 ± 1.56		21.7 ± 1.21		26.5 ± 1.02		15.0 ± 0.97		39.8 ± 1.23		164.7 ± 1.29	
30	14.1 ± 1.11	99.3	47.5 ± 1.89	100	21.6 ± 1.62	99.5	21.7 ± 1.99	81.8	15.0 ± 0.92	100	40.4 ± 1.49	101.5	160.3 ± 1.39	97.3
90	13.8 ± 1.34	97.2	46.7 ± 2.32	98.3	21.0 ± 1.36	96.8	18.4 ± 2.22	69.4	14.9 ± 1.03	99.3	40.8 ± 2.32	102.5	155.8 ± 1.40	94.5
120	13.5 ± 1.78	95.0	46.2 ± 2.67	97.3	20.7 ± 1.03	95.4	15.1 ± 2.57	57.0	14.7 ± 1.16	98.0	39.4 ± 2.67	99.0	150.0 ± 1.31	91.0
180	13.1 ± 1.54	92.3	45.7 ± 2.59	96.2	20.0 ± 1.83	92.2	12.0 ± 1.30	45.2	14.2 ± 1.56	94.6	37.4 ± 2.05	94.0	143.0 ± 1.25	86.8

Table 4
Expired and experimental shelf-life of some flavonols

Tincture (alcohol content, %)	Constituent	Expired shelf-life[a] (days)	Experimental shelf-life[a] (days)
Calendula (40% v/v)	Isorhamnetin-3-*O*-rutgluc (3c)	7	30
Calendula (60% v/v)	Isorhamnetin-3-*O*-rutgluc (3c)	72	65
Milk-thistle (40% v/v)	Silychristin (3m)	40	30
Milk-thistle (60% v/v)	Silychristin (3m)	140	135
Passionflower (40% v/v)	Vicinin-2 (1p)	205	195
	Isovitexin-2″-glc (5p)	25	15
Passionflower (60% v/v)	Vicinin-2 (1p)	260	246
	Isovitexin-2″-glc (5p)	98	90

Means ± SD of three replicates.
[a] For Milk-thistle tinctures the stability was evaluated using a limit of 5% loss from the initial assay value, a limit of 10% was used for the other tinctures.

and validated. This method provided a satisfactory accuracy, specificity, and reproducibility, together with a good separation of all classes of flavonoids including flavones, flavonols, flavanonols and flavanolignans. It can be proposed for the analysis of other phytochemicals containing flavonoids.

Rutin was used as external standard because this compounds is easily available. Good linearity of the calibration curves was achieved between 0.1 and 2.5 μg ($r > 0.99$); the repeatability and reproducibility of the method resulted satisfactory. UV detection was fixed at 350 nm for Calendula flower and Passionflower and at 280 nm for Milk-thistle fruit, as all the constituents of the preparations showed appreciable absorbance at these wavelengths.

From our results it is clear that the solvent mixture did not affect the quali-quantitative profile of the two Calendula tinctures so much. However, from the stability studies, the solvent mixture is very important in maintaining its integrity, since the stability of the flavonoidic constituents of two tinctures was quite different. Thus, the t_{90} shelf life of 60% v/v Calendula tincture was more than 5 months, while that of the 40% v/v one was about 4 months.

Concerning the flavanolignan content of Milk-thistle tinctures, both hydroalcoholic concentrations are stable with respect to the active phytocomplex for at least no more than 3 months.

Concerning the stability of *C*-glycosylflavones of Passionflower tinctures, tinctures at 25 °C was higher than the other investigated tinctures and their t_{90} shelf life for the 60% v/v tinctures was more than 6 months, while t_{90} shelf life of 40% v/v was about 4 months.

In addition, the tinctures submitted to accelerated degradation methods exhibited a decomposition of the phytocomplex but in general the rate of degradation did not obey the Arrhenius law, therefore only long-term experiments can establish the t_{90} shelf life.

Tinctures in some cases they can represent acceptable HDPs or HMPs because its low cost, their versatility, and easy to prepare, but they cannot widely employed in preparations to be stored because their low stability dramatically affected by ethanol content and the nature of constituents.

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References

[1] A.R. Bilia, M.C. Bergonzi, F. Morgenni, G. Mazzi, F.F Vincieri, Int. J. Pharm. 213 (2001) 199–208.

[2] A.R. Bilia, M.C. Bergonzi, G. Mazzi, F.F Vincieri, Drug Dev. Ind. Pharm. (2002) in press.
[3] Eudra/Q/92/021: 'Note for Guidance on stability testing of new active substances and medicinal products'.
[4] P. Maisenbacher, K.A. Kovar, Planta Medica 58 (1992) 351–354.
[5] B. Kommanaboyina, C.T. Rhodes, Drug Dev. Ind. Pharm. 25 (7) (1999) 857–868.
[6] R. Della Loggia, in: Piante Officinali per infusi e tisane: Manuale per Farmacisti e Medici, Calendulae flos; Cardui mariae fructus; Passiflorae herb; OEMF s.p.a., Milan, Italy, 1993, pp. 118–120, 121–123, 363–365.
[7] R. Longo (translated from German Monographs of Kommission E), in: Le monografie tedesche, vols. I–II Studio, Milan, Italy, 1994.
[8] G. Willuhn, Dtsch. Apoth. Ztg. 132 (1992) 1873–1883.
[9] O. Isaac, in: R. Hänsel, K. Keller, H. Rimpler, G. Schneider (Eds.), Hagers Handbuch der Pharmazeutischen Praxis, Calendula, 5th ed., Drogen A-D, vol. 4, Springer-Verlag, Berlin, Heidelberg, 1992, pp. 597–615.
[10] J. van Hellemont, Fytotherapeutisch Ciompendium, 2nd ed., Scheltema & Holkema, Utrecht, 1988, pp. 113–114.
[11] P.R. Bradley, in: British Herbal Compendium, Passiflora, vol. 1, British Herbal Medicine Association, Bournemouth, London, 1992, pp. 171–173.
[12] O. Isaac, Botanik, Chemie, Pharmakologie, Toxicologie, Pharmazie und therapeutische Verwendung, Wissenschaftliche Verlagsgesellschaft, Stuttgart, 1992, pp. 65–66.
[13] J.J.C. Scheffer, Pharm. Weekbl. 114 (1979) 1149–1457.
[14] E. Vidal-Ollivier, R. Elias, F. Faure, A. Babadjamian, F. Crespin, G. Balansard, G. Boudon, Planta Med. 55 (1989) 73–74.
[15] R.F. Weiss, Lehrbuch der Phytotherapie, 7th ed., Hippokrates Verlag, Stuttgart, 1990, pp. 429–433.
[16] H. Wagner, in: J. Beal, E. Reinhard (Eds.), Natural Products as Medicinal Agents, Hippokrates, Stockard, 1981, pp. 217–220.
[17] O. Isaac, in: R. Hänsel, K. Keller, H. Rimpler, G. Schneider (Eds.), Hagers Handbuch der Pharmazeutischen Praxis, Passiflora, 5th ed., Drogen P-Z, vol. 6, Springer-Verlag, Berlin,Heidelberg, 1994, pp. 34–49.
[18] European Pharmacopoeia, 3rd ed., 1997, p. 1647.
[19] A.R. Bilia, D. Salvini, G. Mazzi, F.F. Vincieri, Chromatographia 53 (2001) 210–215.
[20] EMEA/CVMP/814/00 Note for Guidance on Quality of Herbal Medicinal Products.