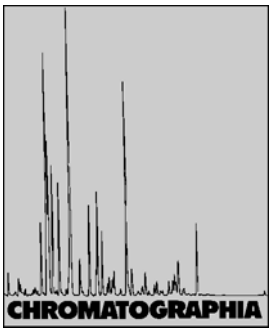
Characterization of Calendula Flower, Milk-Thistle Fruit, and Passion Flower Tinctures by HPLC-DAD and HPLC-MS



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Key Words

Column liquid chromatography
Thin-layer chromatography
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Flavonoids and tinctures
Calendula flower, milk-thistle fruit and passion flower

Summary

As a part of an investigation of the content of herbal drug preparations and herbal medicinal products, we have investigated tinctures (prepared with 40:60 and 60:40 (%, *v/v*) ethanol-water) of calendula flower, milk-thistle fruit, and passion flower, which are, respectively, widely used for their anti-inflammatory properties, to treat hepatic injuries, and to treat tension and difficulty falling asleep. The aim of this work was to evaluate the flavonoid content, because flavonoids are the active constituents or markers of these herbal drugs, and to establish the best solvent for the extraction of the constituents. The findings reported herein both confirm the presence of several flavonoids previously identified in these herbal drugs and report the presence of others not previously described and identified here for the first time. In general the flavonol content was highest in 60% tinctures. A rapid, reversed-phase (RP) HPLC assay was developed and validated and enabled good separation of all classes of flavonoids including flavones, flavonols, flavanonols, and flavanolignans. Aglycones and mono, di, and triglycosides (both *O*- and *C*-derivatives) of the flavonoids are also easily and satisfactorily separated. This method is thus proposed for the analysis of other herbal drugs or herbal drug preparations in which flavonoids are the active constituents or markers.

Introduction

Tinctures, used as herbal drugs and medicines, are the most widely used pharmaceutical form of plant origin; they are administered as the ingredients of many liquid formulations, and can also be evaporated to dryness and used as solid formulations.

The European Pharmacopoeia contains a general monograph about tinctures in which their modes of preparation and the amounts of the herbal drugs in the tinctures are specified [1]. In the herbal drug monographs, however, the amount of alcohol required to facilitate extraction of the phytocomplex is not specified, and thus herbal drug tinctures containing different amounts of alcohol (from 20 to 60%, *v/v*) are marketed.

In this work we have evaluated the flavonoid content of tinctures prepared from calendula flower, milk-thistle fruit, and passion flower; these herbal drugs widely used in traditional medicine and their preparations are also marketed as pharmaceutical specialties or galenicals [2–7]. The aims of the investigation were to identify the constituents of the tinctures and to establish whether 40 or 60% (*v/v*) alcohol was the better solvent for herbal drug extraction.

Experimental

Solvents and Reagents

Acetonitrile, methanol, and absolute ethanol were HPLC-grade from Merck (Darmstadt, Germany); 85% orthophosphoric acid, Chloroform, acetone, glacial acetic acid (AcOH), and ethyl acetate (AcOEt) were analytical grade from Merck; 85% formic acid was obtained from Carlo Erba (Milan, Italy). Water was purified by use of a Milli-Q_{plus} system from Millipore (Milford, MA, USA). Diphenylboric acid, ethanolamine ester (NTS), and polyethylene glycol 4000 (PEG) reagents were from Aldrich-Chemie (Steinheim, Germany).

Standards and Materials

Indena Research Laboratories (Settala, Milan, Italy) kindly provided rutin (batch

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#K12408717, standard purity 88.17%, containing residual solvents, moisture, and other impurities). Homoorientin, isoquercitrin, isorhamnetin-3-*O*-glucoside, isorhamnetin-3-*O*-rutinoside, isovitexin, silybin, taxifolin, and vitexin were obtained from Extrasynthese (Genay, France). The purity of these standards was 89.3, 83.0, 93.5, 90.8, 91.7, 89.0, 92.0, and 90.5%, respectively.

Commercial samples of herbal drugs, kindly provided by Aboca S.p.A. (Sansepolcro, Arezzo, Italy), were *Calendula officinalis* L. (dried flowers, lot n. 61174), *Passiflora incarnata* L. (dried flowering tops, lot n. 60698), and *Silybum marianum* (L.) Gaertn. (dried fruits, lot n. 56313). The flavonoid content of the drugs was in accord with the European and/or the Italian Pharmacopoeias [8, 9].

Sample Preparation

Tinctures were obtained according to methods described in the European Pharmacopoeia [1] – dried plant material (200 g) was macerated with aqueous solutions of ethanol (40 and 60% (*v/v*); 1000 mL).

TLC Analysis

Analytical TLC of the tinctures was performed on 20 cm × 20 cm glass plates precoated with silica gel 60 F_{254} (Merck, Darmstadt, Germany). TLC of calendula and passion flower tinctures was performed with AcOEt-HCOOH-AcOH-H_2O, 100:11:11:26, as mobile phase, that of milk-thistle tincture with $CHCl_3$-acetone-HCOOH, 75:16.5:8.5. NTS/PEG at 365 nm was used for detection [10].

Instrumentation for HPLC-DAD Analysis

HPLC was performed with a Hewlett-Packard (HP; Palo Alto, CA, USA) 1090L instrument with diode-array detector; the equipment was controlled by means of an HP 9000 workstation. Compounds were separated on a 250 mm × 4 mm i. d., 5 μm particle, LiChrosorb RP18 column (Merck) maintained at 26 °C and equipped with a LiChrosorb RP18 precolumn (10 mm × 4 mm i. d.; 5 μm particle; Merck). The mobile phase was a four-step, 40-min, linear gradient prepared from CH_3CN and water (Table I) and containing H_3PO_4 (pH 3.0); the flow rate was 1.3 mL min^{-1}. Before analysis tinctures of calendula flower, milk-thistle fruit, and passion flower were filtered through a cartridge-type sample filtration unit with polytetrafluoroethylene (PTFE) membrane. The volume of sample solution injected was 25 μL. 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 passion flower tinctures and at 280 nm for milk-thistle tinctures. Typical chromatograms obtained from the tinctures are reported in Figures 1–3.

Table I. Composition of the mobile phase used for the HPLC-DAD analysis.

Time (min)	% H_2O	% CH_3CN	Flow (mL min^{-1})
0.10	88.0	12.0	1.30
10.00	82.0	18.0	1.30
15.00	82.0	18.0	1.30
30.00	55.0	45.0	1.30
35.00	0.0	100.0	1.30
42.00	0.0	100.0	1.30
50.00	88.0	12.0	1.30

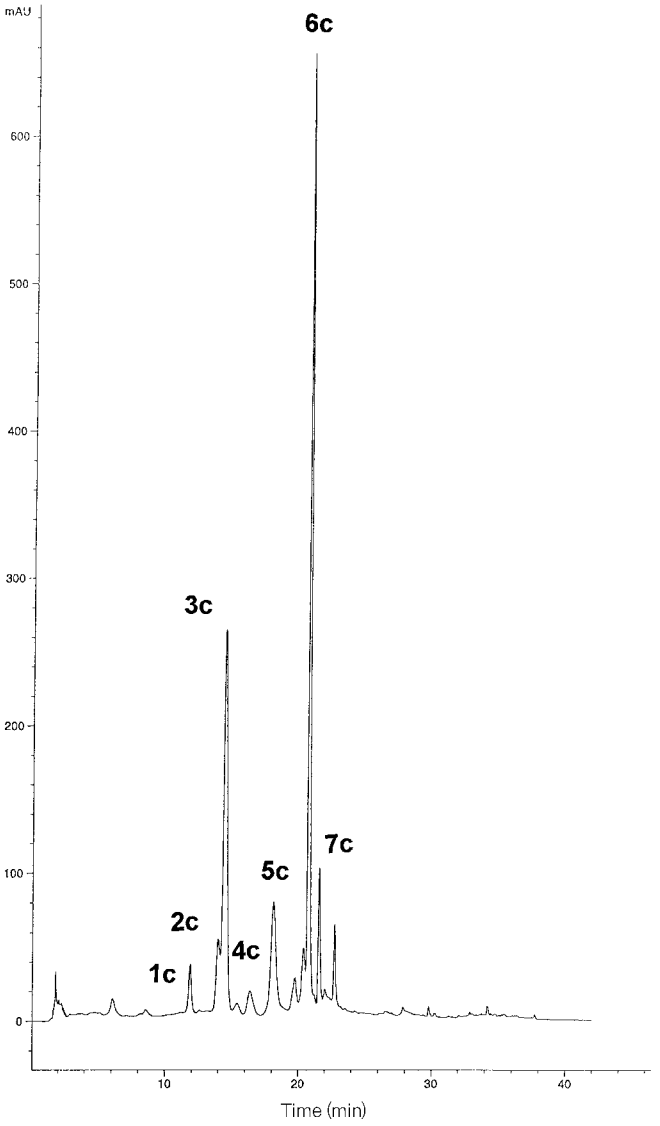

Figure 1. Profile of calendula tincture (60% *v/v*, ethanol) with the HPLC-MS attributions of the components detected. **1c** = quercetin-3-*O*-rutinosylrhamnoside, **2c** = rutin, **3c** = isorhamnetin-3-*O*-rutinosylrhamnoside, **4c** = isoquercitrin, **5c** = isorhamnetin-3-*O*-glucosylglucoside, **6c** = narcissin, **7c** = isorhamnetin-3-*O*-glucoside.

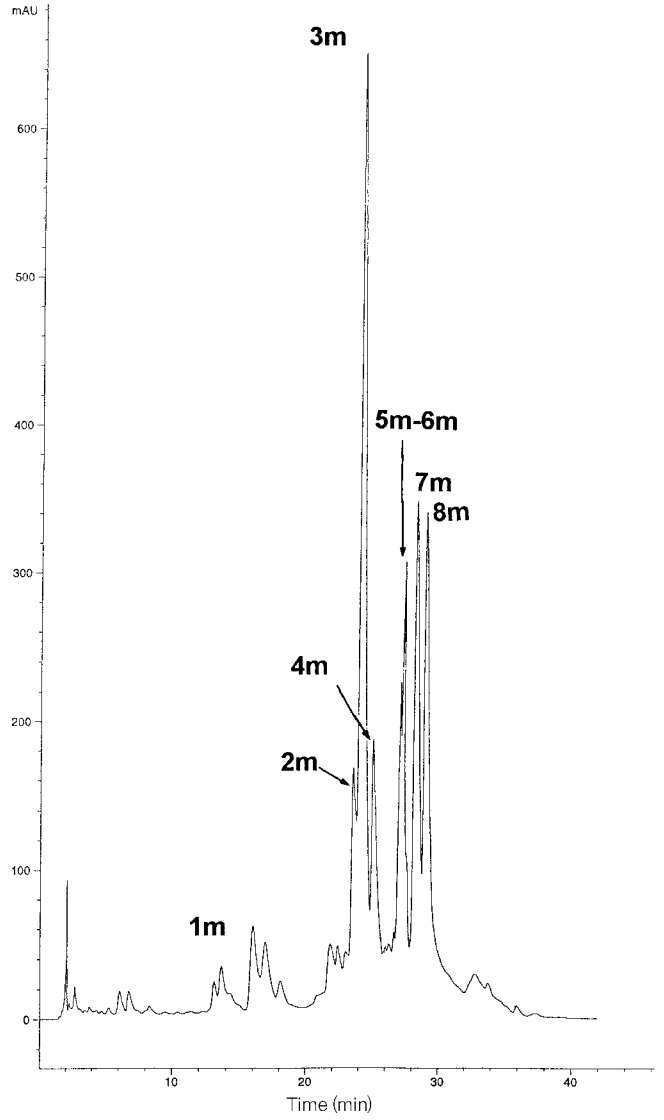

Figure 2. Profile of milk-thistle tincture (60% *v/v*, ethanol) with the HPLC-MS attributions of the components detected. **1m** = taxifolin, **2m** = silydianin, **3m** = silychristin, **4m** = oxy derivative of silybin/isosilybin isomers, **5m** and **6m** = 2,3-dehydro derivatives of silybin/isosilybin isomers, **7m** = silybin, **8m** = isosilybin.

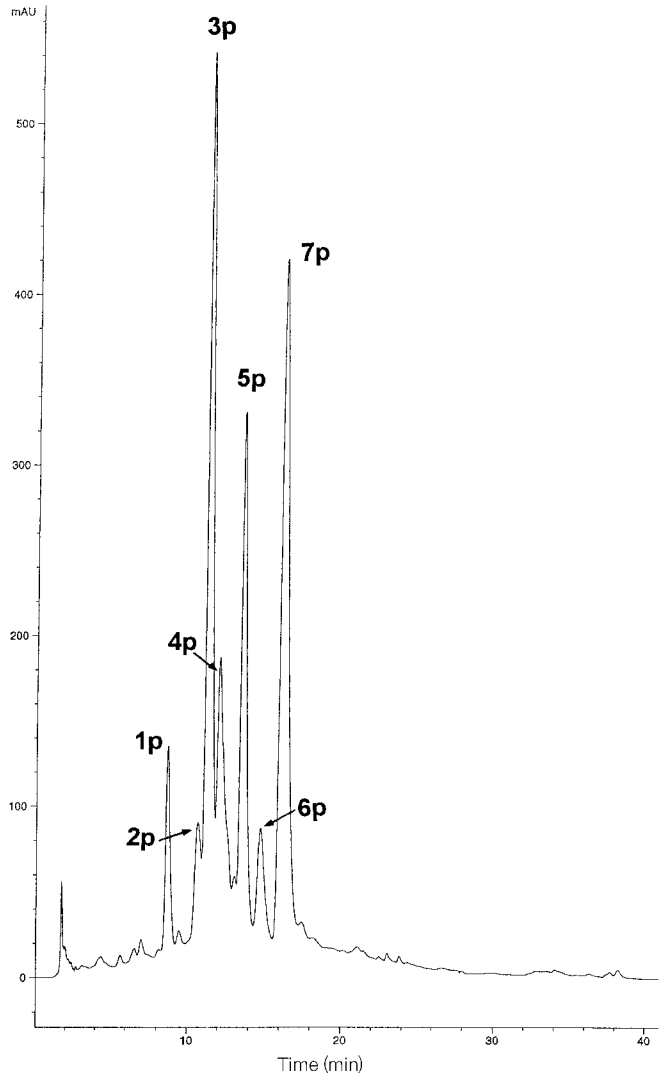

Figure 3. Profile of passion flower tincture (60% *v/v*, ethanol) with the HPLC-MS attributions of the components detected. **1p** = 6,8-diC-glucosylapigenin, **2p** = isoschaftoside, **3p** = schaftoside, **4p** = homoorientin, **5p** = isovitexin-2″-*O*-glucoside, **6p** = vitexin.

Instrumentation for HPLC-MS Analysis

The HPLC system described above was interfaced with an HP 1100 atmospheric pressure ionization (API)-electrospray mass-selective detector (MSD). The interface geometry, orthogonal positioning of the nebulizer relative to the capillary inlet, enabled the use of analytical conditions similar to those used for HPLC-DAD analysis. The conditions used for mass spectrometry (gas temperature 350 °C at a flow rate of 10 L min^{-1}, nebulizer pressure 30 psig, quadrupole temperature 30 °C, and capillary voltage 3500 V) were optimized to achieve the maximum sensitivity of ESI values. The column, time period, and flow rate used were the same as those described above, except that because phosphoric acid was not suitable for HPLC-MS operation, separation was performed with aqueous formic acid (pH 3.0), without appreciable variation of the chromatographic profile. Full scan spectra from *m/z* 100 to 800 were obtained in positive-ion mode; the scan time was 1 s. The volume of sample solution injected was 25 µL.

Identification of Constituents and Peak Purity

Identification of all constituents was performed by HPLC-MS analysis and/or by comparison of the retention times of peaks in the extracts with those of the authentic reference samples. Peak purity was checked by examination the mass spectra and/or by use of HPLC with diode-array detection (UV spectra of the peaks were compared with those of authentic reference samples).

Linearity, Repeatability, and Reproducibility

The linearity range of instrument response was determined by triplicate analysis of each of five different concentrations. Calibration graphs for HPLC were plotted for amounts of sample ranging from 0.10 to

Table II. Flavonoids identified in the tinctures.

No.	Constituent	t_R (min)	UV data (nm)	MS data (*m/z*)	Ref.
1c	Quercetin-3-rutrham	11.7	258, 300(sh), 360	779, 757, 611, 465, 303	10
2c	Rutin	13.6	257, 298(sh), 358	633, 611, 465, 303	
3c	Isorhamnetin-3-rutrham	14.3	257, 271(sh), 300(sh), 354	791, 769, 625, 479, 317	10
4c	Isoquercitrin	15.7	257, 267(sh), 295(sh), 358	487, 465, 303	
5c	Isorhamnetin-3-glcglc	17.4	259, 270(sh), 300(sh), 356	663, 641, 479, 317	12,13
6c	Narcissin	20.7	256, 270(sh), 300(sh), 355	647, 625, 465, 317	
7c	Isorhamnetin-3-glc	21.4	254, 270(sh), 300(sh), 355	501, 479, 317	
1m	Taxifolin	13.7	290, 340(sh)	327, 305	
2m	Silydianin	23.5	290	505, 483, 465, 453, 447, 437, 435, 419.	10
3m	Silychristin	24.1	290	505, 483, 465, 453, 447, 437, 435, 419.	10
4m	Oxy derivative	25.0	290	521, 499, 481, 463, 435	
5m, **6m**	2,3-Dehydro derivatives silybin/isosilybin	27.3, 27.6	290	503, 481	10
7m	Silybin	28.2	290	505, 483, 465, 453, 447, 437, 435, 419	
8m	Isosilybin	28.9	290	505, 483, 465, 453, 447, 437, 435, 419	10
1p	Vicinin-2	8.5	274, 334	617, 595, 577, 559, 541, 529, 523, 499, 475, 457	11
2p	Isoschaftoside	11.1	274, 334	587, 565, 547, 529, 511, 499, 469, 445, 427	10
3p	Schaftoside	11.3	274, 334	587, 565, 547, 529, 511, 499, 469, 445, 427	10
4p	Homoorientin	12.0	255, 267, 293(sh), 346	471, 449, 431, 413, 395	
5p	Isovitexin-2″-glc	13.5	271, 334	617, 595, 433	10
6p	Vitexin	14.1	270, 303(sh), 335.	433, 415	
7p	Isovitexin	16.1	271, 334	455, 433, 415, 397, 379	

2.5 μg ($r > 0.99$). To evaluate repeatability six samples from the same batch of each tincture were analyzed by HPLC and the amount of each constituent was determined to calculate the relative standard deviation. To evaluate the reproducibility of integration standard solutions of rutin (2.5 μg/25 μL) and of each sample of tincture were injected six times and relative standard deviations were calculated.

Quantitation of Flavonoids

All the tinctures were analyzed in triplicate and a calibration graph with six data points was constructed by use of an external standard. The amounts of constituents were calculated taking into account the mean of the response factor of rutin in the reference solutions, i. e. area/concentration$_{(mg/mL)}$ × purity/100, and the response factor of the constituent relative to rutin (RRF). This value was determined by calculating the ratio of the average response factor for each compound to the average response factor for rutin at 350 or 280 nm. All flavanolignans were evaluated as silybin, isorhamnetin diglycosides and isorhamnetin triglycosides were evaluated as narcissin, apigenin-6,8-diC-glycosides were evaluated as isovitexin, and quercetin triglycoside was evaluated as rutin.

Results

The simple reversed-phase HPLC method used optimized the separation of all classes of flavonoid (flavones, flavonols, flavanonols, and flavanolignans). Rutin was used as external standard. Calibration plots with good linearity were obtained between 0.1 and 2.5 μg ($r > 0.99$) and the repeatability and reproducibility of the method were satisfactory. The relative standard deviations obtained from investigation of the repeatability of the method were: homoorientin 2.00%, hyperoside 2.41%, isoquercitrin 2.21%, isorhamnetin 2.58%, isorhamnetin-3-*O*-glucoside 2.32%, isorhamnetin-3-*O*-rutinoside 1.96%, isovitexin 1.73%, quercetin 2.08%, rutin 1.98%, silybin 2.33%, taxifolin 2.19%, and vitexin 2.26%. The relative standard deviations obtained from investigation of the reproducibility of the method were: homoorientin 1.00%, hyperoside 1.11%, isoquercitrin 1.08%, isorhamnetin 1.20%, isorhamnetin-3-*O*-glucoside 0.98%, isorhamnetin-3-*O*-rutinoside 0.99%, isovitexin 0.73%, quercetin 1.01%, rutin 0.85%, silybin 0.96%, taxifolin 1.33%, and vitexin 1.06%.

UV detection was fixed at 350 nm for the calendula flower and passion flower tinctures and at 280 nm for milk-thistle fruit tincture, because the absorbance of all the constituents of the preparations was appreciable at these wavelengths. TLC profiles and chromatograms obtained from herbal drug tinctures containing 40% and 60% (*v/v*) ethanol were similar qualitatively, but not quantitatively.

The flavonoids identified in the tinctures are listed in Table II. Our findings confirmed the presence of the flavonoids reported in the literature and of others not previously reported. Compounds **2c**, **4c**, **6c**, **7c**, **1m**, **7m**, **4p**, **6p**, and **7p** were identified by comparing their t_R values and their UV-Vis and mass spectra with those of the corresponding pure standards. The other constituents were identified by comparison of UV-Vis and mass spectral data with those obtained from standards.

The structures of derivatives **1c**, **3c**, and **5c** were determined mainly by analysis of the fragmentation of quasi-molecular peaks that indicated consecutive loss of two deoxyhexose and one hexose moieties for **1c** and **3c**, and two hexose moieties for **5c**. These data led also to identification of the corresponding aglycone moieties.

Peak **8m** was tentatively identified as isosilybin, because of its similar t_R and the prominent fragments in the mass spectrum (the same ions in the region between *m/z* 400 and 200 i. e. *m/z* 283, 271, 259 which were different from those of silychristin and silydianin). Peak **4m** afforded $[M + H]^+$ and $[M + Na]^+$ ions at *m/z* 499 and 521, respectively. These data indicated that **4m** contained an additional oxygen compared with the flavanolignans **2m**, or **3m**, or **7m** or **8m**; this was also apparent from analysis of the fragmentation pattern of ions between *m/z* 500 and 400, i. e. $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$, and $[M + H - 2H_2O - 2CH_2]^+$ at *m/z* 481, 463, and 435, respectively. In addition, the similarities of prominent fragments in the region between *m/z* 400 and 200 with those of silybin/isosilybin isomers, led to the proposal that **4m** was a derivative of silybin/isosilybin isomers. This milk-this-

Table III. List and amounts (mg/100 mL; means ± SD of results from three replicate analyses) of flavonols present in calendula tinctures.

	60% *v/v* tincture	40% *v/v* tincture
Quercetin-3-rutglc (**1c**)	2.1 ± 0.08	1.8 ± 0.09
Rutin (**2c**)	4.7 ± 0.23	4.0 ± 0.29
Isorhamnetin-3-rutglc (**3c**)	16.6 ± 0.52	18.6 ± 0.80
Isoquercitrin (**4c**)	2.0 ± 0.06	1.6 ± 0.10
Isorhamnetin-3-glcglc (**5c**)	7.1 ± 0.60	9.4 ± 0.64
Narcissin (**6c**)	21.4 ± 0.72	20.4 ± 0.86
Isorhamnetin-3-glc (**7c**)	3.2 ± 0.11	2.6 ± 0.13
Total flavonols	57.1	58.4

Table IV. List and amounts (mg/100 mL; means ± SD of results from three replicate analyses) of flavolignans present in milk-thistle tinctures.

	60% *v/v* tincture	40% *v/v* tincture
Taxifolin (**1m**)	5.5 ± 0.21	2.9 ± 0.17
Silydianin (**2m**)	10.3 ± 0.33	4.8 ± 0.28
Silychristin (**3m**)	52.5 ± 1.06	31.5 ± 0.99
Oxy derivative (**4m**)	18.8 ± 0.84	9.6 ± 0.60
2,3-Dehydro derivatives (**5m**, **6m**)	19.9 ± 0.65	13.8 ± 0.55
Silybin (**7m**)	39.4 ± 0.90	13.5 ± 0.46
Isosilybin (**8m**)	29.4 ± 0.79	15.4 ± 0.70
Total flavanolignans	170.3	88.6

Table V. List and amounts (mg/100 mL; means ± SD of results from three replicate analyses) of flavones present in passion flower tinctures.

	60% *v/v* tincture	40% *v/v* tincture
Vicinin-2 (**1p**)	17.0 ± 0.58	14.2 ± 1.01
Schaftoside/Isoschaftoside (**2p**)	59.6 ± 1.48	47.5 ± 1.56
Homoorientin (**3p**)	25.6 ± 0.89	21.7 ± 1.21
Isovitexin-2″-glc (**4p**)	36.1 ± 1.14	26.5 ± 1.02
Vitexin (**5**)	18.3 ± 0.89	15.0 ± 0.97
Isovitexin (**6p**)	58.9 ± 1.45	39.8 ± 1.23
Total C-glycosylflavones	215.5	164.7

tle metabolite has not previously been described in the literature; its isolation and full identification are currently in progress.

The 6,8-di-*C*-glycosides of the apigenin moieties **2p** and **3p** were suggested by the absence from their spectra of the fragment ions $[M + H - 162]^+$, $[M + H - 146]^+$, or $[M + H - (162 + 146)]^+$ at *m/z* 403, 419, and 257; the identities were confirmed by the presence of the typical fragment ions $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$, and $[M + H - 3H_2O]^+$, 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 observed at *m/z* 499, 469, 445, and 427. The absence of the characteristic $[M + H - 162]^+$ ion at *m/z* 433, (corresponding to the isovitexin) suggested that peak **1p** was a 6,8-di-*C*-glucosides of apigenin. This suggestion was confirmed by the typical fragment ions $[M + H - H_2O]^+$, $[M + H - 2H_2O]^+$, $[M + H - 3H_2O]^+$, and $[M + H - 4H_2O]^+$ present at *m/z* 577, 559, 541, and 523. 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 observed at *m/z* 529, 499, 475, and 457. From these data, peak **1p** was tentatively identified as 6,8-di-*C*-glucosylapigenin (vicenin-2), a known metabolite [11] not previously isolated from *Passiflora incarnata* L. The amounts of all the constituents of the 40% and 60% (*v/v*) tinctures are reported in Tables III–V.

Discussion

Tinctures are usually considered one of the best liquid forms of plant extracts, because of the greater stability of the constituents and lower microbiological contamination. This paper reports our first results from HPLC analysis of the content of tinctures containing 40 and 60% *v/v* ethanol. A direct, rapid, and robust RP-HPLC assay was developed and validated. This method not only afforded acceptable accuracy, specificity, reproducibility, and speed, and good separation of all the classes of flavonoids (including flavones, flavonols, flavanonols, and flavanolignans) but also easily and satisfactorily separated their *O*- and *C*-derivatives and mono-, di-, and triglycosides. This method can thus be recommended for analysis of other herbal drugs or drug preparations containing flavonoids as the active constituents or markers. Seven flavonols were identified from calendula tinctures, eight flavanolignans from milk-thistle tinctures, and seven flavones from passion flower preparations. Among these, isorhamnetin-3-*O*-glucosyl glucoside and vicenin-2 were identified for the first time in calendula and passion flower tinctures, respectively. A new constituent of milk-thistle tinctures, an oxy-derivative of silybin/isosilybin isomers, not previously isolated in nature, was also found.

Although the chromatographic flavonoid profiles obtained from tinctures of each herbal drug containing 40 and 60% ethanol were qualitatively similar, the quantitative flavonoid content was very different. The flavonoid content of 60% *v/v* tinctures was usually higher.

The flavonol content of the 60% *v/v* calendula tincture was 57.1 mg/100 mL tincture, equivalent to 72.5% of the nominal value of the herbal drug. This value was quite similar to that of the 40% *v/v* calendula tincture, 74.1%, although there were some differences between the amounts of the individual constituents. From these data it is apparent that the composition of the solvent mixture had little effect on the quali-quantitatite profile of the two calendula tinctures.

The flavanolignan content of the 60% *v/v* milk-thistle tincture was 175 mg/100 mL tincture, equivalent to ca 88% of the nominal value of the herbal drug. Because the suggested daily dose of silymarin, calculated as silybin, is 200–400 mg, these data suggest that 100–200 mL tincture should be taken daily to have pharmacological effect. The 40% *v/v* milk-thistle tincture contains only 88.6 mg flavanolignans/100 mL tincture, equivalent to only 44% of the nominal value of the herbal drug; this would correspond to 200–400 mL tincture daily. These dosages are, however, considered unacceptable, so tinctures of milk-thistle are unsuitable as a herbal drug preparation because of their very low flavanolignan content.

The *C*-glycosylflavone content of the 60% *v/v* passion flower tincture was

1.078 g/100 mL tincture, equivalent to ca 77% of the nominal value of the herbal drug. The *C*-glycosylflavone content of the 40% (*v*/*v*) passion flower tincture was 823.5 mg/100 mL tincture, equivalent to ca 59% of the nominal value of the herbal drug.

Although tinctures are usually regarded as among the most versatile herbal extracts, and are among the most widely employed, our data suggest they cannot be regarded as acceptable herbal drug preparations or herbal medicinal products because the amount of active constituent is rarely sufficient to afford an active dose. The alcohol content of the solvent used in the extraction can also severely affect the phytocomplex, again reducing the level of the active constituent to below the active dose.

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