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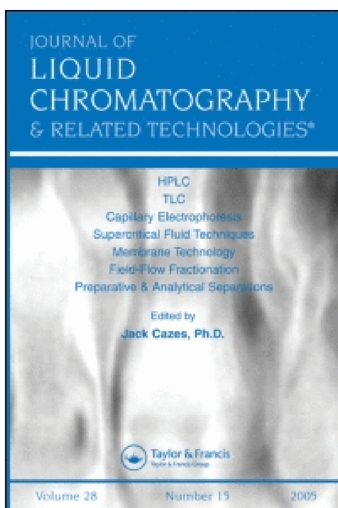

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IDENTIFICATION AND SIMULTANEOUS ANALYSIS OF HARMANE, HARMINE, HARMOL, ISOVITEXIN, AND VITEXIN IN *PASSIFLORA INCARNATA* EXTRACTS WITH A NOVEL HPLC METHOD

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ABSTRACT

A high performance liquid chromatographic method for the simultaneous analysis of two flavonoids (iso-vitexin and vitexin), and three indole alkaloids (harmane, harmine, and harmol) was developed. This method was then utilised to quantitate levels of these five constituents in methanolic extracts of Australian *Passiflora incarnata*.

HPLC analysis was performed using a Waters™ Novapak C_{18} (150 x 4 mm, 4 μm) column, with a gradient solvent system of methanol-water-acetic acid. Detection was achieved by PDA UV (254 nm) and fluorescence (excitation 254 nm, emission 414 nm), utilising the external standard method to obtain quantification.

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INTRODUCTION

Passiflora incarnata has widespread use as a sedative and tranquilizer.(1-3) It is used as an infusion or in the form of extracts, and often in combination with other sedative drugs. *Passiflora incarnata* is characterised by the presence of flavonoids (0.25%), principally C-glycosides of the flavones, apigenin and luteolin, small amounts of cyanogenic glycosides, traces of harmane alkaloids (0.1%), maltol and ethyl maltol (0.05%), and chlorogenic acid.(4,5) . The identity of the pharmacologically active constituent/s is still uncertain, although both the flavonoids and the harmane alkaloids have been considered to be responsible for the observed activity.(6,7)

Qualitative and quantitative analysis of the flavonoids has, to date, been particularly important because they allow identification of several species of *Passiflora*. Two flavonoid derivatives that are typical of *Passiflora incarnata*, are vitexin and isovitexin. Vitexin is known to be a potent inhibitor of thyroid peroxidase,(8) and also to inhibit fast reacting fibres (Na^+-dependent).(9) These two flavonoids have been used as reference constituents for analytical purposes to assay the 'quality' of the crude drug material. In addition to the flavonoids, the harmane alkaloids reportedly produce hallucinatory,(10) inhibition of monoamine oxidase,(11) stimulatory,(12) anthelminthic,(13) antitrypano-somal,(14) and antileishmanial(15) activity. In regard to this reported activity, some researchers suggest(7) that the harmane alkaloids should be regarded as the principal active constituents in *Passiflora incarnata*.

The Swiss Pharmacopoeia (Ph. Helv. VII)(16) previously required a minimal content of 0.01% of harman alkaloids due to this perceived therapeutic acitivity. However, harmane, and norharmane have, in more recent times, been shown to induce tumours in animals, and may also be potential human carcinogen promoters.(17,18) At low concentrations, harmine acts as an inhibitor of the Na^+, K^+-ATPase system.(19) As a direct consequence of this, the levels of harmane, norharmane, harmine, and harmaline should be as low as possible in *Passiflora incarnata* preparations. The French Pharmacopoeia X has set down a limit of 0.8 μg/g harmane in dried plant material, and the Monographie der Kommission E des Bundesgesund-heitsamtes Berlin (1985, 1990) limits the concentration to 0.01%.(7)

The aim of this work was to provide a selective, sensitive, and validated HPLC method for the simultaneous determination of vitexin, iso-vitexin, harmane, harmine, and harmol levels in *Passiflora incarnata* extracts. The developed method was then utilised to study the levels of these constituents in methanolic extracts of dried *Passiflora incarnata* samples that were available to the Australian consumer in 1997-1999.

A number of HPLC methods exist for the separate analysis of the flavonoids (primarily vitexin and iso-vitexin,(20-24) and the harman alka-

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loids.(7,25) However, no methods are available for the simultaneous determination of both these pharmacologically active classes of constituents.

EXPERIMENTAL

Chemicals and Solvents

Methanol was fractionally distilled through glass helices to be of HPLC quality, acetonitrile (HPLC grade), was obtained from BDH Chemicals. Acetic acid was obtained from Ajax Chemicals. Vitexin and isovitexin were purchased from Carl Roth GmbH, while harmane, harmine, and harmol were purchased from Sigma-Aldrich Chemical Co. A UV detection stock standard was made up of iso-vitexin, vitexin, harmol, harmane, and harmine. Working standards were then prepared from this stock solution over the range 0.2 – 60 μg/mL by serial dilution with methanol. A fluorescence stock standard solution was prepared

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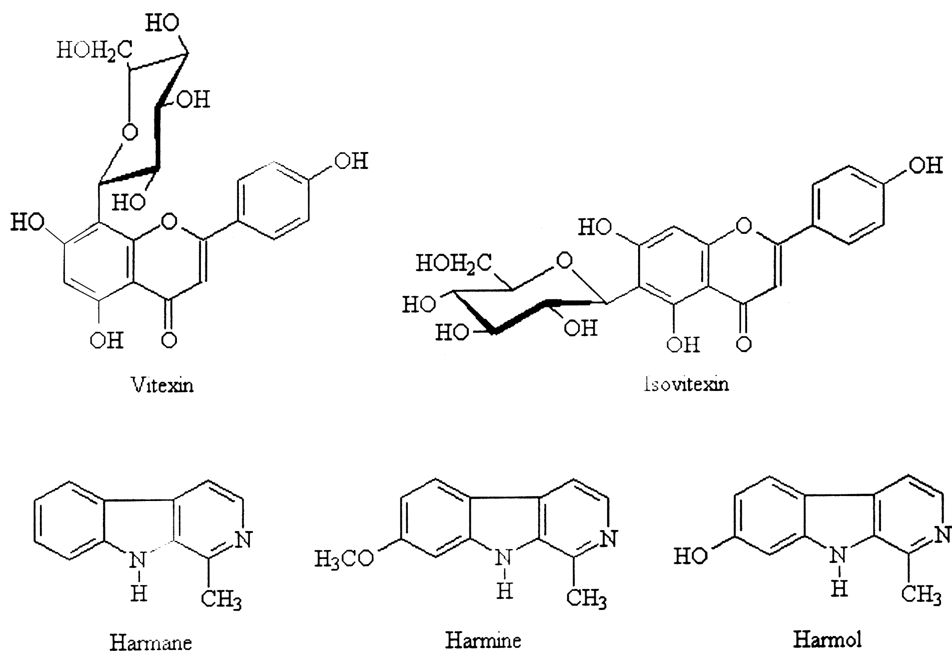

Figure 1. Chemical structures of the two flavonoids and the three harmane alkaloids analysed in the developed HPLC method.

from harmol, harmane, and harmine. PDA-UV and fluorescence detection was calibrated over the appropriate ranges by injection of stock solutions.

HPLC-UV/Fluorescence System

The Waters (Millipore, Milford, MA, USA) HPLC system comprised a 717 autosampler, 600 pump and controller, 996 PDA UV detector, 474 Scanning Fluorescence detector, and Millenium 2010 software. The system was fitted with a Waters Nova-pak® RP-C_{18} (4 µm) 150 x 3.9 mm column coupled to a Waters guard-pak fitted with Nova-pak® C_{18} insert. The system operated at ambient temperature.

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Medicinal Plants

Three major Australian wholesale suppliers of medicinal plants supplied 9 different samples of *Passiflora incarnata* for this study. All samples were stored in a dessicator within an air-conditioned laboratory at 22°C prior to analysis.

Sample Extraction

Dried *Passiflora incarnata* samples (4.0 g, except for sample No. 4 where 1.0 g was used), were subjected to maceration and blending to yield a fine powder. The powdered material was then exhaustively extracted in a soxhlet apparatus with 100 mL of methanol for 6 hours. The extract was then evaporated to dryness, followed by resuspension of the residue in 6.0 mL of methanol. A 100 µL aliquot of this sample was removed and loaded onto a C_{18} maxi-clean (300 mg) cartridge. The methanol was removed by passing nitrogen gas over the cartridge. 10 mL of 85:15 methanol:water was then passed through the cartridge to elute the constituents of interest. The eluent was then passed through a 0.22 µm filter prior to injection onto the HPLC column.

HPLC-UV/Fluorescence Analysis

The initial mobile-phase flow rate was 1.0 mL/min, with UV detection at 254 nm, and fluorescence excitation at 254 nm and emission at 414 nm. The initial solvent ratio was 60:30:10 water:methanol: 5% Acetic acid, with a linear ramp to 45:45:10 over 10 minutes, followed by a linear ramp to 0:95:5 at 30 minutes (flow rate ramp to 1.3 mL/min), followed by a linear ramp to 0:100:0 (1.3

mL/min) over 2 minutes, then a 5 minute hold, followed by a return to initial conditions over 10 minutes (60:30:10, 1.0 mL/min). An equilibration period was employed between runs. Quantitation was obtained by substitution of peak areas of samples into calibration equations generated from external standards. Standards were injected prior to, and after, each duplicate analysis for samples being analysed.

RESULTS AND DISCUSSION

Vitexin, iso-vitexin, harmol, harmane, and harmine were initially all detected using UV-PDA detection. A PDA-UV chromatogram of a standard solution of iso-vitexin, vitexin, harmol, harmane, and harmine at 254 nm is shown in Figure 2, along with elution times from the C_{18} HPLC column. The linearity of the method was validated by injection of the standards over the ranges indicated in the methods section. From the calibration results, the following software-generated equations for the lines of best fit and r^2 values were generated. Equations were (in order of elution from the HPLC column, Y- response variable, and X- amount of standard in μg):

PDA-UV Detection

Isovitexin	$Y = 3.0 \times 10^6X - 2.76 \times 10^4$ ($r^2 = 0.999$)
Vitexin	$Y = 3.0 \times 10^6X - 2.08 \times 10^4$ ($r^2 = 0.999$)
Harmol	$Y = 3.0 \times 10^6X + 3.21 \times 10^3$ ($r^2 = 1.000$)
Harmane	$Y = 3.0 \times 10^6X + 3.21 \times 10^3$ ($r^2 = 1.000$)
Harmine	$Y = 2.0 \times 10^6X + 3.64 \times 10^3$ ($r^2 = 0.999$)

The limit of detection using PDA-UV detection was defined as the concentration of analyte that yields a signal to noise ratio of at least 3:1. These values were calculated from recovery experiments (see Table 1). While the limits of UV detection for harmol, harmane, and harmine in our HPLC system were able to provide detection to levels below those set down for harmane by The French Pharmacopoeia X, and the Monographie der Kommission E des Bundesgesundheitsamtes, these limits were still above those levels determined to be present in our samples under analysis. Therefore, to obtain quantitation of harmol, harmane, and harmine below these levels (at pg levels) we included fluorescence detection in our method.

A fluorescence chromatogram of a standard solution of harmol, harmane, and harmine (excitation: 254 nm, emission 414 nm) is shown in Figure 3. From recovery experiments, the limits of fluorescence detection were determined to be:

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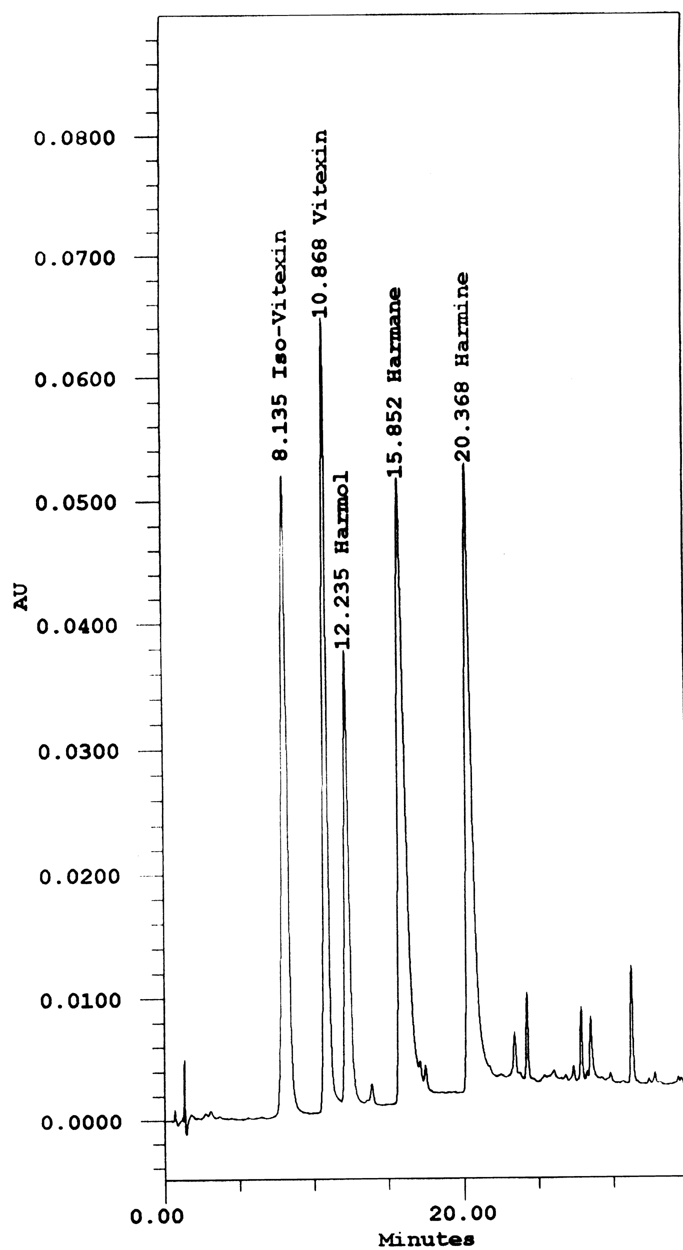

Figure 2. PDA-UV chromatogram of a standard solution of iso-vitexin (0.41 μg), vitexin (0.40 μg), harmol (0.26 μg), harmane (0.59 μg), and harmine (0.78 μg). Wavelength: 254 nm; Injection volume: 10 μL. Conditions as per experimental section.

Table 1. Recovery of Iso-Vitexin, Vitexin, Harmol, Harmane, and Harmine from Spiked *Passiflora incarnata* Samples

Standard	Amount[a] (μg)	% Recovery (Mean)	S.D.	n	Limit of Detection[b] (ng/mL)	Limit of Detection[c] (ng/mL)
Iso-vitexin	500	101	6.7	5	13.0	n.d.
Vitexin	490	121	6.6	5	10.0	n.d.
Harmol	770	78	5.4	5	2.0	0.020
Harmane	650	92	5.1	5	2.0	0.038
Harmine	780	81	3.4	5	3.0	0.008

[a]Amount of standard control *Passiflora incarnata* sample was spiked with for recovery experiments.
S.D. Standard deviation.
[b]Limit of PDA-UV detection, defined as concentration of analyte that yields a signal to noise ratio of 1:3 at 254 nm.
[c]Limit of fluorescence detection, defined as concentration of analyte that yields a signal to noise ratio of 1:3 (excitation 254 nm, emission 414 nm).
n.d. -not detected.

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harmol 20 pg/mL, harmane 38 pg/mL, and harmine 8 pg/mL (see Table 1). The limit of detection using fluorescence detection was again defined as the concentration of analyte that yields a signal to noise ratio of at least 3:1. The linearity of the fluorescence detection method is indicated below with calibration equations and r^2 values:

Fluorescence Detection

Harmane $Y = 1.0 \times 10^{10}X - 3.48 \times 10^{5}$ ($r^2 = 1.000$)
Harmine $Y = 6.0 \times 10^{9}X - 2.05 \times 10^{4}$ ($r^2 = 0.999$)
Harmol $Y = 3.0 \times 10^{9}X - 2.26 \times 10^{4}$ ($r^2 = 1.000$)

The assay was further validated by determining the recovery of iso-vitexin, vitexin, harmol, harmane, and harmine by UV detection, following the spiking of dry *Passiflora incarnata* plant material (1.0 g) with iso-vitexin (500 μg), vitexin (490 μg), harmol (770 μg), harmane (650 μg), and harmine (780 μg), then submitting samples to standard extraction and clean-up procedures. Overall, the mean recoveries were 101% (iso-vitexin), 121% (vitexin), 78% (harmol), 92% (harmane), and 81% (harmine) (see Table 2).

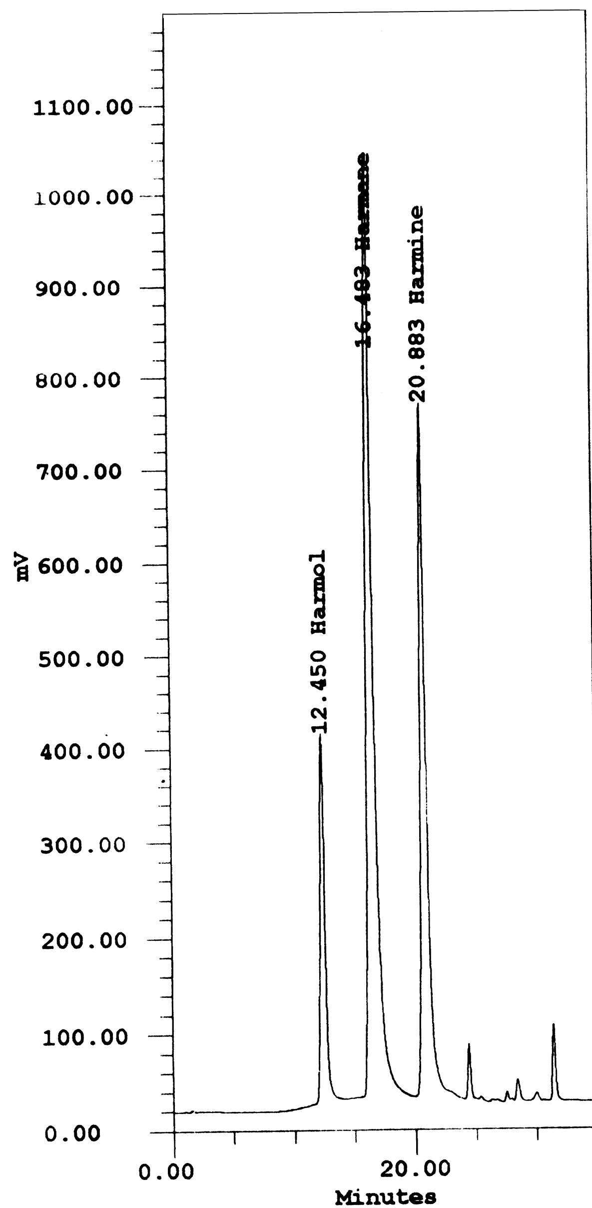

Figure 3. Fluorescence chromatogram of a standard solution of harmol (3.3 ng), harmane (4.1 ng), and harmine (3.4 ng). Excitation: 254 nm; Emission: 414 nm. Injection volume: 10 μL. Conditions as per experimental section.

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Table 2. Analytical Results[a,b] for Levels of Harmol, Harmane, and Harmine in Methanolic Extracts of *Passiflora incarnata*

Sample Number	Iso-Vitexin (μg/g)	Vitexin (μg/g)	Harmol (μg/g)	Harmane (μg/g)	Harmine (μg/g)
1	13.6	169.8	n.d.	0.009	n.d.
2	40.3	194.1	n.d.	0.015	n.d.
3	6.59	97.4	n.d.	0.018	0.005
4	6.62	16.3	n.d.	0.019	0.005
5	8.51	176.9	n.d.	0.018	0.026
6	316.9	1426.4	n.d.	0.112	0.052
7	439.7	2301.5	n.d.	n.d.	0.139
8	n.d.	179.0	n.d.	n.d.	0.271
9	122.9	1421.6	n.d.	n.d.	0.121

[a]Mean values for duplicate analyses.
[b]Micro grams of constituent per gram of dry plant material.
n.d. Not detected above limits of detection.

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In line with the French Pharmacopoeia X level set down for harmane of 0.8 μg/g of dry plant material, none of the samples analysed had levels of harmane exceeding this suggested limit. Harmol was undetected in the samples analysed, and while levels of harmane were reasonably consistent across the samples analysed, levels of harmine varied quite widely. This may not be that unexpected, as levels of harmane alkaloids are reported to vary depending on the stage of development of the plants when harvested.(5) No information was provided by the suppliers of the plant material as to the harvest date of each batch. Five out of the nine samples analysed had harmine levels higher than harmane levels. These levels were below the level of 0.8 μg/g set down for harmane, and therefore, it seems reasonable that these samples, which were commercially available to the Australian public, would not have been of concern at least in regard to these constituents.

CONCLUSIONS

A simple and sensitive HPLC method based on UV and fluorescence detection has been established and optimized. The method possesses acceptable analytical characteristics with respect to recovery and sensitivity. All samples analysed were found to have levels of harmane below that set down by French Pharmacopoeia X of 0.8 μg per g of dry plant material. The method offers the potential to determine levels of recognised therapeutic ‘agents’, along with

potentially harmful 'actives' present in *Passiflora incarnata* samples. The method is, therefore, of particular use in establishing the 'quality' of *Passiflora incarnata* samples and preparations in general.

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