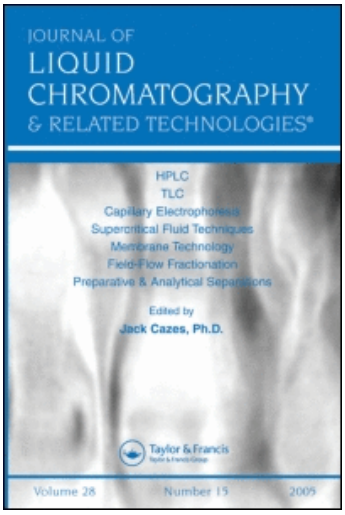

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QUANTITATIVE DETERMINATION OF P-COUMARIC ACID IN ECHINACEA PURPUREA PRESS JUICE AND URGENIN. A VALIDATED METHOD

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ABSTRACT

Echinacea purpurea species have been studied by various authors over the last decade. The starting material was the fresh or dried plant, or an ethanolic extract in which cichoric acid and derivatives were one of the major constituent groups. In our search for a suitable marker for the Echinacea press juice we found that cichoric acid was not the major compound but p-coumaric acid. The amount of p-coumaric acid was therefore determined in Echinacea press juice and Urgenin® and the method was validated.

INTRODUCTION

Urgenin®, which is used for benign prostate hyperplasia, consists of 35% Echinacea press juice and 65% Sabal serrulata extract. E. purpurea

has immunostimulating properties (1) and S. serrulata exhibits a specific anti-androgenic activity by inhibition of 5 α-reductase (2).

In order to standardize the plant preparation and investigate its stability a suitable marker had to be found in the Echinacea press juice and in Urgenin®.

The Echinacea press juice has been studied by various authors over the last decade (3,4,5). The starting material was the fresh or dried plant or an ethanolic extract in which cichoric acid and derivatives were one of the major constituents. In the Echinacea press juice, however,no cichoric acid nor its analogues seemed to be present. Therefore we isolated the main component from the press juice and identified it by NMR and MS as p-coumaric acid.

We developed an analytical HPLC method to quantify p-coumaric acid in the press juice and in the drug Urgenin®. Prior to use, the method was validated.

EXPERIMENTAL

Apparatus

A Hewlett Packard liquid chromatograph, which was equipped with a 20 µl sample loop (manual injection), a HP 1050 series pump and a HP 1040M Series II diode array detector was used. The chromatographic data were processed using HP Chemstation software.

HPLC Conditions

A RP-18 column (Superspher, 5 µm, 250 mm*4mm, LiChroCart, Merck) was used. The mobile phase consisted of a mixture of acetonitrile

(A) and 0.1 M H_3PO_4 (B). Gradient elution was used: within 30 minutes, the composition of the mobile phase changed from 15% acetonitrile (85% 0.1 M H_3PO_4) to 30% (70% 0.1 M H_3PO_4). The flow rate was 0.7 ml/min. The detector was set at 310 nm with a bandwidth of 4 nm and as reference wavelength 380 nm with a bandwidth of 10 nm.

Reagents

The p-coumaric acid was obtained from Fluka (Switzerland, Buchs). Acetonitrile was Labscan for HPLC, phosphoric acid was from J.T. Baker, N.V., (Holland, Deventer). Water used in the chromatographic mobile phase was distilled, deionized (Millipore System) and filtered through a 0.45 µm nylon 66 membrane filter (Alltech). The solvents were degassed by helium before and during the run.

Standard Solutions

The standard solutions were freshly prepared daily by dissolution of p-coumaric acid in a mixture of acetonitrile/water (1/9). The standard solutions were prepared by dilution of stock solutions of approximately 0.25, 0.5, 1, 2.5 and 5 mg/ml. All standard solutions were filtered through a 0.45 µm disposable polyamide (nylon 66, Filterservice) filter before injection.

Sample and Sample Preparation

The Echinacea press juice and Urgenin® were preparations obtained from Madaus s.a. (Germany, Köln).

A 1 ml sample of Echinacea purpurea press juice and Urgenin®, prepared by Madaus, was taken and filtered over a disposable polyamide (nylon 66, Filterservice) filter of 0.45 µm and collected in a vial. The filtrate was injected.

ANALYTICAL RESULTS AND DISCUSSION

Method Validation

Linearity

In order to examine the linearity of the absorbance as a function of the concentration of p-coumaric acid, five solutions with different concentrations of p-coumaric acid, varying from 2.5 ng/µl to 50 ng/µl, were injected twice a day. From every calibration curve the least squares line ($y=a+b*x$), standard error of a (s_a) and b (s_b), the correlation coefficient (r), the significance of the regression coefficient ($\alpha=0.05$) (6) and analysis of variance were performed. The results are presented in Table 1. A linear relationship between the absorbance and the injected concentration of p-coumaric acid was obtained, however, the intercept in some cases significantly differs from the zero point (0,0). The correlation coefficient indicates only a linear positive tendency, but is nevertheless often used to prove the linearity (7).

Analysis of variance (ANOVA) which implies the lack of fit was applied to examine the linearity (8). In the ANOVA, the total sum of squares can be broken up in the sum of squares due to the regression and the sum of squares about the regression or the residual sum of squares. The latter can be broken up in the pure error sum of squares (SS_{PE}) and the lack of fit sum of squares (SS_{LOF}). The SS_{PE} is due to the

TABLE 1

Evaluation of the Linearity

curve	a s_a	b s_b	r	regr. coeff. S/NS	F p
1	13.903	147.630	0.99996	S	2.345
	9.531	0.356			0.190
2	33.805	147.882	0.99997	S	6.680
	11.519	0.377			0.034
3	44.542	146.667	0.99992	S	0.478
	17.624	0.646			0.711
4	61.540	145.868	0.99988	S	20.53
	21.208	0.789			0.003
5	16.000	146.099	0.99989	S	1.136
	20.179	0.758			0.419
6	49.776	143.509	0.99978	S	1.240
	11.060	0.401			0.387

variability within each group of replicate measurements, the SS_{LOF} is due to the variability of group averages about the regression line. The ANOVA of the first curve is given in Table 2, the probability level (p) is the probability that there is no lack of fit.

Sensitivity and limit of detection

The sensitivity (S) is defined as the slope of the calibration curve (9). The range over which the sensitivity can be considered to be constant has lower and upper limits. The lower limit will be the detection limit. For the upper limit no generally accepted definition could be found.

S=146.480±0.401 mAU/ng

TABLE 2

Analysis of variance of Curve 1

Source	SS	Df	Mean Square	F	p
Model	64613347	1	6461335		
Residual	3009.149	8	376.144		
LOF	1759.100	3	586.367	2.345	0.190
PE	1250.049	5	250.010		
Total	64616356	9			

The Kaisers detection limit was calculated (7). If a field blank is unobtainable the lowest detectable instrument signal, y_L, is given by: $y_L=a + k^*s_{y/x}$, in which a is the intercept, $s_{y/x}$ is the standard error of the estimate. Kaiser suggested k=3. The corresponding concentration is: $c_L=k^*s_{y/x}/b$, in which b is the slope.

y_L=199.418 mAU

c_L=1.15 ng/µl

Precision

Five different samples of Echinacea purpurea press juice were injected and the amount of p-coumaric acid was determined. This was repeated on 3 different days to examine the intermediate precision (10) in addition to the repeatability (11). The repeatability expresses the precision of a method under the same operating conditions or a short period of time (within-day). The repeatability is expressed as the

TABLE 3

Repeatability - Intermediate Precision in Echinacea Press Juice and Urgenin®

	Sample	
Parameter	Echinacea press juice	Urgenin
Repeatability		
s	0.61	0.13
s^2	0.38	0.02
RSD	3.92%	2.06%
Intermediate precision		
s	0.99	0.16
s^2	0.99	0.03
RSD	6.38%	2.60%
ANOVA		
F	1.647	0.683
F(critical)	3.885	3.982
p-value	0.233	0.525

TABLE 4

Comparison of the Slope of the Calibration Curve with the Slope of the Standard Addition Curve (α=0.05)

Sample	Echinacea press juice		Urgenin	
Experiment	1	2	1	2
t	2.047	1.0	-0.845	0.146
t_{tab} (d.f.=10)	2.228	2.228	2.228	2.228
S/NS	NS	NS	NS	NS

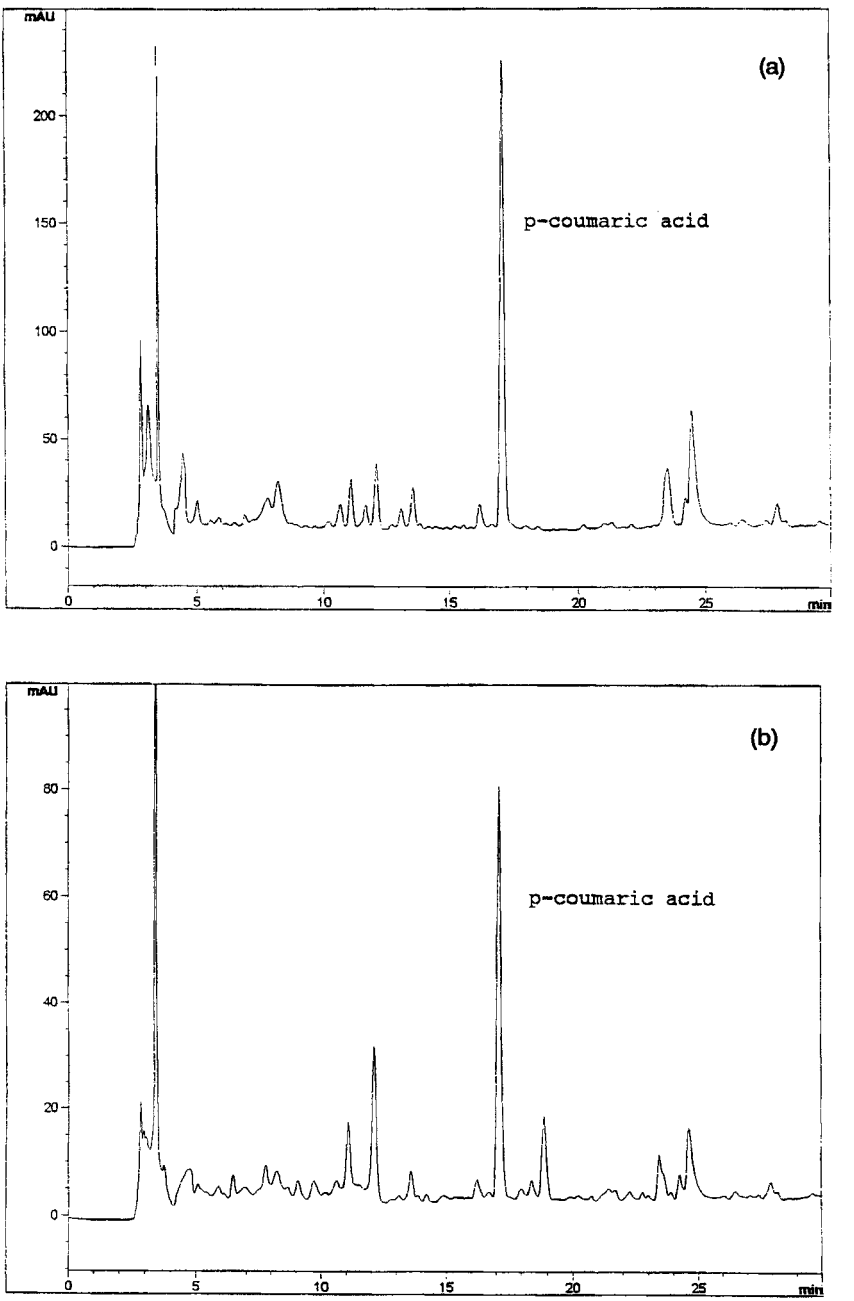

FIGURE 1: typical chromatogram of Echinacea press juice (a) and Urgenin® (b)

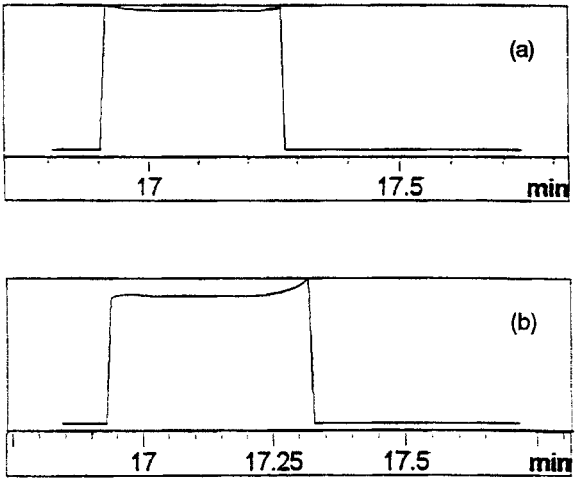

FIGURE 2: shows the absorbance ratio (280nm/330nm) in Echinacea press juice (a) and Urgenin® (b).

repeatability standard deviation (s), the repeatability variation (s^2) and the residual standard deviation (RSD). The intermediate precision i.e. within laboratory variations, different days, different analysts, etc. is expressed as s, s^2, RSD. To investigate whether or not there is a significant difference between the results of the different days a single-factor ANOVA was performed. The results are summarized in Table 3. They indicate that no significant differences between the amounts of p-coumaric acid are found in the samples on different days.

Accuracy

The accuracy can be examined by comparison with another method or by the standard addition method when dealing with plant preparations. Therefore, we preferred to determine the accuracy by the standard

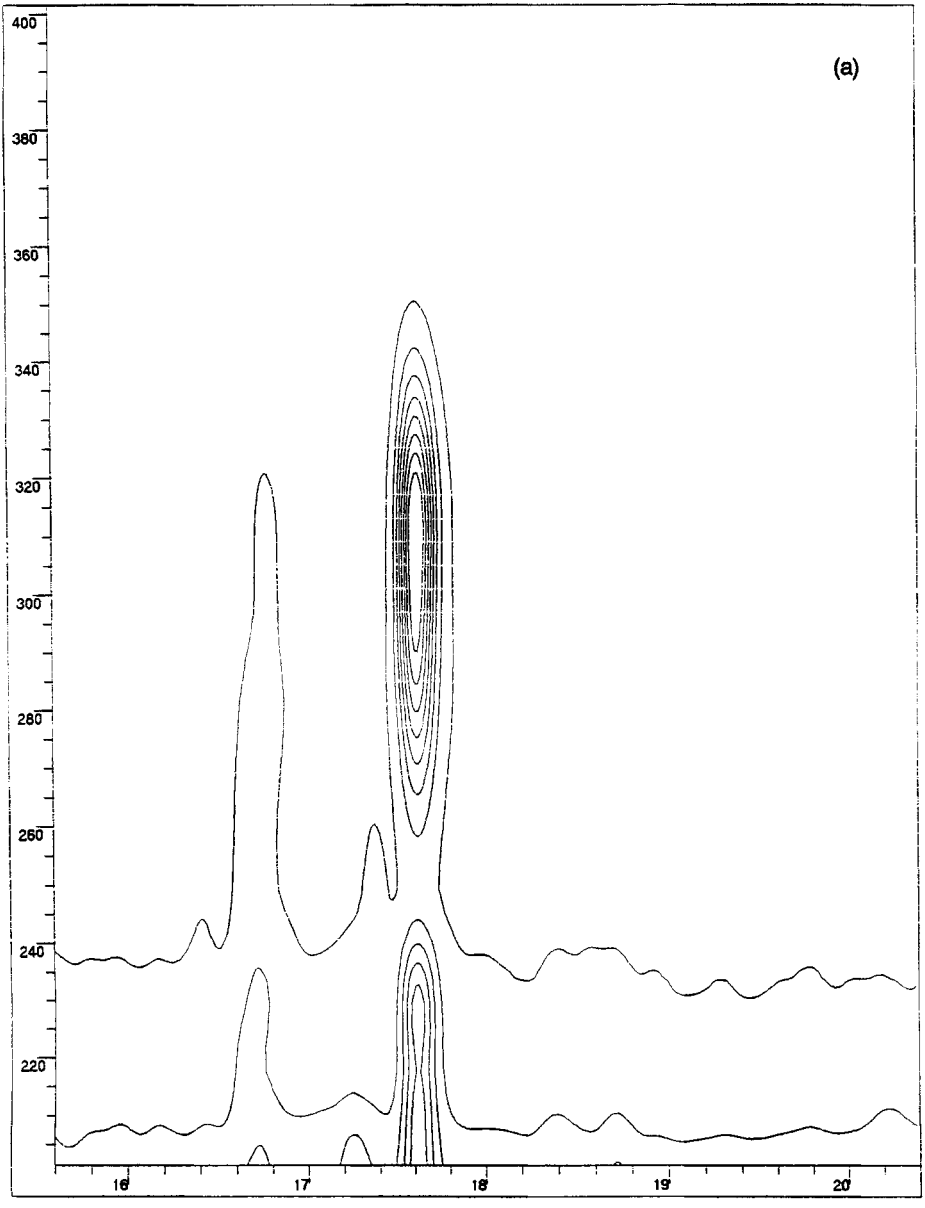

FIGURE 3: shows a contour plot of p-coumaric acid in Echinacea press juice (a) and Urgenin® (b).

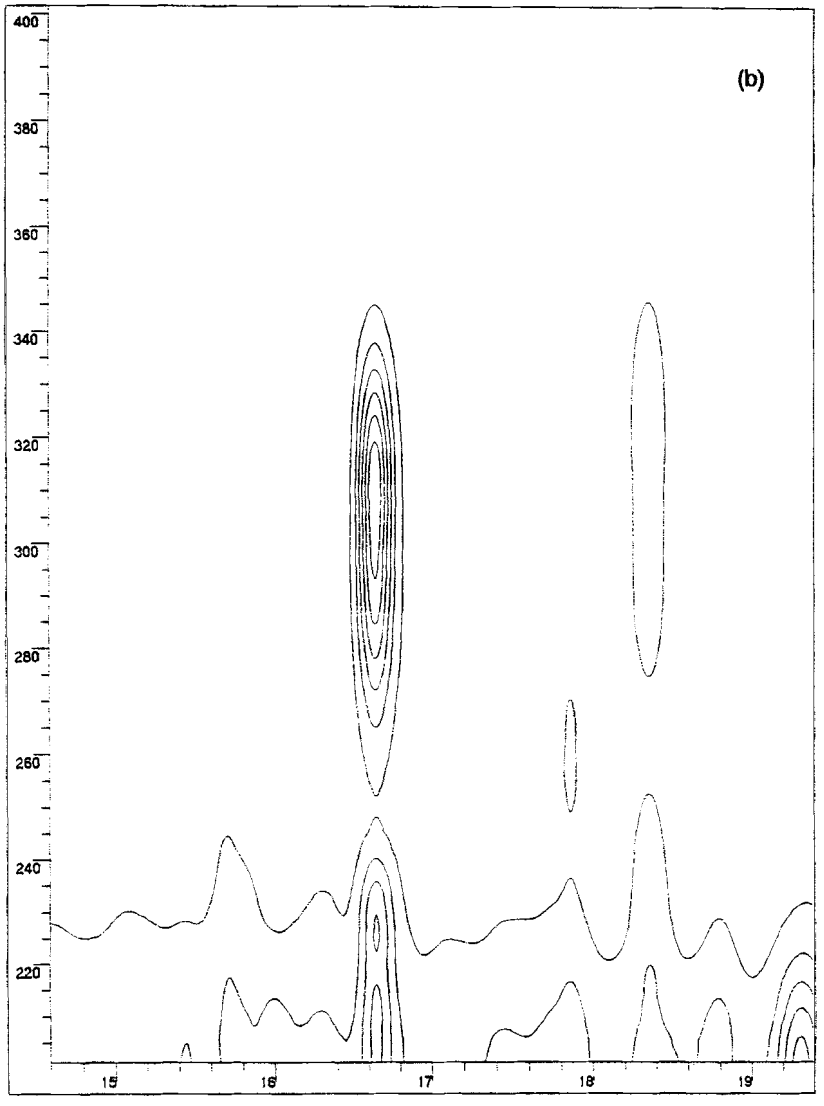

FIGURE 3 (continued)

addition method. Thus, known amounts of p-coumaric acid were added to the sample at 4 different concentration levels (2.5, 5, 15 and 20 ng/µl were added to the sample). The experiment was done twice. We calculated the recovery and compared the slope of the standard addition curve with the slope of the calibration curve (12). The recovery varied in all cases between 96.6% and 101.5%. The results of the comparison of the two curves are given in Table 4.

Selectivity

An analytical determination of a compound is defined as selective, when, with a certain probability and accuracy, the determined compound can be distinguished from related substances, impurities, etc (11). An important tool in the evaluation of the selectivity is the peak purity. We have chosen two visual methods, viz. the absorbance ratios and the contour plot. Although these methods seem to be rather rough, we think that major co-eluting peak(s) can be detected when their UV spectrum is different from that of the major component.

Typical chromatograms of the Echinacea press juice (a) and Urgenin® (b) are given in Figure 1.

The absorbance ratio technique is based on plotting the ratio of absorbances at two wavelengths over the elution profile. The absorbance ratio of a pure compound is constant. Figure 2 shows the absorbance ratio (280nm/330nm) of p-coumaric acid in Echinacea press juice (a) and Urgenin® (b), and indicates the absence of a major co-eluting impurity.

In a contour plot the data are presented as concentric isoabsorptive lines in the (Absorption,time) plane, so that all the data can be observed simultanuously. Major co-eluting peaks, with different UV-spectrum will disturb the normally symmetrical concentric lines of a peak. Figure 3

TABLE 5

Determination of p-Coumaric Acid in Echinacea Press Juice and Urgenin®

Batch Echinacea purp. press juice	Mean amount of p-coumaric acid ng/µl (s)	Batch Urgenin	Mean amount of p-coumaric acid ng/µl (s)
Ia	15.92 (0.25)	Ib	6.07 (0.12)
IIa	16.74 (0.08)	IIb	5.77 (0.14)
IIIa	23.34 (0.68)	IIIb	8.44 (0.19)

shows a contour plot of p-coumaric acid in Echinacea press juice (a) and in Urgenin® (b). In Figure 3, no major impurity is observed.

Analytical Results

The results of the determination are given in Table 5. The amount of p-coumaric acid found in Urgenin® ranges from 90 to 110% of the nominal amount found in the Echinacea press juice, which are the currently accepted limits of the registration authorities for plant drugs in Belgium.

CONCLUSION

As for synthetic pharmaceuticals, medicines of plant origin should be carefully analysed so that standardisation of these drugs can be done. One should, however, take into account that small variations in the

extract composition are inevitable and, therefore, the used analytical methods should be thoroughly validated prior to use on a large scale.

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