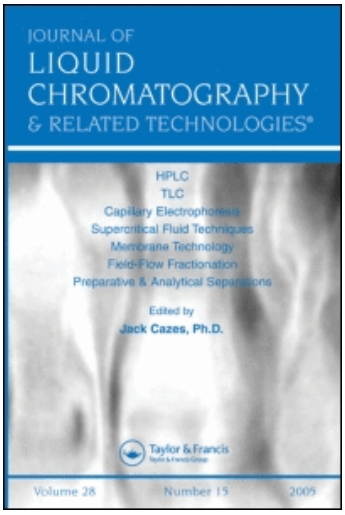

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ISOCRATIC HPLC SEPARATION OF SCOPOLETIN AND *CIS/TRANS* ISOMERS OF FERULIC ACID AS WELL AS ISOSCOPOLETIN AND *CIS/TRANS* ISOMERS OF ISOFERULIC ACID

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ABSTRACT

Cis/trans isomers of ferulic and isoferulic acids and their corresponding coumarins, scopoletin and isoscopoletin, were separated by isocratic High Performance Liquid Chromatography using RP-8 (5 μm) as a stationary phase and aqueous methanol or aqueous acetonitrile as a mobile phase. The UV spectrum of *cis*-isoferulic acid was obtained by a photodiode array detector.

INTRODUCTION

During our study on the light -induced biomimetic synthesis of simple coumarins (1), ferulic and isoferulic acids were used as key building blocks for obtaining scopoletin and isoscopoletin, respectively. For this purpose several solutions of the cinnamic acids were prepared, in different

experimental conditions, in order to study the different parameters that lead to the synthesis of coumarins. In this systematic study a quick identification and estimation of the relative quantities of compounds in the solution, like *cis/trans* equilibrium and coumarins synthesized, were necessary for monitoring the development of the reaction. As expected the HPLC method developed in our earlier work (2) was not restricted only to esculetin and its percursors but it can be extended, with minor adjustments, for the simultaneous analysis of other phenolic and lactonic compounds.

In this work we report an efficient isocratic HPLC separation of *cis/trans* ferulic acids and scopoletin as well as for *cis/trans* isoferulic acids and isoscopoletin using a reversed phase octylsilane packing, an acidified aqueous acetonitrile mobile phase and a UV detection at 290 nm.

In order to select the adequate wavelength for an accurate detection, the UV spectrum of *cis*-isoferulic acid was obtained by a photodiode array detector.

MATERIALS AND METHODS

High Performance Liquid Chromatography

(Equipment and chromatographic conditions)

A Jasco model liquid chromatographic system equipped with a loop injector, a Jasco 875 variable wavelength UV photometric detector and a Varian 4270 integrator was used. The analytical column was a commercially prepacked reversed phase column of 250 mm x 4.0 mm I.D. containing Lichrosorb RP-8 (5 μm) from E. Merck, Darmstadt, West Germany. The UV detector was set at 290 nm. A flow rate of 1.0 ml and a chart speed of 0.5 cm

per minute were used. The volume of injection was 5 µl. The system was operated at room temperature. The mobile phases used were methanol/ aqueous acetic acid (5%), acetonitrile/aqueous acetic acid (5%) and acetonitrile/aqueous acetic acid (1%) (see Results and Discussion) .

The UV spectra were obtained from the test mixture in analysis using a Hewlett-Packard (HP) model 1090M high performance liquid chromatograph fitted with a diode array detector (DAD), a Rheodyne injection valve and a HP 300 computer monitor. The scanning was performed between 210 to 400 nm.The displayed spectra were taken at HPLC peak apices.

Reagents

Trans-ferulic acid and scopoletin (purum) were obtained from Fluka AG, Chemische Fabrik CH-9470 Buchs, Switzerland. *Trans*-isoferulic acid was obtained from Aldrich Chemical Company, Inc., Wisconsin USA . Isoscopoletin was gifted from Prof. Sansei Nishibe, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Higashi Nippon Gakuen University, Japan . Methanol and acetonitrile were Lichrosolv for chromatography (E. Merck). Water used in the chromatographic mobile phase was distilled, desionized and filtered through a 0.2 µm membrane filter. The aqueous solutions were fleshly prepared and degassed by vacuum and sonication before use.

Standard Solutions

The standard solutions were prepared by dissolution of standard reagents in ethanol (0.1 mg/ml), assisted by sonication.

As *cis*-ferulic acid and *cis*-isoferulic acid were not available commercially, they were obtained by exposure of the standard solutions of *trans*-ferulic acid and *trans*-isoferulic acid to diffused daylight for 2 hours. As

expected, a mixture of *cis* and *trans* isomers was formed (3). The standards were stored in sealed containers at 4 °C in darkness.

Sample Preparation

For the study of the chromatographic conditions, test mixtures were prepared by mixing 15 µl of each standard coumarin solutions and 15 µl of each standard *cis*/*trans* cinnamic acids solutions.

RESULTS AND DISCUSSION

Development of Isocratic HPLC Method

Using the binary solvent system (methanol/aqueous acetic acid (5%), 25/75 by volume) *cis*-ferulic acid, scopoletin and *trans*-ferulic acid were found to co-eluted on the column described. The separation of the three compounds is shown in **Fig. 1**. The identification of each component was made by peak picking with standard solutions. The attempts to improve the separation of scopoletin from *trans*-ferulic acid, by studying the influence of solvent composition and flow rate on the chromatographic behaviour, had been failed.Furthermore, in this chromatographic system it was not possible to separate *cis* and *trans* isoferulic acids from isoscopoletin since isoscopoletin and *cis*-isoferulic acid always possessed the same retention time.

In order to observe the chromatographic behaviour of the stereo-isomers involved in the light-induced biomimetic synthesis, a study was carried out with methanol/aqueous acetic acid (5%), 35/65 by volume, as a mobile phase . **Fig. 2** shows that the order of elution of *trans* cinnamic acids isomers is caffeic, ferulic and isoferulic acids, respectively, which is in agreement with their polarity.The analysis of the chromatograms of *cis* isomers **(Fig. 2)** shows that *cis* -caffeic acid was the first to be eluted while *cis*

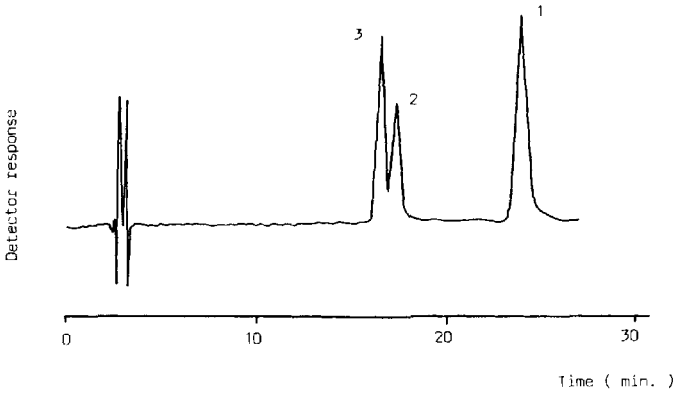

FIGURE 1. Chromatogram of the test mixture containing : Trans-ferulic acid (1), scopoletin (2) and cis-ferulic acid (3). Conditions described in Materials and Methods.

- ferulic acid and *cis* - isoferulic acid, which have similar retention time were eluted later. Again, as expected, the more polar hydroxylated compound is the shorter the retention time it possesses **(Fig. 2)** .Anyhow, the polarity difference observed for *trans* structural isomers was not so evident as for corresponding *cis* isomers.

The experimental determination of the relative pKa of *trans*- cinnamic acids (4) allows us to understand the difference in polarity for the structural isomers, which seems to be related to the relative position of the substituents on the aromatic ring and the possibility of existence of a planar conjugation system with the ethylenic side chain .As for *cis* -ferulic and *cis*- isoferulic acids, which show similar polarities, it seems that the position factor of the substituents does play only a second role. In these compounds the planar conjugation system seems to be disturbed by the molecular spacial arrangement.

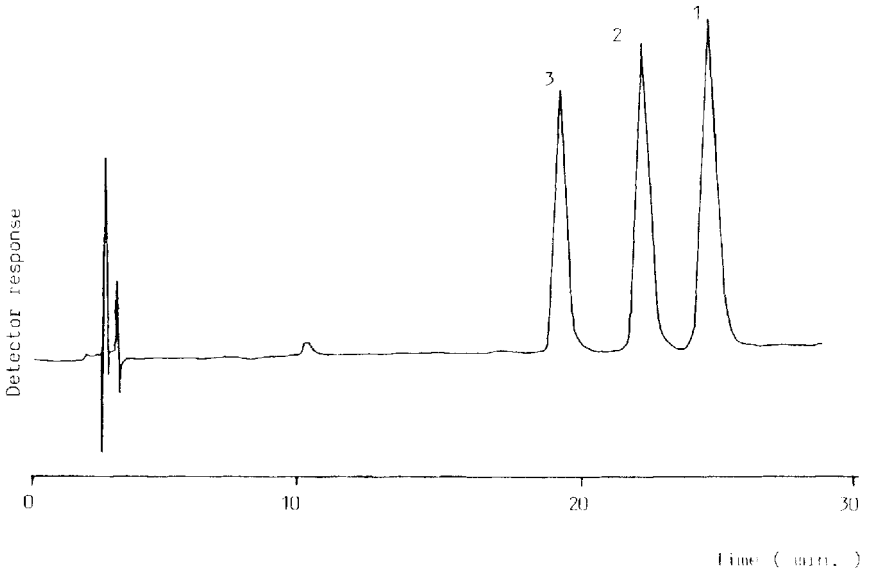

FIGURE 2. Chromatograms of the standard solutions containing :
A- Trans-caffeic acid (1) and cis-caffeic acid (2)
B- Trans-ferulic acid (1) and cis-ferulic acid (2)
C- Trans-isoferulic acid (1) and cis-isoferulic acid (2)
Conditions described in Materials and Methods.

As the polarity and solubility behaviours of *cis/trans* isomers of ferulic and isoferulic acids were different from those of *cis/trans* isomers of caffeic acid, it seems reasonable that the changing from methanol to acetonitrile in a mobile phase would improve resolution and separation of compounds in the test mixtures. In fact, an excellent separation with narrow and symmetrical peaks of scopoletin and *trans*-ferulic acid was obtained by using acetonitrile/aqueous acetic acid (5%) 10/90 by volume as mobile phase **(Fig. 3)**.

With this chromatographic system (acetonitrile/aqueous acetic acid (1%) 15/85 by volume), it was possible to separate and identify isoscopoletin

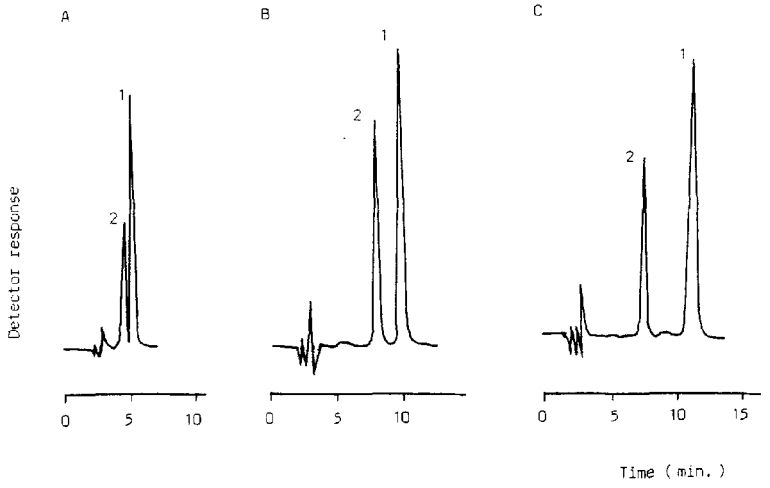

FIGURE 3.Chromatogram of the test mixture containing : Trans-ferulic acid (1), scopoletin (2) and cis-ferulic acid (3). Conditions described in Materials and Methods.

and *cis/trans* isoferulic isomers **(Fig. 4)**. After co-chromatography with the standard solutions it is verified that isoscopoletin was first eluted, followed by *cis* and *trans*-isoferulic acids respectively.

For a selection of the proper wavelength used in the photometric detection, it was necessary to know the UV spectral characteristics of all compounds in analysis. Although the UV data for coumarins (5), *cis/trans* ferulic acids and *trans*- isoferulic acid (6 , 7) were described in the literature, no work has been done for the spectral characterization of *cis*-isoferulic acid, so far. So, we report the UV spectrum of *cis*-isoferulic acid obtained by a photodiode array detector **(Fig.5).**

In summary, a fast and easy HPLC method for the simultaneous analysis of cinnamic acid derivatives and their corresponding coumarins is described. This method requires a small sample volume, short time for

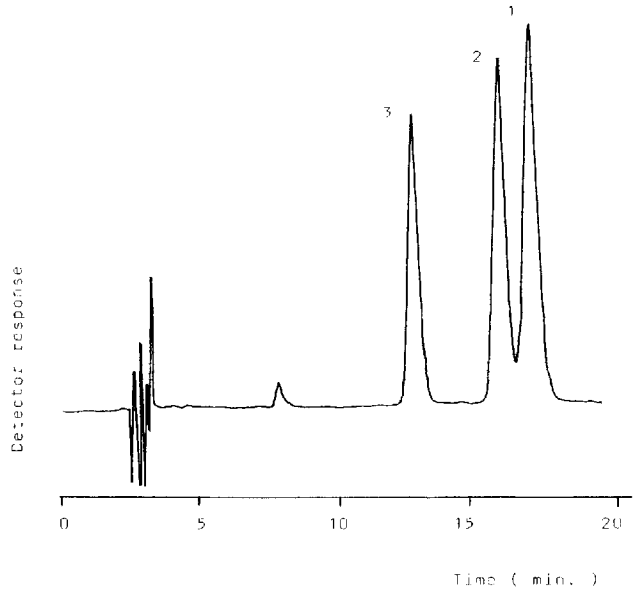

FIGURE 4. Chromatogram of the test mixture containing : Trans-isoferulic acid (1),cis-isoferulic acid (2) and isoscopoletin (3). Conditions described in Materials and Methods.

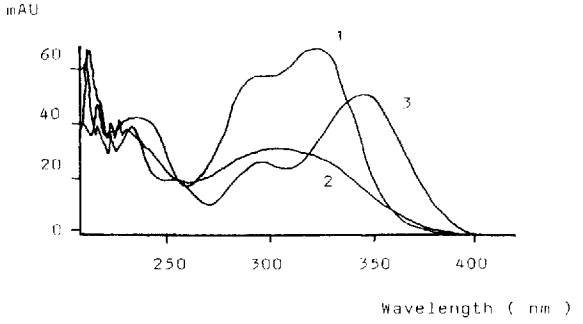

FIGURE 5.The UV spectra of the test mixture containing : Trans-isoferulic acid (1), cis-isoferulic acid (2) and isoscopoletin (3). Conditions described in Materials and Methods.

sample preparation and short chromatogram run compared with the elution time of the TLC system used in the analysis for this class of compounds (2). Another advantage is the use of isocratic conditions which are suitable for the separation of phenolic compounds and coumarins. The results obtained from this work could be of great importance in many fields of organic chemistry, like biomimetic synthesis and phytochemistry for establishing the biogenetic relationships between cinnamic acids and coumarins, either by enzymatic or nonenzymatic experimental conditions.

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