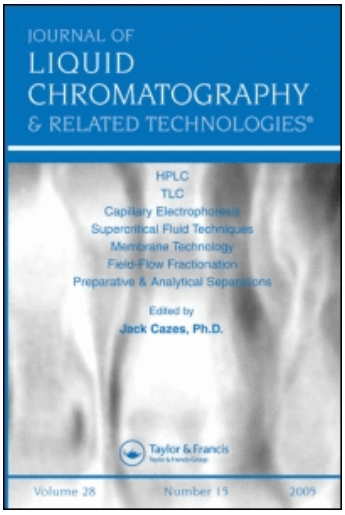

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SIMULTANEOUS ISOCRATIC HPLC SEPARATION OF THE DIASTEREOISOMERS OF CAFFEIC, FERULIC, AND ISOFERULIC ACIDS AND RELATED COUMARINS

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ABSTRACT

A general procedure for the simultaneous analysis of phenolic compounds by a reversed-phase high performance liquid chromatography (RP-HPLC) is described. The series of compounds embraces the *cis/trans* isomers of caffeic, ferulic and isoferulic acids and the coumarins - esculetin, scopoletin and isoscopoletin. In order to establish the experimental conditions, leading to an optimal resolution, several test mixtures were analysed. HPLC separation was carried out on a reversed-phase column (Lichrosorb RP-8, 5 μm), using aqueous tetrahydrofuran as a mobile phase.

INTRODUCTION

Phenolic compounds, which are widespread in Nature, are frequently studied either by their physiological and economic value. At the present time cinnamic acid derivatives and coumarins are of importance in several areas like

chemical ecology (1), foodchemistry (2), pharmacology (3), phytochemistry (4) and organic synthesis (5).

In order to improve isolation, separation and estimation of these compounds from different sources, e.g. plant extracts and beverages, many chromatographic studies have therefore been developed. Although several papers have been reported on separation of hydroxycinnamic acids derivatives and coumarins, as independent entities, a few work seems to be done on the simultaneous analysis of samples containing *Z/E* isomers of phenolic acids and related lactonic compounds.

In our previous papers (6,7) HPLC analysis of simple mixtures containing three compounds - *cis/trans* cinnamic acids and a coumarin - has been described. The present paper reports the chromatographic separation of more complex test mixtures (containing several or all compounds indicated in **fig. 1**) by using the reversed-phase octylsilane packing described in the works above mentioned.

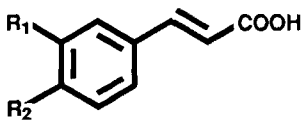
The chromatographic developed system render possible a fast and easy method for the simultaneous determination of the diastereoisomers of some cinnamic acids and structurally related coumarins.This system is of grate value in monitoring the development of the reactions strategically performed, in our lab, for the light-induced biomimetic synthesis of coumarins (8).

MATERIALS AND METHODS

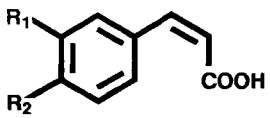
High Performance Liquid Chromatography

(Equipment and chromatographic conditions)

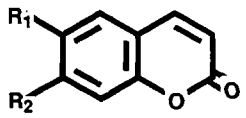
A Jasco model Liquid Chromatographic System equipped with a loop injector, a Jasco 875 variable wavelength UV photometric detector and a Varian 4270 integrator was used. The analytical column was a commercially prepacked reversed phase column of 250 mm x 4.0 mm I.D., containing Lichrosorb RP-8 (5 μm) from E. Merck, Darmstadt, West Germany. The UV detector was set at 290 nm. Samples of each standards and mixtures (5 μl) were injected in the column, equilibrated with the mobile phase, at a flow rate of 1.0 ml/min. A chart speed of 0.5 cm per minute was used.



(1) $R_1 = R_2 = OH$
(2) $R_1 = OCH_3$; $R_2 = OH$
(3) R_1 =OH ; $R_2 = OCH_3$



(4) $R_1 = R_2 = OH$
(5) $R_1 = OCH_3$; $R_2 = OH$
(6) R_1 =OH ; $R_2 = OCH_3$



(7) $R_1 = R_2 = OH$
(8) $R_1 = OCH_3$; $R_2 = OH$
(9) R_1 =OH ; $R_2 = OCH_3$

FIGURE 1

Structures of the diastereoisomers of cinnamic acids and coumarins.

(1) *Trans*-caffeic acid
(2) *Trans*-ferulic acid
(3) *Trans*-isoferulic acid
(4) *Cis*-caffeic acid
(5) *Cis*-ferulic acid
(6) *Cis*-isoferulic acid
(7) Esculetin
(8) Scopoletin
(9) Isoscopoletin

The mobile phase was tetrahydrofuran/aqueous acetic acid (5%) (see Results and Discussion). The system was operated at room temperature.

Reagents

Trans-caffeic acid, *trans*-ferulic acid, esculetin and scopoletin 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. Tetrahydrofuran was 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 (1 mg/ml), assisted by sonication.

As *cis*-caffeic, *cis*-ferulic and *cis*-isoferulic acids were not available commercially, they were obtained by exposure of the standard solutions of *trans*-caffeic, *trans*-ferulic acid and *trans*-isoferulic acid to diffused daylight for 2 hours. As expected, a mixture of *cis* and *trans* isomers was formed (9). The standards were stored in sealed containers, at 4°C in darkness.

Sample Preparation

In order to improve a sequential and rational study several test mixtures were prepared (see **Table 1**), by mixing 50 μl of each standard coumarin solutions and 50 μl of each standard *cis/trans* cinnamic acids solutions.

TABLE 1

Components of the Test Mixtures used in the Analysis

Test mixture	Components
A	3, 6, 9
B	1, 4, 7
C	2, 5, 8
A+B	1, 3, 4, 6, 7, 9
B+C	1,2, 4, 5, 7, 8
A+B+C	1, 2, 3, 4, 5, 6, 7, 8, 9

The identification of each component was made by peak picking with the standard solutions. All the analysis were performed in triplicate.

RESULTS AND DISCUSSION

Development of Isocratic HPLC Method

The main purpose of this work was to develop a HPLC method which could allow the separation and identification of all the compounds shown in **fig. 1.**

In preliminary works (6,7) chromatographic HPLC systems were obtained in order to separate and estimate the components of the test mixtures **A, B** and **C** . Although in the chromatographic analysis a good resolution was achieved for the test mixtures **B** and **C,** with methanolic or acetonitrile aqueous

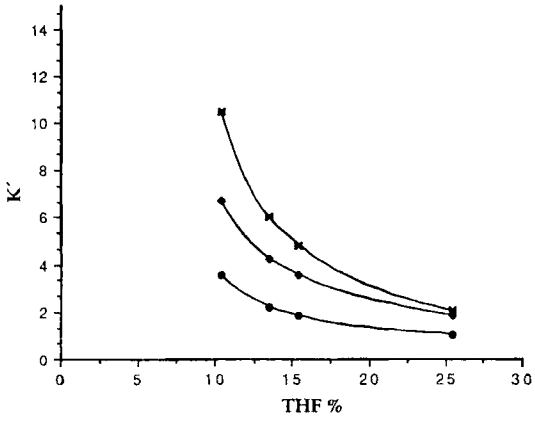


FIGURE 2

Plot of capacity factor (k´) *vs* percent of THF content in the mobile phase for the components of the test mixture **A** *trans*-isoferulic acid (**3**, •), *cis*-isoferulic acid (**6**, ♦) and isoscopoletin (**9**, ⋆).

mobile phases, several problems were found to be related to the separation of the components of mixture **A** .

As the stationary phase seems to be sucessful for the analysis of this kind of compounds, a modification on the mobile phase was thought to be suitable for our purpose. Tetrahydrofuran was chosen as organic modifier instead of methanol or acetonitrile. The effect of variation of percentage of tetrahydrofuran (from 25/75 to 10/90 % v/v tetrahydrofuran/ water: acetic acid (95:5)) on the k´values of the components of mixture **A** was evaluated. **Fig. 2** shows that the variation of the capacity factor (k´) of the components is a function of the concentration of the organic modifier. The analysis of the separation parameters between the solute pairs (**Table 2**) allows to conclue that the solute retention time is influenced by the polarity of the mobile phase, in this reversed-phase liquid chromatography. Indeed the retention time of the compounds increases with the polarity of the mobile phase. These data also

TABLE 2

Effect of Variation of Tetrahydrofuran Concentration on Separation Parameters of the Components of the Test Mixture A.

Mobile phase [a]	R_1[b]	R_2[c]	α_1[d]	α_2[e]
10	2.02	1.38	1.96	1.59
13	1.97	0.94	2.06	1.44
15	1.65	0.72	2.11	1.38
25	0.63	0.09	2.08	1.09

a) Concentration of organic modifier (THF) in the mobile phase, % (v/v)
b) Resolution between isoscopoletin (9) and *cis*-isoferulic acid (6)
c) Resolution between *cis*-isoferulic acid (6) and *trans*-isoferulic acid (3)
d) Separation between isoscopoletin (9) and *cis*-isoferulic acid (6)
e) Separation between *cis*-isoferulic acid (6) and *trans*-isoferulic acid (3)

show that the octylsilane stationary phase possesses an excellent selectivity for the compounds in analysis. Although the differences in the selectivity values in the THF mobile phases are very small, it seems that the increase in water concentration leads to an interesting inverse relationship on the selectivity between the adjacent pairs - isoscopoletin/*cis*-isoferulic acid and *cis*-isoferulic acid/*trans*-isoferulic acid.

These findings indicate that a poor resolution, with severe overlapping of *Z/E* isomers, was obtained when using 25% of tetrahydrofuran in the mobile phase. On the contrary, an optimum resolution,with large k´ values, was achieved with 10/90 % tetrahydrofuran/ water:acetic acid (95:5).

As in practice, an optimum separation for a mixture is often a compromise between maximum resolution and minimum analysis time the

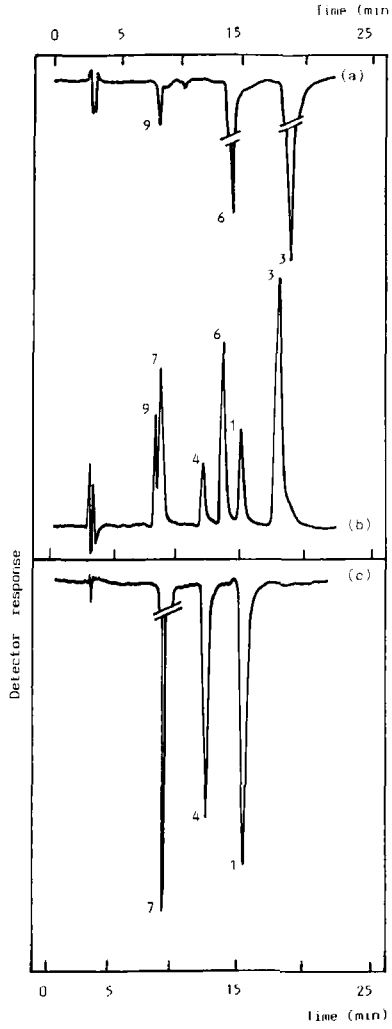


FIGURE 3

Chromatographic profiles of the test mixtures **A**, **A+B** and **B** containing :

a) *Trans*-isoferulic acid (**3**), *cis*-isoferulic acid (**6**) and isoscopoletin (**9**).

b) *Trans*-caffeic acid (**1**), *trans*-isoferulic acid (**3**), *cis*-caffeic acid (**4**), *cis*-isoferulic acid (**6**), esculetin (**7**) and isoscopoletin (**9**).

c) *Trans*-caffeic acid (**1**), *cis*-caffeic acid (**4**) and esculetin (**7**).

The mobile phase is 13/87 % of tetrahydrofuran/water:acetic acid (95:5).
Other conditions described in Materials and Methods.

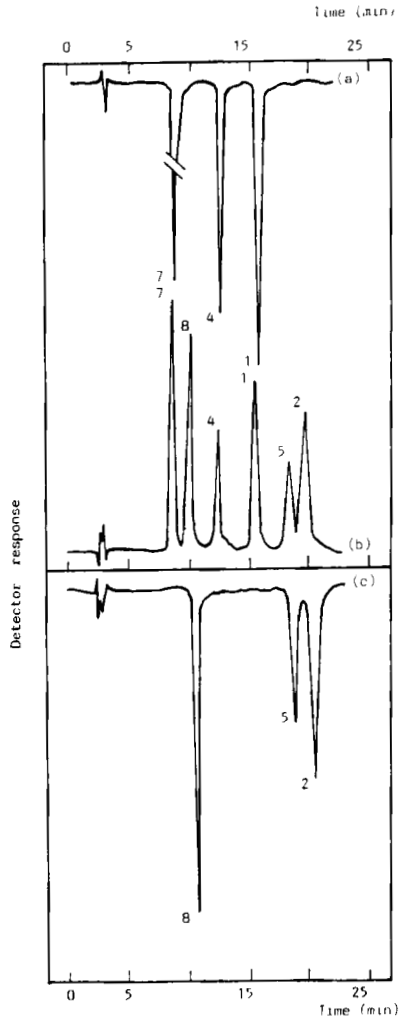


FIGURE 4

Chromatographic profiles of the test mixtures **B**, **B+C** and **C** containing :

a) *Trans*-caffeic acid **(1)**, *cis*-caffeic acid **(4)** and esculetin **(7)**.

b) *Trans*-caffeic acid **(1)**, *trans*-ferulic acid **(2)**,*cis*-caffeic acid **(4)**, *cis*-ferulic acid **(5)**, esculetin **(7)** and scopoletin **(8)**.

c) *Trans*-ferulic acid **(2)**, *cis*-ferulic acid **(5)** and scopoletin **(8)**.

The mobile phase is 13/87 % of tetrahydrofuran/water:acetic acid (95:5).
Other conditions described in Materials and Methods.

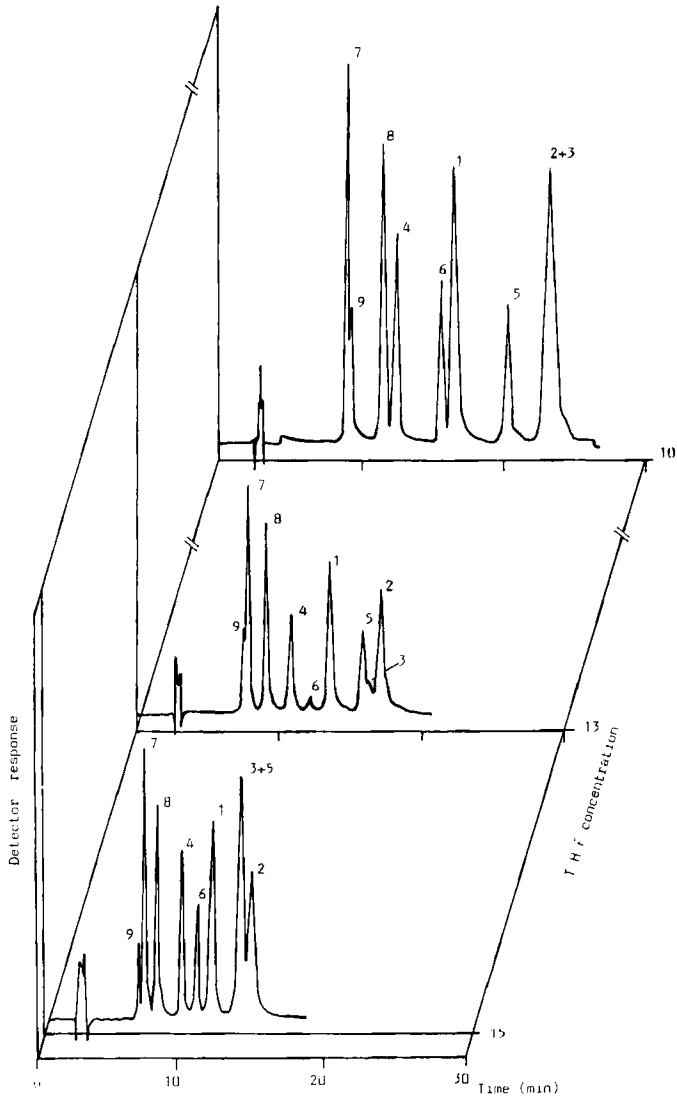


FIGURE 5

Effect of the mobile phase polarity in the analysis of the test mixture **A+B+C**

(**1**) *Trans*-caffeic acid, (**2**) *trans*-ferulic acid, (**3**) *trans*-isoferulic acid, (**4**) *cis*-caffeic acid, (**5**) *cis*-ferulic acid, (**6**) *cis*-isoferulic acid, (**7**) esculetin, (**8**) scopoletin and (**9**) isoscopoletin.

Conditions described in Materials and Methods.

chromatographic conditions obtained with the mobile phase 13/87 % were chosen. **Fig. 3 a** shows that a clean separation of the components of the test mixture **A**, with acceptable capacity factors (> 1), was obtained.

The test mixtures **B** and **C** were also studied in this chromatographic system. **Fig. 3 c** and **4 c** showed that a good separation between *cis/trans* isomers and coumarins was found. When the mixtures **A+B** and **B+C** were analysed in these conditions separation of all the compounds (except for esculetin and isoescopoletin), with symmetrical peak shape, occurred (**Fig. 3 b** and **4 b**).

A more complex situation happened when all the components of this study (**Fig. 1**) were mixed and analysed. The presence of six pairs of diastereoisomers of the cinnamic acids and three structurally related coumarins makes the analysis very difficult, specially when operated under isocratic conditions. Nevertheless the study of chromatographic behaviour of the test mixture (**A +B +C**) using the system described herein has also been done. The variation of the eluent polarity on the separation of the components of the mixture is demonstrated in **Fig. 5.** A better separation was achieved at 13/87 % tetrahydrofuran/ water:acetic acid (95:5). Overlapping between compounds **3** and **5** is found with the less polar phase while overlapping between compounds **2** and **3** occurs with the more polar one. However a poor resolution between isoscopoletin and esculetin was obtained in all of these conditions.

In summary, a fast and easy HPLC method for the simultaneous analysis of several cinnamic acid derivatives and their corresponding coumarins is described. This work also shows that the obtention of an adequate chromatographic resolution among all components of a more complex mixture, in a reasonable time, is more difficult. These chromatographic problems could be decreased by a systematic study of the results obtained in this area. Only after a further thorough treatment of data, a tertiary isocratic system or a rational gradient work could be developed.

However the results obtained from our work could have an important interest, in some scientific areas, since it enable a simultaneous isocratic separation and identification of a series of natural or synthetic compounds that could be found in mixture.

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