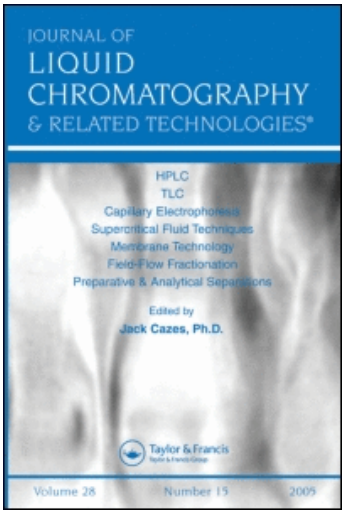

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SEPARATION OF THE DIASTEREOISOMERS OF ETHYL ESTERS OF CAFFEIC, FERULIC, AND ISOFERULIC ACIDS BY THIN-LAYER AND HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

The separation of the diastereoisomers of cinnamoyl derivatives have been studied by thin-layer (TLC) and high performance liquid chromatography (HPLC). TLC separations on two different layers and with two different solvent systems are described. HPLC separation was carried out on a reversed-phase column (Lichrosorb RP-8, 5 μm) using an isocratic elution [acetonitrile : water-acetic acid (95 : 5)]. The series of compounds includes the *cis/trans* isomers of ethyl esters of caffeic, ferulic and isoferulic acids.

INTRODUCTION

During our work on the biomimetic synthesis of simple coumarins the *cis/trans* isomers of ethyl caffeate, ethyl ferulate and

ethyl isoferulate (**Fig. 1**) were identified as side products of the reactions [1].

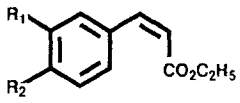
Although a large variety of cinnamic acids derivatives could be found in the literature, only a few papers concerned especially with the separation of their diastereoisomers by HPLC and/or TLC have been published. The present paper reports the chromatographic analysis of some Z/E ethylcinnamates which was carried out by TLC and HPLC. The HPLC analysis developed allows a quick identification and estimation of the relative percentage of the components in solution. Consequently it is of great value for monitoring the development of reactions, performed in our lab, for the light-induced biomimetic synthesis of coumarins.

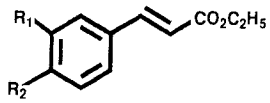
MATERIAL AND METHODS

Thin-Layer Chromatography

Commercially available pre-coated TLC plates were used : silica gel 60 F254 (S_1) and cellulose (S_2), without fluorescent indicator, from Merck, Darmstadt. The layer thickness was 0.2 and 0.1 mm, respectively.

The following solvent systems were used: F_1 - petroleum benzine 40-60 ^{0}C/diethyl ether/ formic acid (5 : 5 : 0.1); F_2 - water/acetic acid (9 : 1). Before development the chambers were allowed to saturate 1h and 3 h, respectively. The time of elution was 1h and 30 min for system S1/F1 and 3 h and 30 min for system S2/F2.





	R_1	R_2		R_1	R_2
1	OH	OH	2	OH	OH
3	OCH_3	OH	4	OCH_3	OH
5	OH	OCH_3	6	OH	OCH_3

FIGURE 1

Structures of the diastereoisomers of the ethylcinnamates
1. Ethyl *cis*-caffeate **2.** Ethyl *trans*-caffeate
3. Ethyl *cis*-ferulate **4.** Ethyl *trans*-ferulate
5. Ethyl *cis*-isoferulate **6.** Ethyl *trans*-isoferulate

Before detection the plates were air-dried (30 min for system S1/F1 and 2 h for system S2/F2). The compounds were visualized under UV light (254 and 366 nm) before and after spraying with 10 ml of an ethanolic solution of KOH (5%), without and with heating (in an oven at 100 °C, during 10 min).

Samples (2.5 µl) were spotted onto the TLC plates by means of a graduated microsyringe. TLC separations were performed at room temperature. Details of other chromatographic conditions can be found in reference [2]. All the analysis were performed in triplicate.

The TLC data are shown in Results and Discussion.

High Performance Liquid Chromatography

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 reversed-phase analytical column was RP-8 with 5 μm particle size (250 x 4.0 mm I.D) from E. Merck, Darmstadt, Germany. The UV detector was set at 290 nm. Samples of each standard (5 μl) were injected in the column, equilibrated with the mobile phase at a flow rate of 1.0 ml /min..

The system was operated at room temperature. A chart speed of 5 mm /min. was used. The mobile phase was acetonitrile/aqueous acetic acid (95:5): system L1 - 30/70 %,v/v; system L2 - 25/75 %,v/v. All the analysis were performed in triplicate.

The HPLC data are shown in Results and Discussion.

Other conditions

The ^1H NMR spectra were recorded on a Bruker pulse (300 MHz) instrument. Chemical shifts (δ) are reported in ppm and coupling constants (J) in Hz. The infrared spectra (IR) were measured with a Perkin Elmer-257 spectrophotometer. Mass spectra (MS) were recorded on a Hitachi Perkin Elmer RMU 6M spectrometer, using a direct inlet probe and an electron energy of 70 eV.

Reagents

All the chemicals used were of analytical-reagent grade. *Trans*-caffeic acid and *trans*-ferulic acid were obtained from Fluka AG, Chemische Fabrik CH-9470 Buchs, Switzerland. *Trans*-isoferulic acid was obtained from Aldrich Chemical Company, Inc., Wisconsin USA . Acetonitrile 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.

Synthesis

The ethyl esters used in this study were synthesized using a modification of the Pearl and Beyer method [3].

General method for the preparation of compounds **2**, **4** and **6** :

The corresponding *trans*-cinnamic acid (2.5 mM) was dissolved in EtOH (50 ml) and H_2SO_4 (0.5 ml) was added. The mixture was refluxed for 3 h. After cooling, the solvent was partially evaporated under reduced pressure. The cooled solution was poured into cold water and neutralized with a sodium bicarbonate solution.

The TLC control of the reaction was carried out with the system S1/F1 and S2/F2 (see Experimental).

Ethyl *trans* 3-(3,4-dihydroxyphenyl)-2-propenoic acid (2). The yellow precipitate obtained was filtered, washed and dried. The residue was recrystallized from aqueous MeOH to give 400 mg of the ester (77 %), as light yellow needles. M.p. 144-147 °C . IRυ_{max} (cm^{-1}) (KBr): 3450, 1660, 1610, 1600, 1520, 1515. ^{1}H NMR (CD_3COCD_3): 7.52 (1H, *d*, J= 15.9, Hb), 7.15 (1H, *d*, J= 2.1, H-2Ar), 7.02 (1H, *dd*, J= 2.1, 8.2, H-6Ar), 6.85 (1H, *d*, J= 8.2, H-5Ar), 6.26 (1H, *d*, J= 15.9, Ha), 4.17 (2H, *q*, J=7.1, OCH_2), 1.25 (3H, *t*, J=7.1, CH_3). MS: m/z 208 (M^+).

Ethyl *trans* 3-(4-hydroxy-3-methoxyphenyl)-2-propenoic acid (4) and **ethyl *trans* 3-(3-hydroxy-4-methoxyphenyl)-2-propenoic acid(6)**. The white precipitates obtained immediately turn to colorless oils. The aqueous emulsions were then extracted with diethyl ether (3x50 ml).

The extracts were washed with H_2O (3x50 ml), dried over Na_2SO_4 and concentrated under reduced pressure. The oil residues were crystallized from diethyl ether/n-hexane to give 440 mg of the ester **4** (79 %) and 490 mg of the ester **6** (88 %), as white needles.

Compound **4** m.p. 49-52 °C. IRυ_{max} (cm^{-1}) (KBr): 3180, 1670, 1630, 1580, 1510. ^1H NMR ($CDCl_3$): δ: 7.58 (1H, *d*, J= 15.9, Hb), 7.04 (1H, *dd*, J= 1.9, 8.2, H-6Ar), 6.99 (1H, *d*, J= 1.9, H-2Ar), 6.88 (1H, *d*, J= 8.2, H-5Ar), 6.26 (1H, *d*, J= 15.9, Ha), 5.99 (1H, *s*, OH), 4.23 (2H, *q*, J= 7.1, OCH_2), 3.88 (3H, *s*, OCH_3), 1.30 (3H, *t*, J=7.1, CH_3). MS: m/z 222 (M^+).

Compound **6** m.p. 53-55 °C . IRυ_{max} (cm^{-1}) (KBr): 3360, 1690, 1640, 1610, 1580, 1510. ^1H NMR ($CDCl_3$): 7.56 (1H, *d*, J= 15.9, Hb), 7.11 (1H, *d*, J= 2.1, H-2Ar), 6.99 (1H, *dd*, J= 2.2, 8.3, H-6Ar), 6.81 (1H, *d*, J= 8.3, H-5Ar), 6.26 (1H, *d*, J= 16.0, Ha), 5.71 (1H, *s*, OH), 4.22 (2H, *q*, J=7.1, OCH_2), 3.89 (3H, *s*, OCH_3), 1.30 (3H, *t*, J=7.1, CH_3). MS: m/z 222 (M^+).

Sample Solutions

The sample solutions of the ethyl *trans*-cinnamates were prepared by dissolution of the synthesized compounds in ethanol (1 mg/ml and 0,1 mg /ml for TLC and HPLC, respectively).

The ethyl *cis*-cinnamates were obtained by exposure of an aliquot of the sample solutions of ethyl *trans*-cinnamates to diffused daylight for 2 hours. As expected, a mixture of *cis* and *trans* isomers was

formed [4]. The solutions were stored in sealed containers, at 4 °C in darkness.

RESULTS AND DISCUSSION

During the study on the biomimetic synthesis of simple coumarins by a photochemical process, besides the Z/E isomers of the cinnamic acids, another pair of fluorescent compounds has been found to appear on the TLC control (system L2/F2) of some solutions. Separation by column chromatography and preparative TLC together with spectroscopic determination have led to the identification of the two compounds as the Z/E isomers of the ethyl esters of the cinnamic acids, used as building blocks for coumarins synthesis. However, these traditional methods of purification are quite tedious and consequently ineffective for our purpose. Eventhough the chromatographic processes were carried out in subdued light, a mixture of the referred Z/E isomers were always found. The long time of analysis for an estimation of the Z/E isomers formed in the reaction made them cumbersome for a systematic study. For this reason an improvement in the chromatographic method, for a quick identification and/or estimation of the diastereoisomers of the cinnamoyl derivatives, was developed.

Thin-Layer Chromatography

Table 1 shows the Rf values of the diastereoisomers of the ethyl cinnamates obtained on silica gel (S1) and cellulose (S2), developed on

TABLE 1. TLC and HPLC Data of Ethyl Cinnamates

		TLC [a]		HPLC [b]	
Nº	Compound	S_1/F_1	S_2/F_2	L_1	L_2
1	Ethyl *cis*-caffeate	39	67	8.29	11.40
2	Ethyl *trans*-caffeate	34	38	9.21	13.32
3	Ethyl *cis*-ferulate	62	67	15.89	24.23
4	Ethyl *trans*-ferulate	47	43	17.88	28.68
5	Ethyl *cis*-isoferulate	46	68	14.36	22.32
6	Ethyl *trans*-isoferulate	44	36	16.95	28.36

a) For TLC systems see Experimental. The Rf values are in Rf .100.

b) For HPLC systems see Experimental. The Rt are in min.

the system F1 and F2, respectively. The system S2/F2 gave a better separation of isomers than the system S1/F1, although it was quite time consuming (see Experimental).

Concerning the pair of diastereoisomers **3-4** it was found that their chromatographic behaviour, i.e. in Rf and fluorescence color, seems to be similar in both chromatographic systems (**Tables 1, 2** and **3**). The pairs of diastereoisomers **1-2** and **5-6** seem to have similar chromatographic separation (**Table 1**).

Table 2 and **Table 3** show the fluorescence colours of the compounds (UV light 366 nm), before and after KOH treatment. It was observed that the original fluorescence produced by the compounds changed after spraying with the chromogenic reagent. The effect of the spraying reagent on the fluorescence colours of the cinnamates, both before or after heating, is undoubtedly helpful for a rapid detection and characterization of the ethylcinnamates.

High Performance Liquid Chromatography

In order to observe the chromatographic behaviour of the Z/E isomers of ethyl esters a study was carried out with sample solutions (see Experimental).

Using the binary solvent system, acetonitrile/aqueous acetic acid and the reversed-phase octylsilane packing used in the analysis of the corresponding cinnamic acids [5-7] it was possible to obtain the separation and estimation of the diastereoisomers of the cinnamates derivatives.

The results of this chromatographic study were shown in **Table 1**. These findings allow us to conclude that with the mobile phase L1 the

TABLE 2. Colour of Fluorescence of the Ethyl Cinnamates Spots on TLC, System S1/F1[a], With/Without the Chromogenic Reagent.

Nº	Compound	Spot appearance [b]		
		UV	KOH/UV	
			before heating	after heating[c]
1	Ethyl *cis*-caffeate	lt-b	bn	bn-y
2	Ethyl *trans*-caffeate	lt-b	bn-y	bn-y
3	Ethyl *cis*-ferulate	b	lt-gn	lt-b
4	Ethyl *trans*-ferulate	b	lt-gn	lt-b
5	Ethyl *cis*-isoferulate	lt-b	bn	lt-bn
6	Ethyl *trans*-isoferulate	lt-b	lt-bn	lt-bn

a) For TLC system see Experimental.

b) Colour of the fluorescence at 366 nm : b, blue; lt-b, light blue; bn-y, brownish yellow; lt-gn, light green; lt-bn, light brown; bn, brown

c) The plates were sprayed with the chromogenic reagent and observed immediately, after which the colours tend to darken.

TABLE 3. Colour of Fluorescence of the Ethyl Cinnamates Spots on TLC, System S2/F2[a], With/Without the Chromogenic Reagent.

Nº	Compound	Spot appearance [b]		
		UV	KOH/UV	
			before heating	after heating[c]
1	Ethyl *cis*-caffeate	b	y-gn	y
2	Ethyl *trans*-caffeate	b	y-gn	y
3	Ethyl *cis*-ferulate	lt-b	lt-gn	b
4	Ethyl *trans*-ferulate	lt-b	lt-gn	b
5	Ethyl *cis*-isoferulate	p	y	y
6	Ethyl *trans*-isoferulate	p	y	y

a) For TLC system see Experimental.

b) Colour of the fluorescence at 366 nm : b, blue; lt-b, light blue; p, purple, y-gn, yellowish green; lt-gn, light green; y, yellow.

c) The plates were sprayed with the chromogenic reagent and observed immediately, after which the colours tend to darken.

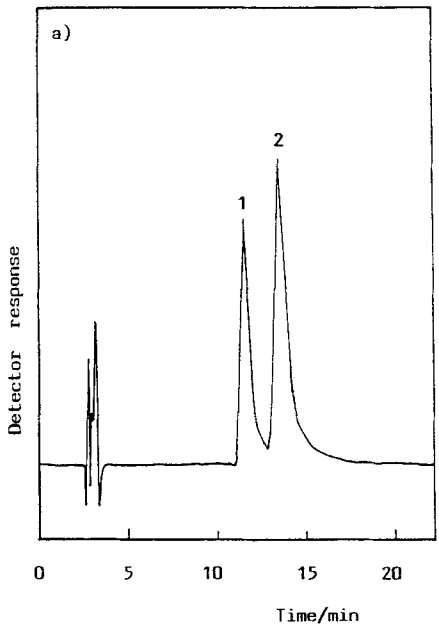


FIGURE 2

Chromatogram of the sample solutions containing:

a) **1.** Ethyl *cis*-caffeate ; **2.** Ethyl *trans*-caffeate (Mobile phase : L2)

b) **3.** Ethyl *cis*-ferulate ; **4.** Ethyl *trans*-ferulate (Mobile phase : L1)

c) **5.** Ethyl *cis*-isoferulate ; **6.** Ethyl *trans*-isoferulate(Mobile phase : L1)

Other conditions described in Experimental

separation between the pairs of the diastereoisomers **3-4** and **5-6** was achieved. To improve resolution the polarity of the mobile phase was increased (system L2), leading to an excellent separation between the geometrical isomers **1-2**. In this system the separation between the Z/E isomers of the methoxy cinnamate derivatives was also improved, although it involved a longer time of analysis.

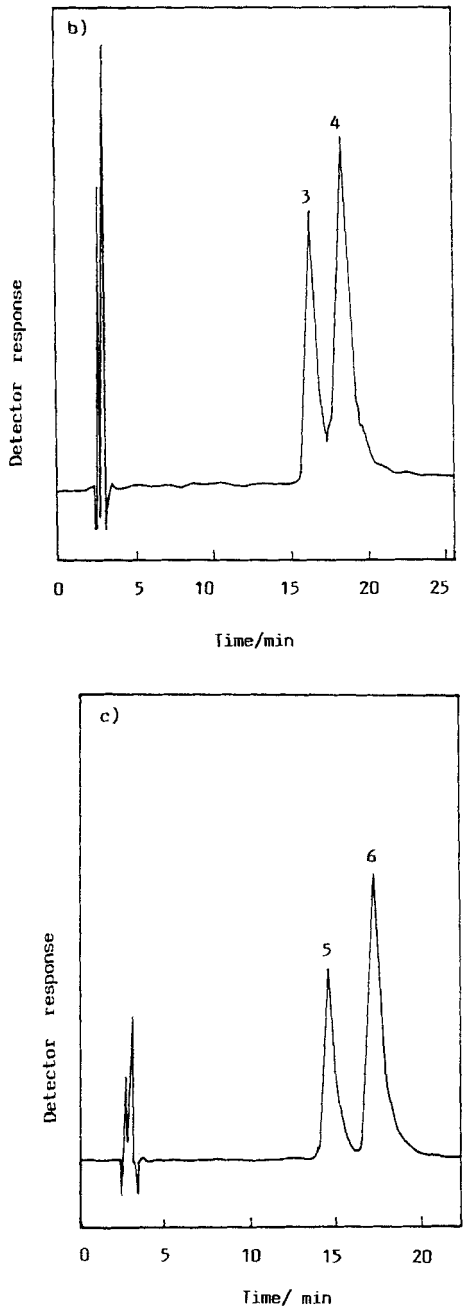


FIGURE 2 (continued)

Although the differences in the observed selectivities are not striking (1.11; 1.12; 1.18 with L1 and 1.16; 1.18; 1.27 with L2) the variation found on the retention times is relevant when a simultaneous isocratic separation and identification of a mixture of natural or synthetic compounds was carried out.

The expected elution pattern [5-7] in this reversed-phase system is observed : the *cis* isomers are first eluted , while the *trans* isomers have longer retention times. **Fig. 2** shows the isocratic elution profile of the Z/E isomers of the cinnamic acid derivatives. The separation took less than 20 min.

The HPLC method presented appears to be a good alternative to TLC. It provides a mean for a fast, simultaneous, sèmi-quantitative screening of the composition of the solutions performed in our work.

Although designed specifically for the simultaneous determination of the diastereoisomers of cinnamic acid ethyl esters of synthetic mixtures, the chromatographic systems developed herein could be a valuable contribution for food chemistry [8].

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