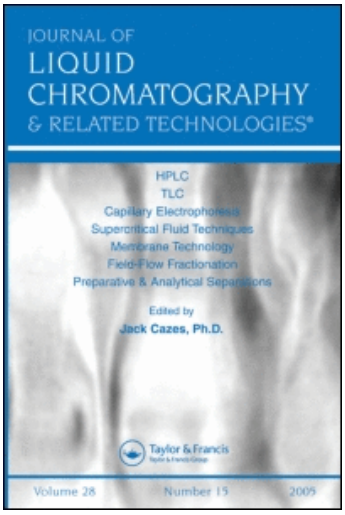

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Quantitative Analysis of Kielcorins in Biomimetic Synthesis by Liquid Chromatography/UV Detection

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ABSTRACT

An HPLC/UV assay was developed for separation and quantification of isomeric kielcorins in different synthetic crude products. *trans*-(±)-Kielcorin C, *cis*-(±)-kielcorin C, *trans*-(±)-kielcorin D, *trans*-(±)-isokielcorin D, and *trans*-(±)-kielcorin E were obtained by oxidative coupling of coniferyl alcohol and an appropriate xanthonic building block, 3,4-, 1,2-, and 2,3-dihydroxyxanthone, respectively, with silver carbonate, silver oxide, and potassium hexacyanoferrate (III). The isomeric kielcorins were separated and quantified directly in the nine crude

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products obtained. The assay was validated with respect to specificity, linearity, range, limits of quantification, and detection.

Key Words: Liquid chromatography; Isomeric separation; Kielcorins; Oxidative coupling.

INTRODUCTION

Kielcorins are 1,4-benzodioxane derivatives belonging to the xanthonolignoids, with a skeleton in which a phenylpropane unit is linked to a xanthone nucleus through a dioxane bridge.[1] We have previously achieved a biomimetic synthesis of natural kielcorin and kielcorin B and evaluated their biological activities.[2,3] These compounds exhibited an interesting hepatoprotective activity against *tert*-butylhydroperoxide-induced toxicity in isolated rat hepatocytes.[4] In order to obtain compounds belonging to the kielcorin's family for structure/activity relationship studies, we have synthesized *trans*-(±)-kielcorin C (1), *cis*-(±)-kielcorin C (2), *trans*-(±)-kielcorin D (3), *trans*-(±)-isokielcorin D (4), and *trans*-(±)-kielcorin E (5).

Prior work on the synthesis of 1,4-benzodioxans showed the influence of several oxidising agents in the nature and amounts of the products formed in oxidative coupling type reactions.[5–7] Concerning biomimetic synthesis of xanthonolignoids, little information has been accumulated,[2,3,8] hence, the importance of investigating the oxidative coupling reaction in the presence of several oxidizing agents. The synthetic approach of these constitutional isomers was based on biomimetic pathway by oxidative coupling of coniferyl alcohol[6] with an appropriate xanthone:[2,3] 3,4-dihydroxy (7), 1,2-dihydroxy (8), and 2,3-dihydroxyxanthone (9), for C, D, and E series, respectively (Figure 1), using different oxidising agents: silver carbonate (Ag_2CO_3), silver oxide (Ag_2O), and potassium hexacyanoferrate (III) ($K_3[Fe(CN)_6]$).

Attempts to resolve the isomeric mixtures of kielcorins 1-2 and 3-4 by preparative thin layer chromatography were unsuccessful in separating *trans*/*cis* or normal/iso forms, respectively, in a quantitative mode. Hence, we considered developing a method for isomeric resolution and quantification of kielcorins 1–5 that allow us to compare the results obtained from different synthetic conditions.

In recent years, high-liquid chromatography in reversed-phase mode (RPHPLC) has been applied successfully to a number of isomeric separations due to the selectivity and sensitivity of this chromatographic technique and to faster separations of positional[9–11] and stereoisomers.[12,13] Although, there is no data concerning the application of HPLC to xanthonolignoids,

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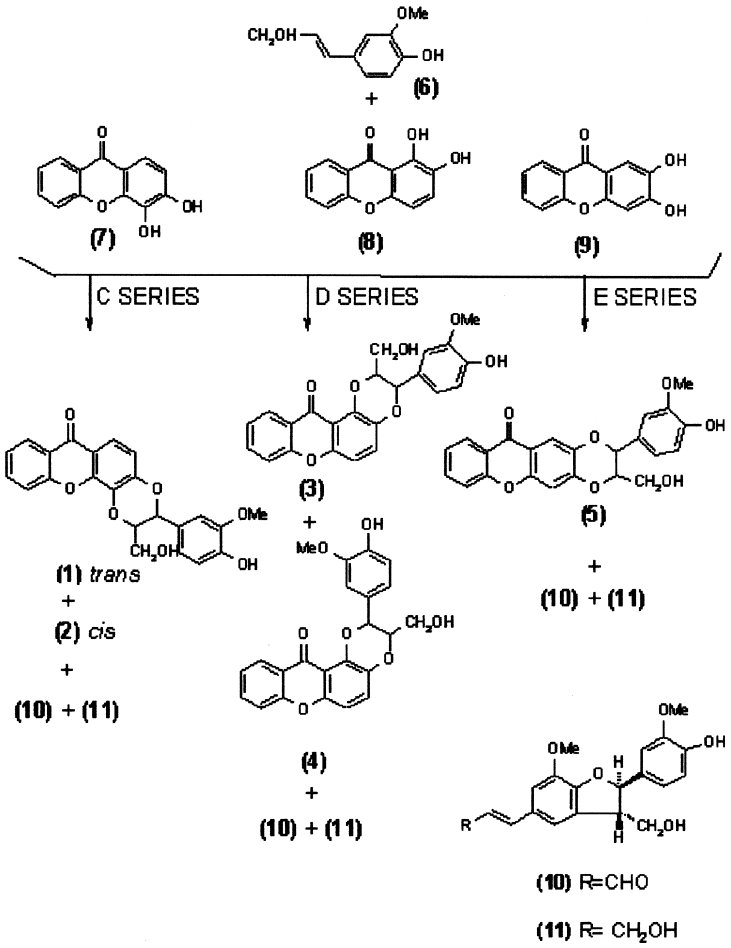

Figure 1. One-step biomimetic synthesis of kielcorins: *trans*-(±)-kielcorin C (1), *cis*-(±)-kielcorin C (2), *trans*-(±)-kielcorin D (3), *trans*-(±)-isokielcorin D (4), and *trans*-(±)-kielcorin E (5).

this technique has been used to separate compounds with similar features, namely xanthones[14–16] and 1,4-benzodioxane derivatives.[5]

In this work, we present the development and validation of an RPHPLC method with UV detection that allows the direct quantification of isomeric kielcorins from the crude product. This method was applied to the analysis of the isomeric kielcorins obtained in nine crude products from a comparative

study using different oxidising agents (silver carbonate, silver oxide, and potassium hexacyanoferrate (III)).

EXPERIMENTAL

Instrumental and Operating Conditions

A model 880 PU (Jasco, Japan) equipped with a 875/UV detector (Jasco, Japan), a Rheodyne 7125 injector, and a CSW 1.7 integrator was employed. Analysis was performed at 25°C on a C 18 Nucleosil column (5 μm, 250 × 4.6 mm I.D.), from Macherey-Nagel (Düren, Germany) equipped with a Macherey-Nagel guard precolumn. Separations of the crude products were performed in isocratic work at a flow rate of 1 mL/min with detection on 239 nm for C and D series, and 241 nm for E series. The sample injection volume was 20 μL. For compounds 1–2, separation was achieved with methanol/1% acetic acid in water (6 : 4 *v*/*v*), for compounds 3–4, with acetonitrile/1% acetic acid in water (5 : 5 *v*/*v*), and for compound 5, with methanol/1% acetic acid in water (7 : 3 *v*/*v*). The column void time (t_0) was considered to be equal to the peak of the solvent front and was taken from each particular run.

Reagents and Materials

Silver carbonate, sodium acetate, and potassium hexacyanoferrate (III) were supplied by Merck (Darmstadt, Germany). Coniferyl alcohol and silver oxide were obtained from Sigma (Steunheim, Germany). Acetone, toluene, and acetic acid glacial 100% were of analytical grade from Merck. Methanol and acetonitrile were of HPLC grade from Merck. HPLC ultrapure water was generated by a Milli-Q system (Millipore, Bedford, MA, USA). The mobile phases were degassed for 15 min in an ultrasonic bath before use. All samples were filtered through a hydrophilic Durapore-GV membrane of 0.45 μm pore size from Millipore before injection.

General Procedure for the Synthesis of Kielcorin Derivatives

With Silver Carbonate/Silver Oxide

The appropriate dihydroxyxanthone (7–9) (315 mg, 1.4 mmol) and coniferyl alcohol (6) (250 mg, 1.4 mmol) were taken in toluene/acetone (1 : 1) and stirred with 2.0 mmol of the oxidizing agent (550 mg of silver carbonate/460 mg of

silver oxide) in the dark for 2–3 days. The suspension was filtered, the filtrate evaporated, and the crude product was analysed by HPLC.

With Potassium Hexacyanoferrate (III)

To a stirred solution of the appropriate dihydroxyxanthone (7–9) (315 mg, 1.4 mmol) and coniferyl alcohol (6) (250 mg, 1.4 mmol) in acetone/water (1 : 1), sodium acetate (0.9 g, 11 mmol, in 25 mL of water), and then potassium hexacyanoferrate (III) solution (1.0 g, 30 mmol in 25 mL of water) was added at room temp. After reaction, the mixture was slightly acidified with 10% hydrochloric acid and diluted with water. The product was extracted with dichloromethane; the extract was washed with water, dried, and the solvent evaporated to dryness. The crude product was analyzed by HPLC.

Sample Preparation

The nine crude products obtained (ca. 600 mg) were accurately weighed and dissolved in 500 mL of methanol. Serial dilutions in methanol were made to the following final concentrations: 100, 200, and 300 μg/mL, for use in determination of precision assays.

Stock standard solutions of each kielcorin 1–5 were prepared by dissolving 2.5 mg accurately weighed in 25 mL of methanol. Standard solutions were obtained by the dilution of stock solutions with methanol at least to five different concentrations over the range of interest, in this case, 5.0, 25, 50, 75, 100, and 200 μg/mL for *trans*-kielcorin C (1), 0.5, 2.5, 3.75, 12.5, and 50 μg/mL for *cis*-kielcorin C (2), 10, 25, 50, 75, and 100 μg/mL for *trans*-kielcorin D (3), 0.25, 0.5, 1.0, 2.5, 5.0, 10, and 25 μg/mL for *trans*-isokielcorin D (4), and 0.5, 1.0, 2.5, 5.0, and 25 μg/mL for *trans*-kielcorin E (5).

RESULTS AND DISCUSSION

Method Development

The present assay was developed for the analysis of three series of kielcorins: C series (1–2), D series (3–4), and E series (5) (Fig. 1). The separations of the kielcorin isomers were performed on RPHPLC and were optimised for each series to the described experimental conditions used for the validation procedure (Figs. 2 and 3). Chromatographic factors are listed in Table 1. Several kinds of mobile phase compositions were investigated,

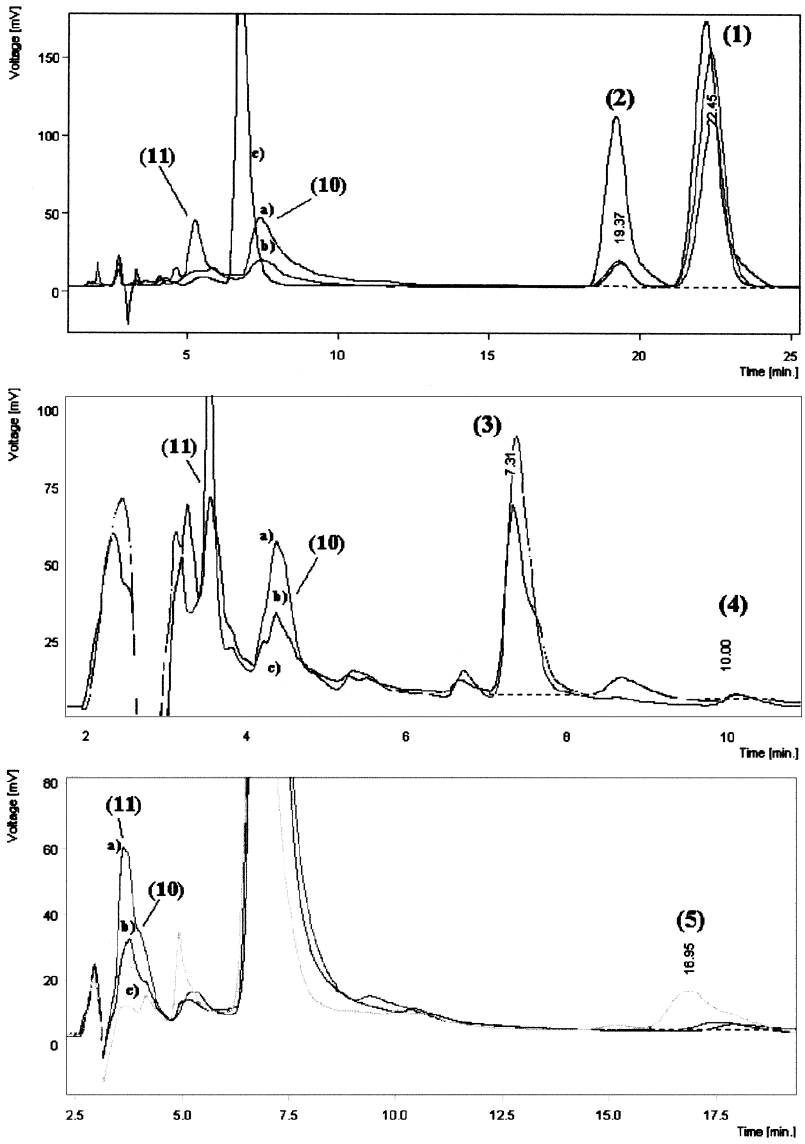

Figure 2. Chromatograms of 200 g/mL solutions of the crude products obtained in C, D, and E series using as oxidizing agent: a) Ag_2CO_3, b) Ag_2O, and c) $K_3[Fe(CN)_6]$.

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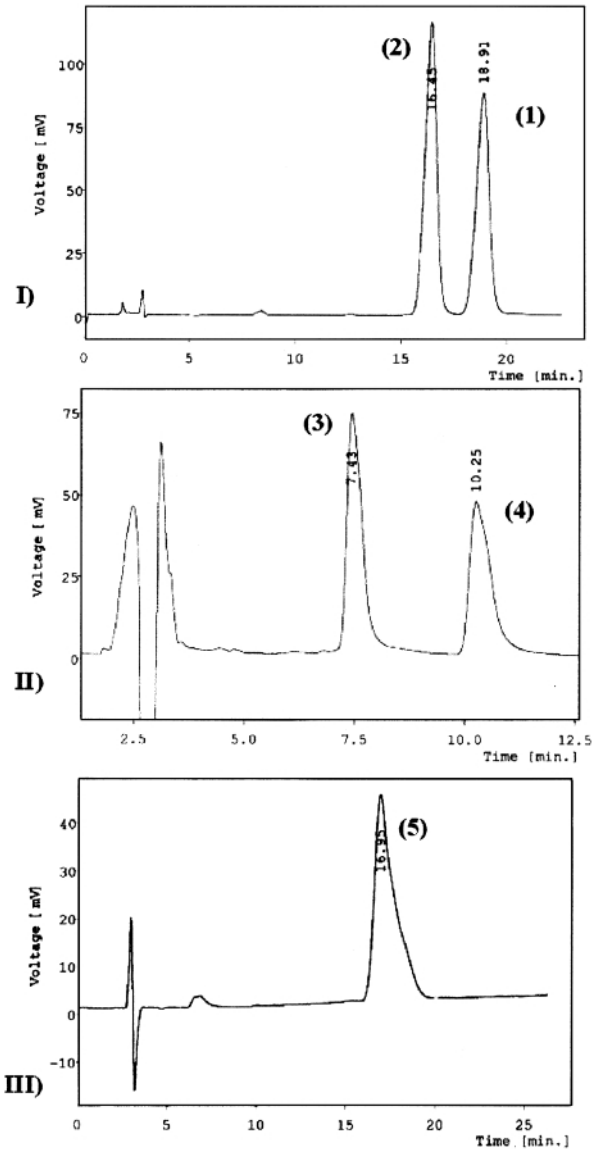

Figure 3. Chromatograms of standards//conditions: I) trans-(±)-kielcorin C (1) and cis-(±)-kielcorin C (2)// methanol/1% acetic acid in water (6 : 4), II) trans-(±)-kielcorin D (3) and trans-(±)-isokielcorin D (4)// acetonitrile/1% acetic acid in water (5 : 5), and III) trans-(±)-kielcorin E (5)// methanol/1% acetic acid in water (7 : 3).

Table 1. Separation performance:[a] retention factors (k'_1), separation factors (α) and resolution factors (R_s), of the investigated kielcorin derivatives 1–5 in optimised chromatographic conditions.[b]

	Mobile phase	Oxidising agent	k'_1	α	R_s
1	MeOH/HOAc 1% in H_2O (6 : 4)	Ag_2CO_3/Ag_2O	4.3	1.2	3.5
		$K_3[Fe(CN)_6]$	4.3	1.2	3.6
2	MeOH/HOAc 1% in H_2O (6 : 4)	Ag_2CO_3/Ag_2O	3.5	4.9	12.7
		$K_3[Fe(CN)_6]$	3.5	5.6	20.4
3	MeCN/OHAc 1% in H_2O (5 : 5)	$Ag_2CO_3/Ag_2O/K_3[Fe(CN)_6]$	1.2	1.1	2.2
4	MeCN/HOAc 1% in H_2O (5 : 5)	Ag_2CO_3/Ag_2O	2.1	1.3	3.3
		$K_3[Fe(CN)_6]$	2.1	1.6	3.2
5	MeOH/HOAc 1% in H_2O (7 : 3)	$Ag_2CO_3/Ag_2O/K_3[Fe(CN)_6]$	3.8	2.1	7.3

[a]The values are the mean of three experiments. The column void time (t_0) was considered to be equal to the peak of the solvent front and was taken from each particular run. It was about 2.2 min in MeOH/HOAc 1% in H_2O (6 : 4), 2.5 min in MeCN/HOAc 1% in H_2O (5 : 5) and 2.6 min MeOH/HOAc 1% in H_2O (7 : 3).
[b]Chromatographic conditions as in Figure 2 (200 μg/mL solutions of the crude products).

and under optimised conditions, kielcorins 1–5 were fully resolved ($R_s > 2$) and their retention factors (k') satisfactory (1.2–4.8).[17]

The standard compounds 1–5 were previously isolated and purified from crude products and characterised by spectroscopic methods. Detailed synthesis and complete assignment will be published elsewhere.

Assay Validation

The assay was validated with respect to specificity, linearity, range, limits of quantification and detection, and precision. Accuracy was inferred once precision, linearity, and specificity had been established. The terms are defined according to ICH guideline 3AQ[18] and to U. S. Pharmacopeia 24.[19]

The specificity of the analytical method in this study was determined by the analysis of the side products of the oxidative coupling reactions,[20] phenylcoumaran (10) and dehydrodiconiferyl alcohol, (11) and the xanthonic precursors (7–9) (Figure1) in standard solutions, which absorb in the same λ values. Under the applied conditions, no interference from the components

of the crude product was observed at the retention time for kielcorins 1–5 (Fig. 2).

Linearity was evaluated in triplicates of at least five calibration standard solutions of the compounds 1–5. The range of the calibration curves was set at 80 to 120% of the kielcorins concentration in the crude product solution.[18,19] The regression line was calculated as $y = a + bx$, where x was the kielcorin concentration (μg/mL) and y the response (peak area expressed as mAU). The calibration curves were obtained using the linear least squares regression procedure. The calibration data of the kielcorin derivatives 1–5 is summarized in Table 2. The RSD (relative standard deviation) values for the response factors of three experiments were in the range 0–5% (3.6, 3.6, 4.0, 3.7, and 2.0 % for compounds 1–5, respectively), and they were considered adequate for verifying the linearity of the regression.[21] Linear relationships with regression coefficients (R) of at least 0.999 were found for all substances, hence, there were linear relationships between the amounts of kielcorins 1–5 and the detector response.

The limits of detection (LODs) and quantification (LOQs) were evaluated by analysing ten blank samples. LODs and LOQs were expressed as the standard deviation of these responses, divided by the slope and multiplied by a factor of 3.3 for LODs and 10 for LOQs.[17,18] The LODs were found to be 0.050 μg/mL, 0.051 μg/mL, 0.052 μg/mL, 0.054 μg/mL, and 0.010 μg/mL for *trans*-kielcorin C (1), *cis*-kielcorin C (2), *trans*-kielcorin D (3), *trans*-isokielcorin D (4), and *trans*-kielcorin E (5), respectively. The LOQs were found to be 0.15 μg/mL, 0.15 μg/mL, 0.16 μg/mL, 0.16 μg/mL, and 0.03 μg/mL for *trans*-kielcorin C (1), *cis*-kielcorin C (2), *trans*-kielcorin D (3), *trans*-isokielcorin D (4), and *trans*-kielcorin E (5), respectively.

The precision of the assay was investigated with respect to repeatability and intermediate precision. For intra/day precision three concentrations of the crude product were analysed within 24 hours. Within each series, every sample was injected at least five times. For intermediate precision, samples from

Table 2. Calibration data of the kielcorin derivatives 1–5.

	Range (μg/mL)	*y*-Intercept[a]	Slope[a]	Correlation coefficient (R)
1	5.00–200	-56.45 ± 2.03	141.4 ± 5.09	0.9991
2	0.50–50.0	93.14 ± 3.35	137.8 ± 4.96	0.9988
3	10.0–100	-76.60 ± 3.06	105.2 ± 4.21	0.9990
4	0.25–25.0	13.58 ± 0.50	129.6 ± 4.79	0.9993
5	0.50–25.0	44.99 ± 0.90	136.8 ± 2.74	0.9993

[a]The values are the mean ± standard deviation of three experiments.

three concentrations of the crude products were analysed on five consecutive days assaying, in quintuplicates. Precision was expressed as relative standard deviation (RSD) given by the standard deviation value of the peak area divided by the mean.[18] Table 3 summarizes the RSDs for repeatability and intermediate precision. The RSD values of results from replicate analyses ranged between 0.95 and 10.4% showing that the precision of the method was acceptable.

Application

The aim of our work was the analysis of kielcorin derivatives directly in crude products obtained in the oxidative coupling type reactions. To evaluate the influence of oxidizing agents in the nature and quantities of the isomeric kielcorins, we developed a rapid method for the separation of these isomeric compounds. This method was applied in a comparative study using as oxidizing agents silver carbonate, silver oxide, and potassium hexacyanoferrate (III).

Data of the quantification results are summarized in Table 4. In C series, none of the oxidizing agents used afforded stereoselectivity, although

Table 3. Precision data for HPLC-UV assay of the kielcorin derivatives 1–5.

	Concentration of the crude product (mg/mL)	Repeatability RSD (%) ($n^a = 15$)	Intermediate precision RSD (%) ($n^a = 30$)
1	0.100	10.43[b]	4.569[c,d]
	0.200	1.602[b]	3.153[c,d]
	0.300	1.418[b]	6.147[c,d]
2	0.100	7.768[b]	3.541[c,d]
	0.200	1.120[b]	3.081[c,d]
	0.300	1.354[b]	4.433[c,d]
3	0.100	2.364[c]	3.752[b,d]
	0.200	4.735[c]	1.336[b,d]
	0.300	6.216[c]	4.082[b,d]
4	0.100	4.500[c]	9.270[b,d]
	0.200	2.571[c]	2.682[b,d]
	0.300	7.741[c]	2.851[b,d]
5	0.100	1.189[c]	2.134[b,d]
	0.200	1.008[c]	0.846[b,d]
	0.300	1.020[c]	0.947[b,d]

[a]n = number of injections.
[b,c,d]RSD values for peak area of compounds 1–5 obtained in the oxidative coupling using Ag_2O, Ag_2CO_3, and $K_3[Fe(CN)_6]$ as oxidizing agents respectively.

Table 4. Quantification of compounds 1–5 in the crude products.[a]

	Ag_2CO_3[b]	Ag_2O[b]	$K_3[Fe(CN)_6]$[b]
1	27%	36%	39%
2	3.1%	3.5%	11%
3	22%	20%	34%
4	0.4%	0.7%	1.1%
5	2.4%	1.9%	10.4%

[a]The values are represented in yields of the oxidative coupling reactions.
[b]Oxidizing agents used in the comparative study.

when using silver carbonate and silver oxide, less amounts of the *cis* isomer 2 was obtained. Two dimeric compounds, phenylcoumaran (10) and dehydrodiconiferyl alcohol (11) were also found as secondary derivatives (Figure 2) in all the reactions.[3] By carrying out the oxidative coupling in potassium hexacyanoferrate (III), the quantities of 10 and 11 were markedly reduced and higher yields of kielcorins were obtained. The most efficient method involved the use of potassium hexacyanoferrate (III) as oxidizing agent in the oxidative coupling reaction (Table 4).

CONCLUSIONS

A method based on RPHPLC/UV has been developed for the separation of isomeric xanthonolignoids in crude products reactions. Baseline separations were successfully achieved for all kielcorin derivatives under study. The assay was found to be precise, sensitive, and calibration curves were linear within the studied concentration range, allowing the quantification of these compounds. The method was applied to a comparative study using different oxidizing agents in oxidative coupling type reactions. Further investigations, namely enzymatic oxidation and solid phase reactions, are under study in order to improve yields and regio/stereoselectivity in kielcorins synthesis. Hence, HPLC methodology can be successfully applied for direct analysis of xanthonolignoids and quantitative studies in synthetic crude products.

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