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Assay of 2,3-dihydroxybenzoic acid and related compounds in plant materials by high-performance liquid chromatography

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Abstract

Salicylic acid and its putative biosynthetic precursors were assayed isocratically by RP-HPLC with UV detection at 280 nm. Optimum resolution was provided by an HPLC mobile phase consisting of MeOH–1% aqueous HOAc (40:60, v/v), at pH 4. Furthermore, for the analysis of 2,3-dihydroxybenzoic acid (2,3-DHBA) in *Catharanthus roseus* cell cultures after elicitation, a mobile phase consisting of acetonitrile–1% aqueous HCOOH containing 0.25% trichloroacetic acid (1:5, v/v), at pH 2, was used. The recovery for the free form of 2,3-DHBA was about 80% after a one-step extraction of the cells. The detection limit of 2,3-DHBA was 3 μg by using saligenin as an internal standard.

Keywords: Plant materials; *Catharanthus roseus*; Dihydroxybenzoic acid; Benzoic acids; Salicylic acid

1. Introduction

Salicylic acid (SA) is widely distributed in angiosperms and has an important regulatory role in plants. It serves for example as an endogenous inducer of thermogenesis and it acts as an endogenous signal for the induction of systemic acquired resistance in plants infected by pathogens [1,2]. Therefore, the biosynthetic pathway of SA is intensively studied in different plants. SA is thought to be derived from phenylalanine via *trans*-cinnamic acid (CA), through either *o*-coumaric acid (*o*-COA) or benzoic acid (BA) as an intermediate [3,4]. However, little is known about the biosynthetic pathway of a variety of other substituted BA derivatives, such as 2,3-dihydroxybenzoic acid (2,3-DHBA) and 2,5-dihydroxybenzoic acid (2,5-DHBA) in plants. In previous studies on the biosynthesis of hydroxybenzoic acids it was shown that those two compounds might originate from SA, as this compound administered to plants was hydroxylated in the 3- or 5-position giving rise to 2,3-DHBA and 2,5-DHBA [5].

In the course of our studies on the biosynthetic pathway of SA and the closely related compound 2,3-DHBA in plants, we used *Catharanthus roseus* cell suspension cultures as a model. This system has several advantages, since a high production of 2,3-DHBA was observed after elicitation of *C. roseus* cell cultures with a cell wall preparation of the fungus *Pythium aphanidermatum* [6]; SA can also be

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produced in yeast-elicited cells. To conduct our studies, we needed assays for the determination of these compounds.

Several HPLC methods have been reported for the determination of SA and other BA derivatives. In most cases, the plant phenolic acids were detected using an RP-HPLC system with UV detection [7,8]. In these systems, BA and CA were assayed separately with UV detection at 280 nm, whereas SA and *o*-COA were quantified fluorimetrically at different wavelengths. For biological samples, such as human plasma, an HPLC separation coupled with an electrochemical detector has been used in order to measure SA, 2,3-DHBA and 2,5-DHBA [9,10]; it offers the advantage of a high sensitivity (about 0.1 pmol of the analytes). However, SA can not be analysed at the same potential as its hydroxylated products.

In the present study, we describe the development of two HPLC systems: one for the analysis of SA and its biosynthetic precursors, another for the analysis of 2,3-DHBA and 2,5-DHBA.

2. Experimental

2.1. Chemicals

All solvents used for HPLC were either of analytical or HPLC grade. Methanol and acetonitrile (ACN) were purchased from Rathburn (Walkerburn, UK). Acetic acid and formic acid were from Baker (Deventer, Netherlands), and trichloroacetic acid (TCA) from Merck (Darmstadt, Germany). All of the reference compounds were obtained from Sigma (St. Louis, MO, USA).

2.2. HPLC conditions

All the data were obtained using an isocratic RP-HPLC system consisting of a 515 HPLC pump from Waters (Milford, MA, USA) equipped with a Gilson model 232 autosampler (sample injection volume 5 μl), and a 2158 Uvicord SD UV detector from LKB equipped with an 8-μl flow cell operating at 280 nm. The analyses were carried out at room temperature using a 125×4 mm I.D. LiChrospher 60 RP-select B column (Merck), with a particle size of 5 μm, and a flow-rate of 0.8 or 1.0 ml/min. A guard column (LichroCart 4-4, Merck) was used in combination with the analytical column.

Several mobile phases were examined for the determination of benzoic acid derivatives. The first group tested was a mixture of various percentages of MeOH (Fig. 1) in 1% aqueous HOAc adjusted to pH 4.0 with 6 *M* NaOH. Subsequently, mobile phases consisting of MeOH–1% aqueous HOAc (40:60, v/v) adjusted to various pH values (Fig. 2) were tested. Another group of mobile phases consisted of a mixture of various percentages of ACN in 1% aqueous HCOOH containing 0.25% TCA adjusted to pH 2.0 (Fig. 5). The last group of mobile phases consisted of ACN–1% aqueous HCOOH containing 0.25% TCA (1:5, v/v) set to various pH values (Fig. 6) with either 6 *M* NaOH or phosphoric acid. The mobile phases were filtered through a nylon filter, pore size 0.45 μm (Sigma–Aldrich Chemie, Zwijndrecht, Netherlands) and degassed under vacuum before their use.

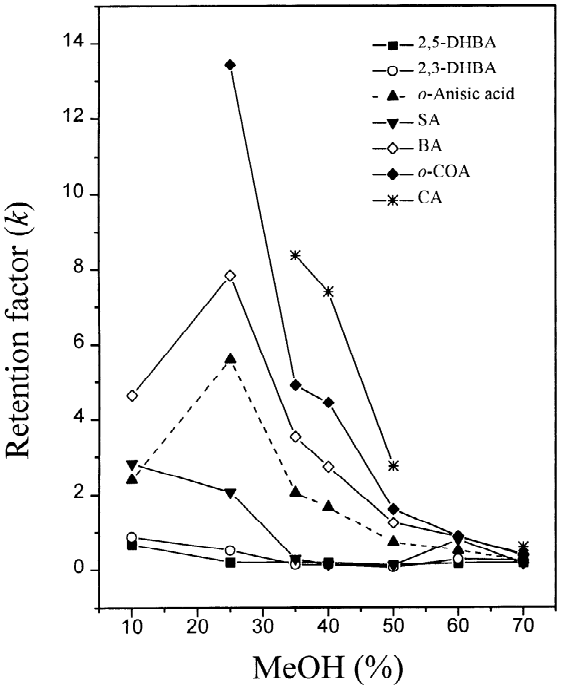

Fig. 1. Graph of *k* values vs. percentage of MeOH in 1% aqueous HOAc, at pH 4. Abbreviations of acids: DHBA= dihydroxybenzoic acid, SA=salicylic acid, BA=benzoic acid, *o*-COA=*o*-coumaric acid and CA=cinnamic acid.

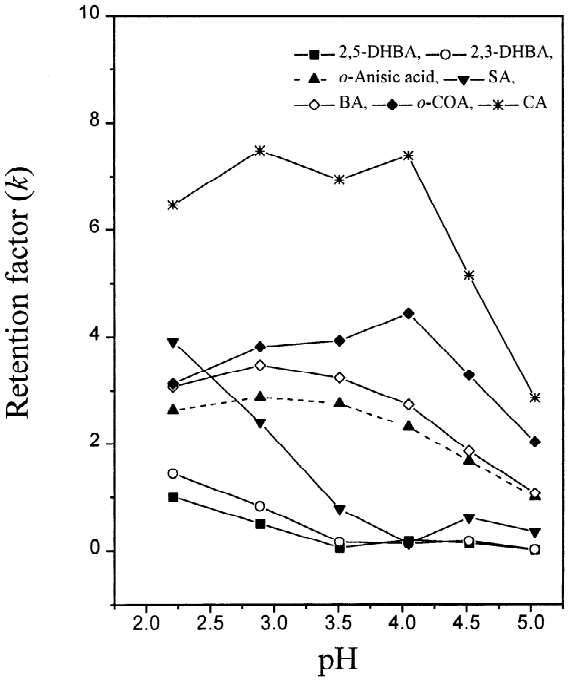

Fig. 2. Graph of *k* values vs. pH of the mobile phase consisting of MeOH–1% aqueous HOAc (40:60, v/v).

2.3. Plant material and elicitation

Cell cultures of *C. roseus* (L.) G. Don (Madagascar periwinkle) were grown under conditions as previously described [11]. For the preparation of the elicitor, the fungus *Pythium aphanidermatum* (CBS 313.33) was maintained on MS [12] agar medium, containing 3% sucrose, at 27°C in the dark for a period of 7–10 days. One week after transferring the fungus to liquid medium, the fungal cells were separated from the medium by filtration and the filtrate was autoclaved. The filtrate was directly used as an elicitor or was kept at −20°C until further use. The elicitation was carried out by addition of the elicitor preparation (10 ml per flask) aseptically to 5-day-old *C. roseus* cell cultures.

2.4. Assay of 2,3-DHBA in C. roseus cell cultures

The level of 2,3-DHBA was measured both in the cells and in the medium after elicitation as described above. A sample of frozen *C. roseus* cells was pulverized in liquid nitrogen using a pestle and mortar; then 5 ml of acidified MeOH (90% MeOH and 10% water acidified with phosphoric acid, pH 3) containing saligenin (500 μg) were added to 1 g of the fine cell powder. The mixture was homogenized with a vortex mixer for about 1 min and further sonicated for 10–15 min. The supernatant was separated from the pellet after centrifugation at 3500 rpm for 15 min. The supernatant was evaporated under reduced pressure until dryness, and then dissolved in 500 μl of the mobile phase consisting of ACN–1% aqueous HCOOH containing 0.25% TCA (1:5, v/v) before analysis.

The bound form of 2,3-DHBA in the cells was measured after enzymatic hydrolysis using a slightly modified version of the method of Enyedi et al. [13]. A sample of about 0.5 g of frozen cells in liquid nitrogen was extracted with 2.5 ml of acidified MeOH containing saligenin (250 μg) as an internal standard. The supernatant was separated from the pellet after centrifuging the mixture at 14 000 rpm for 5 min, and was then evaporated under reduced pressure until dryness. Subsequently, the residue was resuspended in 250 μl of 0.2 m*M* sodium acetate buffer (pH 5.5), containing 2 mg of β-glucosidase (Sigma, sweet almond, 9 U/mg). After incubation at 37°C for 90 min, the reaction was stopped by addition of 0.5 ml of 10% TCA. The resulting mixture was centrifuged at 14 000 rpm for 3 min, and subsequently submitted to HPLC.

To quantify 2,3-DHBA in the medium of the *C. roseus* cell cultures, a sample of the medium after filtration was centrifuged at 3500 rpm for 1 min. The pH of the medium was adjusted to pH 3.5–4 with phosphoric acid and the resulting solution was injected directly into the HPLC system. Saligenin (125 μg/ml of sample) was added as an internal standard.

2.5. Determination of extraction recovery

We examined different substances as an internal standard which have previously been used by other workers such as *o*-anisic acid or 3,4-DHBA [7,9]. Moreover, we tested saligenin. Stock solutions were prepared by dissolving 100–150 mg of 2,3-DHBA in 100 ml of acidified MeOH together with one of the internal standards tested. A calibration curve was

made from the stock solutions by preparing various concentrations of the solutions by suitable dilution. For the determination of the extraction recovery, samples with a range of concentrations (50–500 μg) of 2,3-DHBA and an internal standard in 5 ml of acidified MeOH were added to 1 g of non-elicited cells (not containing free 2,3-DHBA). Subsequent manipulations were performed as for the elicited cells. The preparation was then analysed by HPLC and the extraction recovery was determined.

3. Results and discussion

3.1. The effects of the percentage of organic modifier and pH on retention time

The dependence of the retention factor of a solute (k) on the organic solvent content of a mobile phase has been used by some workers as a strategy to develop a simple isocratic HPLC system for the determination of a series of compounds [14,15].

We used this approach for optimizing the HPLC separation of some phenolic acids. The dependence of the k values of SA, 2,3-DHBA, 2,5-DHBA and their precursors such as CA, *o*-COA and BA, and of *o*-anisic acid, tested as internal standard, on the percentage of MeOH in the mobile phase is shown in Fig. 1. A wide range of k values ($0.1<k<14$) was obtained by varying the percentage of MeOH in the mobile phase. Most of the compounds tested can be separated in a mobile phase with MeOH levels of 25–40%, at pH 4.0. However, the resolution of these compounds becomes poor in a mobile phase containing about 50% MeOH or more. The changes of the MeOH amount in the mobile phase did not affect the resolution of the hydroxybenzoic acids to a large extent.

The effect of the pH on the retention time of the phenolic acids was studied in the mobile phase containing MeOH–1% aqueous HOAc (40:60, v/v). Fig. 2 shows that the k values of BA, *o*-COA, CA and *o*-anisic were not much affected by increasing the pH from 2.0 to 4.0, whereas the k values of the dihydroxybenzoic acids remained more or less constant, but low. Thus, 2,3-DHBA and 2,5-DHBA can not be separated with this mobile phase. From these results it was concluded that under isocratic conditions none of those systems was capable of separating all the BA derivatives. However, two different systems were found to be suitable for the two major analytical problems, i.e. the separation of SA and its biosynthetic precursors, and the analysis of 2,3-DHBA in the presence of SA, a possible biosynthetic precursor of 2,3-DHBA.

Fig. 3 shows the separation of SA and its precursors using the mobile phase MeOH–1% aqueous HOAc (40:60, v/v) at pH 4.0. The simultaneous determination of SA, BA, CA and *o*-COA could be achieved with this isocratic HPLC system in less than 15 min with the UV detector set at a single wavelength (280 nm). The detection limits (taken as a peak height of five times the noise level) were: SA=1.5 μg, CA=0.25 μg, *o*-COA=0.5 μg and BA=25 μg. Under this condition SA is eluted very early (k=1.6), which might give a problem when the compounds from plant materials — a complex matrix — must be analysed. However, the k value of SA can be increased further by reducing the MeOH

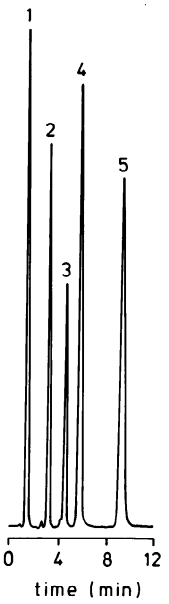

Fig. 3. Chromatogram obtained for phenolic acids on LiChrospher 60 RP-select B. Peaks: 1=SA (1.3 min), 2=*o*-anisic acid (3.1 min), 3=BA (4.6 min), 4=*o*-COA (5.8 min) and 5=CA (9.4 min). Mobile phase: MeOH–1% aqueous HOAc (40:60, v/v), pH 4. Flow rate: 1 ml/min. UV detection at 280 nm.

content of the mobile phase from 40 to 35%, and by lowering the pH to 3.5. We applied this system successfully for the determination of SA in tobacco leaves (*Nicotiana tabacum* L. c.v. Samsun NN), as shown in Fig. 4.

We encountered a problem in the analysis of 2,3-DHBA and 2,5-DHBA because of their similar properties (see Figs 1 and 2). Previous reports on the determination of these hydroxybenzoic acids also showed that these compounds were rapidly eluted in an HPLC system using a mobile phase containing 25% of MeOH and 75% of 20 m*M* sodium acetate buffer, at pH 5.0 [8].

As the MeOH-containing solvents were not satisfactory for the separation of 2,3-DHBA and 2,5-DHBA, a series of mixtures of ACN and 1% aqueous HCOOH containing 0.25% TCA, pH 2, were tested. Fig. 5 shows the improved band spacing of SA, 2,3-DHBA and 2,5-DHBA in the mobile phases containing 10–30% ACN; the *k* values are ranging from 2 to 21. In the mobile phase containing 10% ACN, we observed peak broadening, but all of the compounds tested were perfectly resolved. However, a good separation with better peak shapes was obtained with the mobile phases containing 15–20% ACN. The effect of the pH of the mobile phase on the resolution of SA, 2,3-DHBA and 2,5-DHBA was also studied, using the mobile phase consisting of ACN–1% aqueous HCOOH containing 0.25% TCA (1:5, v/v). The pH values ranging from 1.8 to 5.0 were set using 8 *M* NaOH. As shown in Fig. 6, the two dihydroxybenzoic acids and SA can be separated at pH 2–3.

Eventually, we developed a suitable isocratic method in which 2,3-DHBA can be determined separately from 2,5-DHBA and SA. As shown in Fig. 7, using the mobile phase consisting of ACN–1% HCOOH containing 0.25% TCA (1:5, v/v), at pH 2.0, 2,5-DHBA and 2,3-DHBA were eluted at about 4 and 5 min, respectively, while SA was eluted at about 12.5 min. The detection limit of 2,3-DHBA was 3 μg. This method was applied to quantify

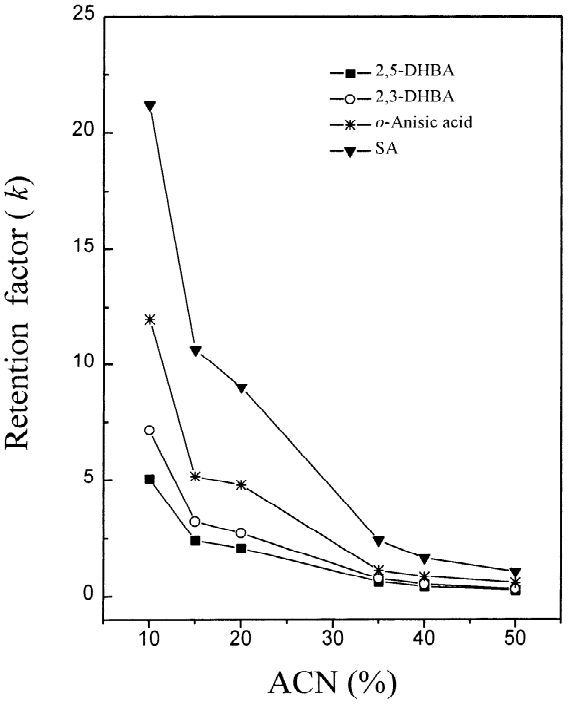

Fig. 5. Graph of *k* values vs. percentage of ACN in 1% aqueous HCOOH containing 0.25% TCA, at pH 2. Abbreviations of acids as in Figs. 1 and 2.

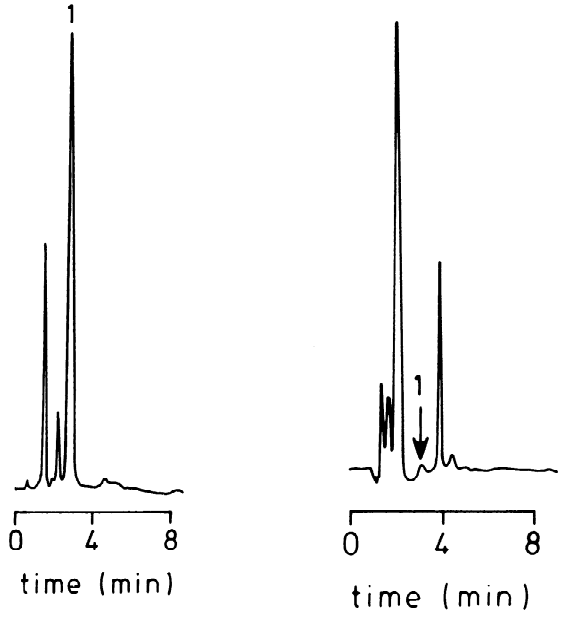

Fig. 4. Chromatograms obtained for salicylic acid (peak 1; 3.6 min) extracted from tobacco leaves with (left) and without (right) acid hydrolysis. Mobile phase: MeOH–1% aqueous HOAc (35:65, v/v), pH 3.5. Flow rate: 1 ml/min. UV detection at 280 nm.

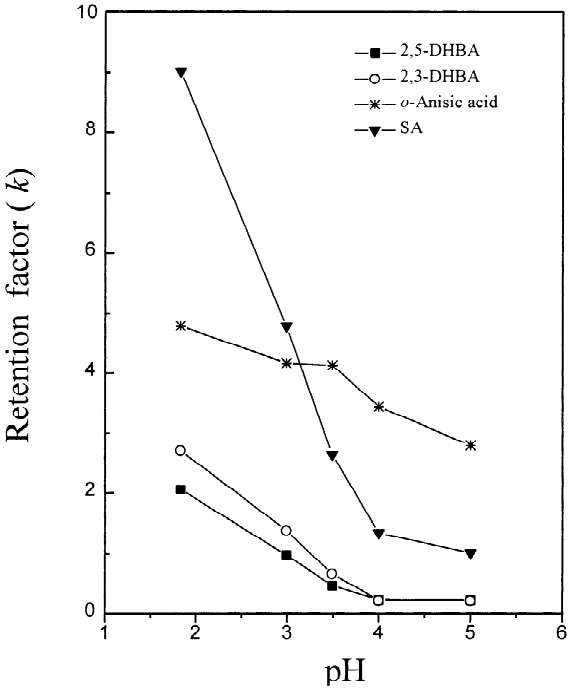

Fig. 6. Graph of *k* values vs. pH of the mobile phase consisting of ACN–1% aqueous HCOOH containing 0.25% TCA (1:5, v/v).

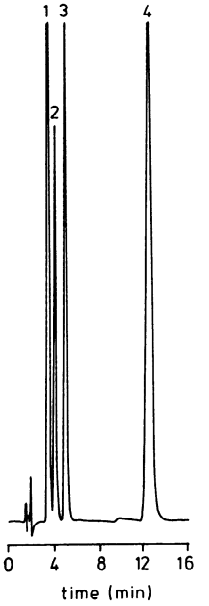

Fig. 7. Chromatogram obtained for phenolic acids on LiChrospher 60 RP-select B. Peaks: 1=saligenin (3.4 min), 2=2,5-DHBA (4.0 min), 3=2,3-DHBA (5.0 min) and 4=SA (12.5 min). Mobile phase: ACN–1% aqueous HCOOH containing 0.25% TCA (1:5, v/v), pH 2. Flow rate: 0.8 ml/min. UV detection at 280 nm.

2,3-DHBA in the biomass and in the medium of *C. roseus* cell cultures (Fig. 8).

3.2. Quantitative assay of 2,3-DHBA in plant materials

Cell suspension cultures of *C. roseus* accumulated 2,3-DHBA after elicitation with a cell wall preparation of *P. aphanidermatum* [6]. To be able to quantify endogenous 2,3-DHBA in such *C. roseus* cell cultures, several substances were tested in order to find a suitable internal standard. We found that 3,4-DHBA was not suitable as internal standard for the assay of 2,3-DHBA in *C. roseus* cells, since it was eluted very early (at about 2.7 min) in our system. It was therefore decided to test the suitability of saligenin and *o*-anisic acid as an internal standard.

For the determination of the extraction recovery, non-elicited *C.roseus* cells (not containing free 2,3-DHBA) were spiked with a wide range of concentrations of 2,3-DHBA (50–500 μg per g of cells, fresh weight) together with the same range of

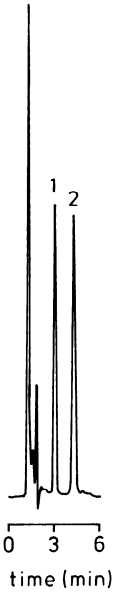

Fig. 8. Chromatogram obtained for 2,3-dihydroxybenzoic acid from the medium of elicited *Catharanthus roseus* cell cultures using the same conditions as described in Fig. 7. Peaks: 1= saligenin (internal standard, 3.2 min), 2=2,3-DHBA (4.7 min).

concentrations of either saligenin or *o*-anisic acid. Extraction losses were calculated for the concentrations used for 2,3-DHBA and the internal standards. The recovery patterns of both 2,3-DHBA and saligenin were similar (about 80%) up to addition of 150 μg of these substances per g of cells (fresh weight). However, the recovery of saligenin decreased to about 60% at higher concentrations. This might be due to the limited solubility of this compound in 90% MeOH used as extraction solvent. For the other compound tested as an internal standard, i.e. *o*-anisic acid, we observed a different extraction recovery pattern compared with that of 2,3-DHBA. The recovery of 2,3-DHBA was about twofold higher than that of *o*-anisic acid, and it varied along the applied range of concentrations.

For this reason, we could not use *o*-anisic as an internal standard for the determination of 2,3-DHBA in the *C. roseus* cultures. Thus saligenin was chosen as internal standard for the quantification of 2,3-DHBA in these cultures, since — in addition — it was not present as endogenous compound in elicited *C. roseus* cell cultures and it was stable along the period of analysis. Subsequently, organic solvent extractions either with ethyl acetate or acidified MeOH (pH 3.5–4.0) were compared. In accordance with a previous study [8], the DHBAs were unstable at neutral pH at room temperature. Although a somewhat higher recovery of 2,3-DHBA was obtained with ethyl acetate (results not shown), acidified MeOH was chosen since this solvent provided a higher stability for 2,3-DHBA.

A single step extraction of 2,3-DHBA from the cells was preferred, using acidified MeOH, since the recovery was not improved after a second extraction. In contrast to the determination of 2,3-DHBA in the cells, the levels of 2,3-DHBA in the medium of the *C. roseus* cell cultures could be assayed directly after acidification of the medium to pH 4. The advantage of the fast approach was that losses of 2,3-DHBA during extraction due to its instability could be avoided.

4. Conclusion

Simple isocratic reversed-phase HPLC methods for the assay of some phenolic acids have been developed with UV detection at 280 nm. The mobile phase consisting of MeOH–1% aqueous HOAc (40:60, v/v), pH 4.0, is suitable for the analysis of SA and its precursors CA, *o*-COA and BA. A mobile phase consisting of ACN–1% aqueous HCOOH containing 0.25% TCA (1:5, v/v) at pH 2.0 gives a satisfactory result for 2,3-DHBA, 2,5-DHBA and SA. By using the latter mobile phase, the free form of 2,3-DHBA in medium of *C. roseus* cell cultures elicited by a fungal preparation can be quantified using saligenin as an internal standard.

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