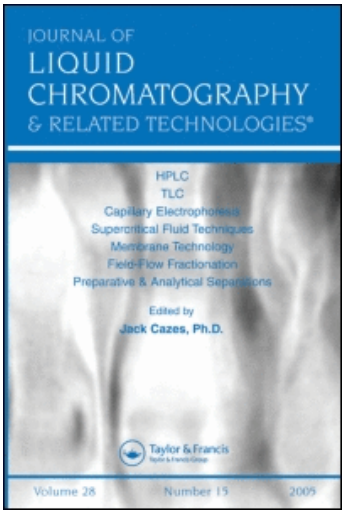

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Abstract: A simple single step ion exchange chromatography method has been developed for purification of SA from plant cell culture extracts. The optimum washing and elution solvents are 25 mM sodium phosphate (pH 7.0–7.5) and 0.3 M HCl in 60% acetonitrile, respectively. Using the developed method the SA recoveries were found 69–80% calculated from the whole purification process. In particular, in the second 10 mL elution, 70 μg SA in 1 g resin yielded 65% SA recovery, which provided a well-detectable amount of SA with a relatively low level of impurities as analyzed by 400 MHz ^{1}H-NMR.

Keywords: Salicylic acid, Plant cell cultures, Ion exchange chromatography, ^{1}H-NMR

INTRODUCTION

Salicylic acid has several physiological functions in plants[1] and serves as a precursor for chelating agents in bacteria.[2] One of the important functions of SA in plants is to induce systemic acquired resistance (SAR) generated by biotic or abiotic stress. SAR is characterized by increased levels of some acidic pathogenesis related (PR) proteins and phytoalexins, which have antimicrobial activities.[3] SA is a C6C1 compound that is synthesized from chorismate, the end product of the shikimate pathway and a substrate for 5

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enzymes leading to a diversity of secondary metabolites in plants [reviewed in ref. 4]. By chorismate mutase (EC 5.4.99.5), chorismate is transformed into prephenate and subsequently (by some steps) into phenylalanine. SA in plants is thought to be derived from phenylalanine, whereas microorganisms employ the enzymes isochorismate synthase (EC 5.4.99.6) and isochorismate pyruvate-lyase (IPL) to transform chorismate into isochorismate and then directly into SA [reviewed in ref. 5]. However, it might also be possible that the isochorismate pathway leads to SA in plants,[6] which was indirectly proven in *Arabidopsis*.[7] Under normal conditions, plants produce SA only in trace amounts, but the level of SA rapidly increases upon pathogenic attack by e.g., tobacco mosaic virus in tobacco.[6] SA and some other phenolics are also found in *Catharanthus roseus*, which are produced as a defense response against biotic- or abiotic stress,[8] for example elicitation of *Catharanthus roseus* (L.) G. Don cell cultures with *Pythium aphanidermatum* extract causes the induction of SA biosynthesis (unpublished results) and 2,3-dihydroxybenzoic acid via the isochorismate pathway.[9]

The trace amounts of SA in plants have made it difficult to find a suitable method for analysis of this compound in plants. Up to date either gas chromatography (GC) or high performance liquid chromatography (HPLC) has been used for the analysis of SA from plants.[10–12] Among the previous methods, an HPLC separation combined with fluorescence detection offers the optimum for the quantitative SA analysis in terms of selectivity (detects fluorescence compounds only) and sensitivity (in nanograms level). However, this method is not satisfactory for biosynthesis studies on SA. Such studies require the isolation of sufficient SA for NMR spectrometric measurements to determine incorporation positions of e.g., ^{13}C-labels, similar to that done for 2,3-dihydroxybenzoic acid.[9] Such studies will allow analysis of carbon fluxes through the pathway(s) involved. Thus, the goal is to develop a simple purification method of SA from a large amount of cells that gives a sample of SA for which signals can be observed in the NMR, without overlap of other compounds.

In developing a purification method, the physicochemical properties of the target compound should be taken into account. SA belongs to the group of phenolic acids. This structure allows the compound to be separated from an aqueous extract under acidic conditions by partitioning with a non-polar solvent or purification by ion exchange chromatography (IEC). For example, IEC was used to extract benzoic acid from landfill leachate, a liquid formed by the degradation of the organic matters present in landfill, which can contaminate the soil, superficial, and ground water.[13] Also, anion exchange chromatography was employed to further clean up an aliquot fraction obtained from prepurification of plant extracts using polyamide column chromatography.[14] These purification steps were part of the procedure developed for a GC/HPLC analysis of a series of phenolic acid glucosides such as 4-β-D-glucosides of vanillic-, syringic-, 4-hydroxybenzoic-, protocatechuic-, and gallic

acid, salicylic acid 2-*O*-β-D-glucoside, and the 1-*O*-β-D-glucose esters of 4-hydroxybenzoic-, vanillic-, and syringic acid from plant extracts.

Considering the property of SA as an acid with a moderately low pKa of 3.0,[15] we chose IEC using a strong anion exchanger Dowex 1WX2 (100 mesh) as the resin for purification of this compound from plant cell culture extract to obtain a relatively clean extract for NMR analysis as a tool in a retro-biosynthetic study of SA. In the present study, we developed and optimized an IEC system using pure SA and subsequently applied this method to plant extracts. The aim of this purification step is to obtain a relatively pure and well detectable amount of SA in the fraction for analysis by ^{1}H-NMR spectrometry.

EXPERIMENTAL

Chemicals

Chemicals Used for the Medium of Cell Suspension Cultures

The chemicals used in Macro Murashige and Skoog/M&S salts[16] or Macro Gamborg B5 salts:[17] $CaCl_2$ (min. 99%), KH_2PO_4 (min. 99.5%), KNO_3 (min. 99%) and NH_4NO_3 (min. 99%) were obtained from Merck (Darmstadt, Germany) and $MgSO_4$ exsiccatus BP was purchased from OPG Farma, BUVA BV (Uitgeest, The Netherlands). The chemicals used in Micro M&S or Micro Gamborg B5 salts: $FeSO_4 \cdot 7H_2O$ (Brocades-ACF groothandel NV, Maarssen, The Netherlands) and H_3BO_3, $MnSO_4 \cdot H_2O$, Na_2EDTA, $ZnSO_4 \cdot 7H_2O$ (Merck) were dissolved in one solution, whereas others including $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, KI, $NaMoO_4 \cdot 2H_2O$ (Merck) were dissolved in another solution due to the problem of solubility. Glycine (99.7%), 1-naphthaleneacetic acid (NAA), and nicotinic acid (99.5%) were from Merck (Schuchardt, Germany). D(+)-glucose (>99.0%) was obtained from Fluka Chemie (Buchs, Germany). Myo-Inositol (>99.0%) and sucrose (99.7%) were from Duchefa Biochemie (Haarlem, The Netherlands). Pyridoxine-HCl was from Sigma-Aldrich Chemie (Steinheim, Germany). Thiamine-*di*-HCl was from Janssen Chimica (Geel, Belgium).

Chemicals Used for Extraction, Separation, and Analysis of Salicylic Acid

Dowex 1WX2 100 mesh (replacement for 1X2100) and salicylic acid were purchased from Sigma-Aldrich (St. Louis, MO, USA). Methanol (MeOH, >99.8%), acetonitrile (AcCN, >99.8%), and ethyl acetate (EtOAc, >99.8%) were from Biosolve BV (Valkenswaard, The Netherlands). Cyclohexane (>99%), acetic acid (100%), and hydrochloric acid (HCl, 36–38%) were purchased from Mallinckrodt Baker BV (Deventer, The Netherlands).

Di-sodiumhydrogenphosphate 2-hydrate (99%), sodium acetate trihydrate (99.5–101.0%), *ortho*-phosphoric acid (85%), and trichloroacetic acid (TCA, >99.5%) were from Merck. Sodium hydroxide (>99%) was from Boom (Meppel, The Netherlands). CH_3OH-d_4 was from C.E. Saclay (Gif-Sur-Yvette, France).

Plant Cell Cultures

C. roseus line A12A2 was grown in M&S medium[16] without growth hormones, whereas *C. roseus* line A11 was grown in Gamborg B5 medium[17] containing NAA (1.86 mg/L). Both media were supplemented with 2% of D(+)-glucose monohydrate as the carbon source. The cells were grown in 250 mL-Erlenmeyer flasks containing 100 mL medium and cultivated at 24 ($\pm$1)°C under continuous light (500–1500 lux), on a shaker at 100 rpm. The lines A12A2 and A11 were subcultured every week and every 3 weeks, respectively, by addition of the same volume of fresh medium to the cell cultures.

Elicitation

Pythium aphanidermatum (Edson) Fitzpatrick CBS 313.33 was used as an elicitor. This fungus was maintained on malt extract agar plate medium, at 25°C, in the dark, and subcultured every week. The solid culture was aseptically cut to pieces and two pieces (each about 1 cm^2) were transferred into a 250-mL Erlenmeyer flask containing 100 mL M&S liquid medium with 3% sucrose. This culture was then cultured at 27°C on a shaker at 100 rpm for 7 days. It was then sterilized in an autoclave and subsequently filtered (to separate extract from broken cells/mycellia) under aseptic conditions. The filtrate was used as the elicitor. Ten or 20 mL of the *Pythium* extract was added to 100 mL of 5 days old *C. roseus* suspension cells. The cultivation conditions for the treated cultures were the same as that for culture maintenance. The elicited cells were harvested 24 h and/or 48 h after treatment by vacuum filtration, using a P2 glass filter to separate the cells from medium. The cells were frozen in liquid nitrogen and used for the SA extraction.

Salicylic Acid Extraction

Non-elicited- and elicited cells of *C. roseus* A11 and A12A2 were weighed and homogenized using a mortar and pestle in the presence of liquid nitrogen. For 10 g of homogenized material (placed in a 50 mL-analytical tube), 2 × 25 mL of 100% MeOH were used for the extraction. The solid phase was separated from the liquid phase by a centrifugation (2500 rpm,

15°C, 10 min) using a Varifuge 3.0 R (Heraeus Sepatech, Germany). The MeOH extract was then evaporated using a rotary evaporator at maximum temperature, 40°C, after the addition of 200 μL of 0.2 M NaOH. Subsequently, the concentrated extract (±5 mL) was acidified with 5 mL of 5% trichloroacetic acid (TCA) to pH 2.0, before partitioning with 10 mL of EtOAc-*n*-hexane (1:1), twice. In order to obtain SA from SA-glucoside (SAG), 3 mL of 8 M HCl was added to the TCA fraction resulting in pH 0-1 and placed in a water bath at 80°C, 1 h, to hydrolyze the SAG. A partitioning with 15 mL of EtOAc-*n*-hexane (1:1) was performed twice to extract the liberated-SA. The non-polar extracts were pooled and evaporated using a rotary evaporator at room temperature. This extraction of SA was performed based on the method described by,[12] in which the free SA was extracted prior to acid hydrolysis for a separate quantitative analysis of free SA and SAG. However for extraction of total SA, acid hydrolysis can be performed on the concentrated MeOH extract prior to partitioning of the total SA. The concentrated extract (around 0.5 mL) was dissolved in 1–2 mL of 25 mM sodium phosphate (pH 7.0–7.5):MeOH (90:10 v/v) before application to the IEC column.

Ion Exchange Chromatography

Application to SA Standard

Ten grams, 2 g or 1 g of Dowex 1WX2 (100 mesh) were placed separately in glass columns (i.d. 1 cm) providing a height of about 15, 3, and 1.5 cm, respectively. The resin was rinsed and preconditioned with 25 mM sodium phosphate (pH 7.0–7.5) before loading with SA standard. This buffer complemented with 10% to 20% MeOH, was employed as the loading solvent. Elution of SA was performed using solvents containing different concentrations of HCl in aqueous AcCN (40–60%) or MeOH (15–60%). Fractions were collected, both from the washing steps (2 × 10 mL of the phosphate buffer without MeOH) and the elution steps (10 × 10 mL). Those obtained from the washing step were vortexed, centrifuged, and analyzed by HPLC. Also, the fractions of the elution step were vortexed and subsequently sampled (20–100 μL each). HPLC buffer was added to the samples, neutralized by the addition of 10 μL of 0.5 N NaOH (to adjust a pH 5–7 in the final volume of 500 μL of a sample) and centrifuged before injection into the HPLC system. A range of concentrations of SA standard solution (0.025–1.0 μg/mL) was used in every set of measurements.

Application to Plant Extracts

Samples of plant cell extracts containing different amounts of SA were loaded (using the same loading buffer mentioned as for experiments with pure SA) on

separate columns to provide the systems with different ratios of the amounts of SA/resin. After washing each column with 30 × 10 mL of 25 mM sodium phosphate (pH 7.0–7.5), the SA was recovered with 10 × 10 mL of 0.3 M HCl in 60% AcCN. Preparation of the samples for HPLC analysis obtained from the washing step and the elution step was similar to that used for pure SA. The fraction containing most SA of the highest ratio of SA/resin system was evaporated using a rotary evaporator at room temperature. After evaporation, the remaining acidic aqueous extract (about 4 mL) was partitioned with 5 mL of EtOAc-*n*-hexane (1:1), twice. The a-polar phases were pooled and washed with the same volume of deionized water, twice. Sodium-sulphate was added to remove the remaining acid/water. Subsequently, the a-polar extract was separated from sodium-sulphate by a glass pipette into a round flask and evaporated at room temperature to dryness. The dried extract was redissolved in CH_3OH-d_4 for NMR analysis.

HPLC Analysis

HPLC analysis of SA was performed based on the system described by.[12] The column, a Phenomenex column type LUNA 3 μ C18 (2) 150 × 4.60 mm equipped with a SecurityGuard from Phenomenex (Torrance, CA, USA) was used. The mobile phase, 10 mM sodium acetate buffer in 10% MeOH (pH 5.5), was pumped using an LKB 2150 HPLC pump (Bromma, Sweden), at a flow rate of 0.80 mL/min. The injection (20 μL) of the samples was performed using a Gilson 234 auto-injector (Villiers Le Bel, France). The detection was carried out using a Shimadzu RF-10AxL spectrofluorometric detector (Tokyo, Japan), at an emission wavelength of 407 nm and an excitation wavelength of 305 nm, which was connected to a CR 501 Chromatopac printer (Shimadzu, Kyoto, Japan). The SA peak appears at around 13 min. HPLC equipment was used to check the profile of impurities present in the washing fractions. This HPLC consisted of a Waters 600 (Milford, MA, USA) pump, a Gilson sample injector model 231 with a Gilson dilutor model 401 (Villiers Le Bel, France), a Waters photodiode array (PDA) detector type 990 (Milford, MA, USA) connected to a personal computer, and a Waters 5200 printer plotter (Milford, MA, USA).

^{1}H-NMR Analysis

NMR spectra were recorded on a Bruker AV 400 MHz spectrometer equipped with an Indy Silicon graphics computer. For each sample, 128 scans were recorded with the following parameters: 0.17 Hz/point, pulse width (PW) = 4.0 μsec. (30°C), and relaxation delay (RD) = 1.0 sec. FIDs were Fourier transformed with LB = 0.30 Hz. The NMR analyses were performed

on the fractions containing most SA of the highest ratio of SA/resin experiment and of the SA standard dissolved in CH_3OH-d_4.

RESULTS AND DISCUSSION

Application to SA Standard

A sample containing 100 μg of SA standard was applied to 10 g of the resin. There was no SA detected in the washing step but 10 mL of the elution solvent (0.1 M HCl in 15% MeOH, pH = 1) could elute only about 1% of the total SA applied. Changing the concentration of HCl to 0.25 M increased the SA eluted to about 2% of the total SA loaded per 10 mL elution. When the concentration of MeOH was increased to 40% (containing 0.25 M HCl), the SA amount increased to about 4.2% of the total SA loaded per 10 mL elution, and this amount was slightly improved to 4.6% when the HCl concentration was doubled (0.5 M). Increasing the concentration of MeOH improved the affinity of SA for the elution solvent, but not sufficiently for a good recovery. The SA elution profile obtained from the 10 g resin column pointed to a huge overcapacity of the column, which could be decreased to improve recovery. Therefore, we investigated the elution profile of 50 μg SA standard loaded on 1 g and 2 g of the resin employing 25 mM sodium phosphate (pH 7.0–7.5) as the washing solvent and 0.2 M HCl in 40% MeOH as the elution solvent. The result is presented in Figure 1A.

Since bigger ratio of amounts of SA/resin seemed to provide the most efficient elution, we investigated the influence of MeOH concentration on the SA (50 μg) elution profile of 1 g of the resin (data not shown). It was clear that higher HCl and MeOH concentration provided a more efficient SA elution. Also, some experiments using 50 μg of SA standard applied on 2 g of the resin were done to study the elution profiles affected by different concentrations of HCl and MeOH or AcCN (Figure 1B). The use of AcCN was considered because AcCN has almost the same polarity as MeOH, 5.8 and 5.1, respectively,[18] but a bigger eluotropic strength than that of MeOH. This shows AcCN to be a better solvent modifier for analytes compared to MeOH, not only in RP-HPLC but also in some ion exchangers, affinity phases, and chiral phases, due to the hydrophobic interaction between analytes and the adsorbents.[19] Increasing only the concentration of HCl from 0.3 M to 0.4 M, but not the concentration of MeOH, did not improve the SA elution. Figure 1B shows that for 50 μg of SA applied to 2 g Dowex 1WX2 (100 mesh), the affinity of SA to the elution solvent increased by a higher concentration of HCl and of the organic solvent. Changing the organic solvent from MeOH to AcCN resulted in the highest level of SA present in the first 10 mL elution. The cross linked polystyrene matrix of the resin might have hydrophobic interactions with the undissociated SA, from which the SA could be eluted more efficiently by AcCN than by MeOH.

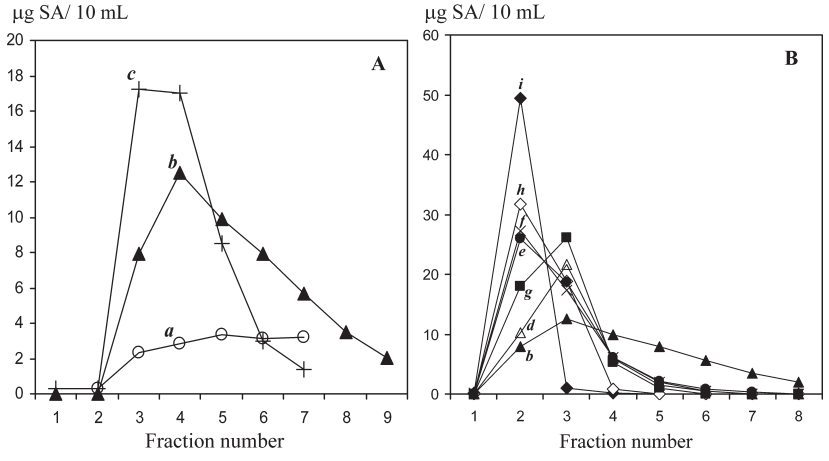

Figure 1. A: The elution profiles of SA standard applied on separate columns with different conditions. Ratio of amounts of SA/resin *a*: 10 μg/g (10 g resin used), *b*: 25 μg/g (2 g resin used), *c*: 50 μg/g (1 g resin used). The elution solvent employed was 0.25 M HCl in 40% MeOH for *a*, whereas 0.2 M HCl in 40% MeOH was used for *b* and *c*. Fraction no.1–2: the washing step. Fraction no. 3–9: the elution step. B: The elution profiles of SA standard applied on separate columns with ratio of amounts of SA/resin was 25 μg/g (2 g resin employed) using different concentrations of HCl and MeOH or AcCN, *b*: 0.2 M HCl in 40% MeOH, *d*: 0.2 M HCl in 60% MeOH, *e*: 0.3 M HCl in 60% MeOH, *f*: 0.4 M HCl in 60% MeOH, *g*: 0.2 M HCl in 40% AcCN, *h*: 0.2 M HCl in 60% AcCN, *i*: 0.3 M HCl in 60% AcCN. Fraction no.1: the washing step. Fraction no. 2–8: the elution step. The resin was Dowex 1WX2, 100 mesh and the washing solvent was 25 mM sodium phosphate (pH 7.0–7.5).

Application to Plant Cell Extracts

For 2 g Dowex 1WX2 (100 mesh) loaded with 50 μg SA standard, after washing with a 25 mM sodium phosphate pH 7.0–7.5, the elution solvent 0.3 M HCl in 60% AcCN provided the most efficient SA elution profile since almost all of the SA applied (97%), eluted in the first 10 mL elution. The total amount of SA in the MeOH extract or in the non-polar extract before evaporation was determined as 100%. The SA recovery is affected by the loss of SA not only from the washing step but also from the whole process, including evaporation and sample loading. An experiment using an acid-hydrolysis extract of 33 g fresh weight (FW) of *C. roseus* A11 elicited cells containing only 7.2 μg SA, resulted in 69% SA recovery in the elution solvent. Using the same washing and elution solvents, some experiments employing different amounts of endogenous SA (in different extracts of *C. roseus* A12A2 elicited cells) applied per gram of the resin were performed to study the SA recoveries and elution profiles. The results are shown in Figure 2A.

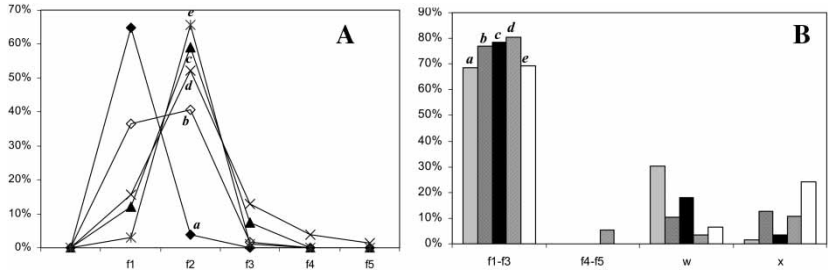

Figure 2. A: Effect of ratio of amounts of SA/resin on the elution profile of SA from plant extract. Expressed as % of SA recovered in the first 5 × 10 mL elution (fraction f1–f5) using Dowex 1WX2 100 mesh as the resin, 25 mM sodium phosphate pH 7.0–7.5 as the washing solvent and 0.3 M HCl in 60% AcCN as the elution solvent (*a*: 3.6 μg/g, *b*: 16.7 μg/g, *c*: 19 μg/g, *d*: 45 μg/g, *e*: 70 μg/g, the amounts of resin used in *a*: 2 g, *b*: 2.5 g, *c*: 3.5 g, *d*: 4 g, *e*: 4 g, the amount of SA before IEC application = 100 %). B: % of SA recovered in total first 3 × 10 mL elution (f1–f3) and after the first 30 mL elution (f4–f5), % of SA lost in the washing step (w) and % of SA lost in other steps (x).

The SA retention volume changes depending on the ratio of the amounts of SA/resin. Low ratio of SA/resin provided fast elution (in fraction 1), whereas late elution (in fraction 2) was obtained at a high ratio of SA/resin. The application of an extract to the system provides a recovery of 69–80% (in total 3 × 10 mL first elution) from the whole purification process, as shown in Figure 2B. Not only a high SA recovery is necessary (because of the low level of SA produced by plants), but also a low degree of impurities after the separation is also important to allow a further NMR analysis of the purified SA.

HPLC diode array detection (DAD) was used to check the profile of impurities in the washing step, which showed many huge-peaks after 30 min in the chromatograms (data not shown). Despite the selectivity of the fluorescence detector, the fluorescence chromatograms of the crude extracts showed many peaks at 2–5 minutes, which were highly reduced, or often disappeared, in the fluorescence chromatograms of the most SA containing fractions after purification. One of those small peaks of impurities detected at around 2–5 minutes in the HPLC fluorescence chromatogram of the most SA containing fraction after purification, was the peak of 2,3-dihydroxybenzoic acid (data not shown). This compound is present in a relatively large amount as compared to SA in a MeOH extract of *C. roseus* elicited cells (unpublished result). By increasing the volume of the washing buffer from 50 mL to 300 mL, the impurities were almost completely removed as could be detected by the HPLC DAD analysis of the last 10 mL of the washing step, or in the HPLC fluorescence chromatogram of the major SA fractions of the purification method (data not shown). The ^{1}H-NMR spectrum shows that

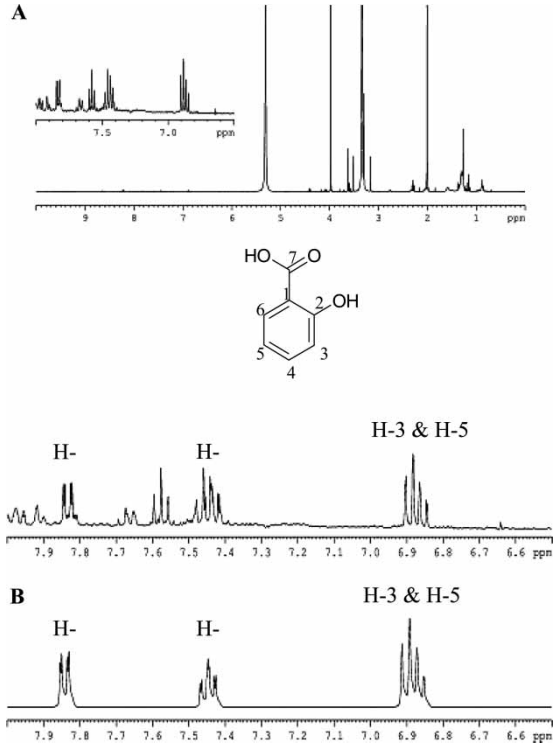

Figure 3. A: The ^{1}H-NMR spectra (CH_3OH-d_4) of the most SA containing fraction after an ion exchange chromatographic separation (184 μg of endogenous SA determined by HPLC). B: The ^{1}H-NMR spectra of 180 μg of SA standard dissolved in CH_3OH-d_4.

the IEC system used was relatively selective for SA. In a typical experiment, 65% of the SA was recovered in the second 10 mL elution of a plant extract containing 280 μg of total SA extracted from 200 g fresh weight *C. roseus* elicited cells (4 g of resin was used, providing SA/resin ratio of 70 μg/g). This amount of SA provided well detectable SA signals with a low level of impurities in the 400 MHz ^{1}H-NMR spectra (Figure 3).

CONCLUSION

An ion exchange chromatographic system consisting of 2 g of Dowex 1WX2 (100 mesh) as the resin, 25 mM sodium-phosphate pH 7.0–7.5 as the washing solvent, and 0.3 M HCl in 60% AcCN as the counter ion solution using 50 μg SA standard, provided an efficient SA elution profile since almost all SA (97%) eluted in the first 10 mL eluent. The application of an extract to the

system provides a recovery of 69–80% (in total 3 × 10 mL first elution) calculated from the whole purification process. Depending on the amount of SA in an extract, the amount of the resin can be determined to obtain the best elution profile with most of SA concentrated in one fraction. The amount of SA (184 μg) in the most SA containing fraction after purification of an extract of *Catharanthus roseus* elicited cells (containing 280 μg of total SA, extracted from 200 g FW elicited cells) provided well detectable SA signals with a low level of impurities in the 400 MHz ^{1}H-NMR spectra. This method is, thus, suited as a single step concentration and purification method of SA, as trace compound in *Catharanthus roseus* cell cultures.

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