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Reversed-phase liquid chromatographic separation and simultaneous profiling of steroidal glycoalkaloids and their aglycones

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Abstract

Improved and simplified reversed-phase liquid chromatographic conditions for the separation and simultaneous profiling of both steroidal glycoalkaloids and their aglycones, having solanidane- or spirosolane-type structures, are described. The most reproducible retention behavior for these ionizable compounds on C_{18} columns was achieved under isocratic and gradient elution conditions using acetonitrile in combination with triethylammonium phosphate buffer at pH 3.0, when basic functional groups of solutes and silanol groups on the silica are fully protonated minimizing ionic interactions. Gradient elution was the only feasible approach for the simultaneous separation of steroidal glycoalkaloids and their aglycones. A Zorbax SB C_{18} column, specially designed for low-pH separations, showed good performance in critical separations. The impurities of the commercial tomatine and tomatidine standards were studied and confirmed using mass spectrometric, liquid chromatographic and thin-layer chromatographic methods.

Keywords: Steroidal glycoalkaloids; Glycoalkaloids; Alkaloids; Aglycones; Toxins

1. Introduction

Steroidal glycoalkaloids (SGAs) are naturally occurring nitrogen-containing toxins found in the *Solanaceae* family [1–3]. These compounds are also found in common food crops such as in potato and tomato. SGAs contain three structural portions: a polar, water-soluble sugar moiety with three or four monosaccharides; a non-polar steroid unit and a basic portion with either indolizidine or oxa-azaspirodecane structure (Fig. 1). Spirosolane-type alkaloids are weaker bases than solanidane-type alkaloids depending on the inductive effect of the oxygen atom attached to the same carbon atom as the nitrogen atom. Approximate pK_a values of solasodine and solanidine, determined in 60% alcohol–water solution, are 7.70 and 8.62, respectively [4]. UV absorbance spectra of SGAs and steroidal glycoalkaloid aglycones (SGAAs) are very simple and similar. They lack conjugated unsaturation and therefore they exhibit only end absorbance near 200 nm. This means that they have low UV sensitivity and can be detected and identified by diode array detection (DAD) UV only when present relatively high amounts (5–10 ng/injection).

Wild *Solanum* species that generally contain high

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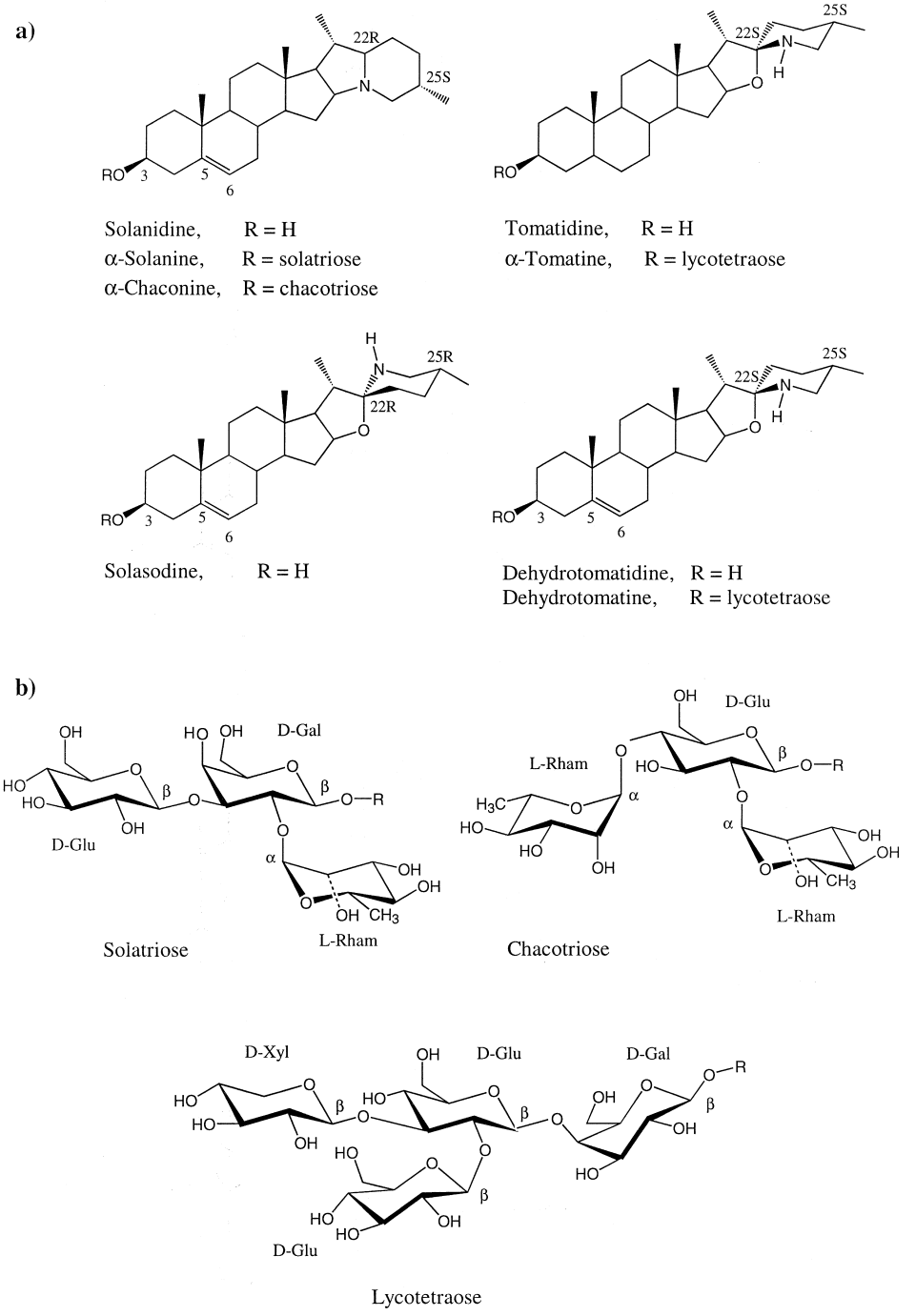

Fig. 1. The structures of (a) steroidal glycoalkaloid aglycones (SGAAs) and (b) sugar moieties present in steroidal glycoalkaloids (SGAs) evaluated in this report.

amounts of glycoalkaloids are widely used in potato breeding as a valuable genetic material to introduce desirable characteristics such as biotic and abiotic stress resistance into cultivated potato. This may result in high levels of SGAs in the tubers of potato breeding lines. Therefore, it is very important to be able to determine the whole SGA profile and find new potentially toxic compounds. It has been reported that only small structural differences in the steroid or basic portion as well as the nature, number and order of attachments of sugar units in the carbohydrate moiety, influence the biological activity of the SGAs [5–7].

Separation and determination of these structurally closely related compounds present an enormous analytical challenge. The methods in current use for the analysis of SGAs include: colorimetry [8–10]; thin-layer chromatography (TLC) [11]; gas chromatography (GC) [12–16]; mass spectrometry (MS) [17–19]; combined GC–MS [20,21]; high-performance liquid chromatography (HPLC) [22–31]; liquid chromatography (LC)–MS [32] and immunoassays [33–36]. It must be pointed out that each method has its relative advantages and disadvantages, and no method is able to analyze the whole set of complex compounds in many types of samples. Currently the most used method to analyze SGAs and SGAAs is LC, which can separate and quantify these compounds individually directly without derivatization. Bushway et al. were the first to separate α-solanine and α-chaconine, the major glycoalkaloids in commercial *Solanum tuberosum* cultivars using an LC method [22]. Afterwards, a great many different LC methods have been reported to determine glycoalkaloids.

An important part of our research program on potato glycoalkaloids involves the rapid profiling of SGAs and SGAAs of wild and cultivated potatoes used in plant breeding as well as the hybrids between them. Therefore, the main aim of this study has been to investigate the effect of chromatographic conditions on LC behavior of SGAs and SGAAs and to develop a simple method for the simultaneous profiling of individual SGAs and SGAAs, using commercial model compounds, which have solanidane or spirosolane structures (Fig. 1). Currently no such method is available in the literature.

2. Experimental

2.1. Chemicals and reagents

α-Solanine (purity approx. 95%), α-chaconine (approx. 95%), solanidine (purity 98%), tomatine (no label purity), tomatidine hydrochloride (purity 88%, cont. 9% solasodine) and solasodine (approx. 99%) obtained from Sigma (St. Louis, MO, USA) were used as reference compounds.

Distilled water was further purified with Gelman's Water I apparatus (Gelman Sciences, Ann Arbor, MI, USA). HPLC-grade acetonitrile and methanol (Labscan, Dublin, Ireland) were filtered through a 0.45-μm membrane filter (Millipore, Milford, MA, USA) and thoroughly degassed by ultrasonication.

Orthophosphoric acid (85%) was from Merck (Darmstadt, Germany). 1.0 *M* tris(hydroxymethyl)aminomethane hydrochloride (Tris), 1.0 *M* triethylammonium acetate (TEAA) and 1.0 *M* triethylammonium phosphate (TEAP) buffers in water were obtained from Fluka (Buchs, Switzerland). The 25 mmol Tris eluent with pH 7.4, 25 mmol TEAA eluent with pH 7.0 and 25 mmol TEAP eluent with pH 3.0 were prepared by diluting 25 ml of 1.0 *M* Tris, TEAA and TEAP to 1000 ml with water. The pH of the buffers was measured before addition of the organic modifier.

For TLC experiments 95% ethanol (99.5%, ETAX Aas, Primalco, Rajamäki, Finland) and dichloromethane (Labscan, Analytical Sciences) were used. TLC spraying solutions were: 3.0 g of ammonium thiocyanate (NH_4SCN) and 1.0 g of cobalt (II) chloride [$Co(II)Cl_2$] in 20 ml of water or sulfuric acid (95%)–methanol (1:1).

2.2. Preparation of standards

The stock solutions were prepared by dissolving 1.0 mg of each reference compound in 1 ml of methanol or ethanol (99.5%). For the simultaneous determination of SGAs and SGAAs 50 μl of α-solanine, α-chaconine, solanidine and solasodine stock solutions were combined with 100 μl of tomatine and 150 μl of tomatidine hydrochloride stock solutions. All the LC samples were filtered

with Acrodisc-CR 0.45-μm cartridges (Gelman) before the injections.

The tomatidine base was obtained by dissolving commercial tomatidine hydrochloride in methanol at 60°C and adjusting the pH to 10 with 25% ammonia solution. The free base was extracted with 2×2 ml of dichloromethane (HPLC-grade, Labscan). The organic phase was dried with anhydrous Na_2SO_4 and evaporated to dryness under a gentle stream of argon.

For the comparison of the aglycones of the commercial tomatine with those of the commercial tomatidine, the hydrolysis of the tomatine was performed as follows: 5 mg of tomatine was dissolved in 2 ml of methanol and to this solution 0.5 ml of concentrated HCl (>25%) was added and the solution was heated, magnetically stirring at 70–80°C for 2.5 h. Finally the pH was adjusted to 10 with 25% ammonia solution and this basic solution was extracted with 2×2 ml of dichloromethane. The organic solution was dried with Na_2SO_4 and evaporated to dryness under a gentle stream of argon. The residue was reconstituted in 2 ml of methanol (HPLC-grade) for the analyses.

2.3. Instrumentation and separation conditions

A Hewlett-Packard HP 1090A liquid chromatographic system (Waldbronn, Germany) with autoinjector, a built-in diode-array detector HP 1040A, HP 3392A integrator, HP 85B computer control and HP 9121D disk memory was used. Another apparatus consisted of a Waters 600 HPLC pump, a Rheodyne 7725i injector with a 5-μl loop (Cotati, CA, USA), a Waters column heater module, a Waters 996 photodiode array detector and Millenium 2010 chromatography manager (Waters, Milford, MA, USA). The effluent from the column was monitored at 205 nm. The on-line UV absorbance spectral data were collected from 190 to 400 nm with the DAD system. The applied chromatographic columns are shown in Table 1.

Under isocratic conditions the concentration of acetonitrile varied from 25 to 60%. The gradient elution with acetonitrile–buffer followed the linear gradient of 20–70% acetonitrile in 20 min or stepwise gradient of 20, 25, 35, 45 and 65% acetonitrile at time 0, 12, 15, 17 and 25 min. The columns were operated at constant temperatures of between 25 and 50°C. The flow-rates of eluent with columns of 4.0–4.6 mm diameter were 1.0–1.5 ml/min and with the column of 2.1 mm diameter the flow-rate was 0.5 ml/min. All results are the mean value of at least three injections.

Mass spectra of dehydrotomatidine, tomatidine and solasodine were obtained on a Jeol JMS-SX 102 two-sectors (BE (B stands for magnetic field and E for electric field)) mass spectrometer at 70 eV with direct sample inlet at a source temperature of 200°C. Fast atom bombardment (FAB) MS spectra of dehydrotomatine and tomatine were recorded on a Finnigan Mat 8200 two-sectors (BE) mass spectrometer by bombardment the samples (dissolved in glycerol matrix) with Xe. A resolution of approximately 3000 was used.

2.4. TLC procedure

Normal-phase TLC was performed on pre-coated (0.25 mm) silica gel 60 F_{254} sheets (Merck, Darmstadt, Germany). The TLC plates were developed in a chamber saturated with 95% ethanol or methanol–

Table 1
LC columns used in this study

Column name	Column dimensions (mm)	Particle size (μm)	Pore diameter (Å)	Carbon loading (%)	Supplier
Zorbax-Rx C_{18}	250×4.6 mm	5	80	12	Hewlett-Packard, Rockland Technologies, Newport, DE, USA
Zorbax-SB C_{18}	250×4.6 mm	5	80	10	Hewlett-Packard, Rockland Technologies, Newport, DE, USA
Inertsil ODS-3	250×4.6 mm, 150×4.0 mm	5	100	15	GL Sciences, Tokyo, Japan
Hypersil ODS	200×2.1 mm	5	120	10	Shandon Southern Products, Astmoor, UK
LiChrosorb Hibar RP-18	250×4.0 mm	5	100	16	Merck, Darmstadt, Germany

chloroform (2:1). The plates were sprayed with aqueous cobalt (II) thiocyanate solution or with sulfuric acid (95%)–methanol solution (1:1). After spraying with sulfuric acid the plates were allowed to dry and subsequently were heated until the spots became visible. If TLC plates are heated too long the color differences between the spots disappear and all the spots become brown. The first reagent has the disadvantage that it does not produce any color differences between the spots of different steroidal alkaloids and the spots disappear in 10 min.

3. Results and discussion

3.1. Chromatographic conditions and separation

The aim of this work was to find chromatographic conditions suitable for as many compounds as possible, keeping the number of individual LC methods as few as possible. A number of difficulties were found in the chromatography of these basic compounds depending on the fact that solute retention in reversed-phase (RP)-LC is the result of a variety of complex interactions between solute, mobile phase and stationary phase species and these interactions are not yet well understood. The degree of separation for SGAAs and SGAs was very strongly dependent on chromatographic conditions.

Only silica-based C_{18} columns (Table 1) were used in this study, although amino columns have been generally used for carbohydrate analyses and also for the analyses of glycoalkaloids [26,37,38]. We did not use them, because they are notorious for their instability. Further, non-silica-based columns (e.g., polymer- or zirconium-based columns) might have been useful, because they have no upper pH limit and unwanted silanol interactions are absent, but according to literature they generally produce worse separations than silica-based columns. LiChrosorb Hibar RP-18 column was from the older generation of RP columns, whereas Hypersil ODS, Inertsil ODS-3, Zorbax Rx C_{18} and Zorbax SB C_{18} were selected from the newer generation of RP columns with highly pure and low acidity silica, low residual silanols and homogeneous bonded-phase coverage. Zorbax SB C_{18} column is specially designed for low-pH separations and usable at elevated temperatures. Silica-based columns had to be operated in the pH 2–8 range that does not suppress the ionization of SGAs and SGAAs.

Acetonitrile was employed as the basic organic modifier of the mobile phase because of its low UV cut-off, which allowed the use of 200–205 nm as detection wavelength for SGAs having only end absorption in their UV absorbance spectra. The weak solvents of the mobile phase were: water, acidified to pH 3.0 with 85% orthophosphoric acid, 25 m*M* Tris buffer (pH 7.4), 25 m*M* TEAA buffer (pH 7.0), and 25 m*M* TEAP buffer (pH 3.0). Our experiments revealed that acetonitrile–25 m*M* TEAP buffer (pH 3.0) was the best solvent system eluting reproducibly both SGAs and SGAAs under both isocratic and gradient elution conditions from all the columns of this study, apart from LiChrosorb Hibar column, which is based on the older silica chemistry and interacts more strongly with bases than newer columns. This column gave broad and tailing peaks with varying retention times to SGAAs.

As the weak solvent of the mobile phase was water, acidified to pH 3.0 with 85% orthophosphoric acid, both SGAs and SGAAs eluted as broad and tailing peaks at varying retention times or even not at all. Acetonitrile–Tris buffer (pH 7.4) gave reproducible and baseline separations ($R_s > 1.5$) only for the SGAs but not for the SGAAs, which were eluted as very broad and tailing peaks. Further, TEAA buffer (pH 7.0) resulted in the noisy base line and its UV transparency was not so good at low wavelength range 200–205 nm. At low-pH separations the retention times decreased strongly as compared to the medium-pH separations, necessitating lower concentrations of acetonitrile. The optimal isocratic solvent conditions were 25–30% acetonitrile in TEAP buffer (pH 3.0), but for the separation of SGAAs 40–60% acetonitrile in TEAP buffer was necessary. By using a low-pH mobile phase, both basic functional group of glycoalkaloids and acidic silanol groups on the silica support are fully protonated minimizing ionic interactions between them and providing more reproducible separations. In addition, the use of triethylammonium ions contributed to better chromatography. Isocratic elution was ineffective for the separation of SGAs and SGAAs during a single run. Therefore, gradient elution was employed.

The role of column temperature has usually been neglected in LC, although retention relates directly to temperature and temperature control is nevertheless important part of separation efficiency. The rule of thumb is that a 1°C change in temperature results in a 1–3% change in retention for isocratic RP separations [39]. Fig. 2 shows the results from isocratic runs of SGAs and SGAAs performed at six temperatures. It can be seen that all the compounds, apart from solasodine, eluted earlier as the temperature increases. The absolute retention times were highly reproducible (the average RSD 0.15%) under nominally unchanged chromatographic conditions. The retention varied by approximately 0.6%/°C for SGAAs and 0.2%/°C for SGAs, much less than generally. Solasodine showed minor increase in retention between temperatures of 25 and 35°C and a decrease between 35 and 50°C (Fig. 2b). Inspection of the plots in Fig. 2 reveals no significant changes in selectivity when temperature varies, apart from solasodine, which moves closer to tomatidine and finally co-elutes as temperature increases. Thus the best selectivity is obtained at 30°C for the SGAAs. The different effect of temperature changes on retention of solasodine is possibly due to its different stereochemistry in the F-ring when compared to tomatidine and dehydrotomatidine that may produce different conformational changes as a function of temperature.

Temperature influences retention in gradient elution as well as in isocratic separation. Fig. 3 shows plots of the retention times of the SGAs and SGAAs against temperature at three column temperatures under linear and stepwise gradient elution conditions. The gradient retention times were also highly reproducible under nominally unchanged conditions. The average variability of linear and stepwise gradient retention times for all the compounds at three temperatures was 0.05% and 0.3%, respectively. Increase in the temperature from 30 to 50°C decreased the retention time of SGAs and SGAAs only 2–5% (on average 0.2%/°C) under linear gradient elution conditions (Fig. 3a), but under stepwise gradient elution conditions the decrease was 1–2% (on average 0.1%/°C) for SGAs and 11–16% (on average 0.7%/°C) for SGAAs (Fig. 3b). The relative peak spacing does not appear to change significantly.

Fig. 4 shows representative chromatograms of the

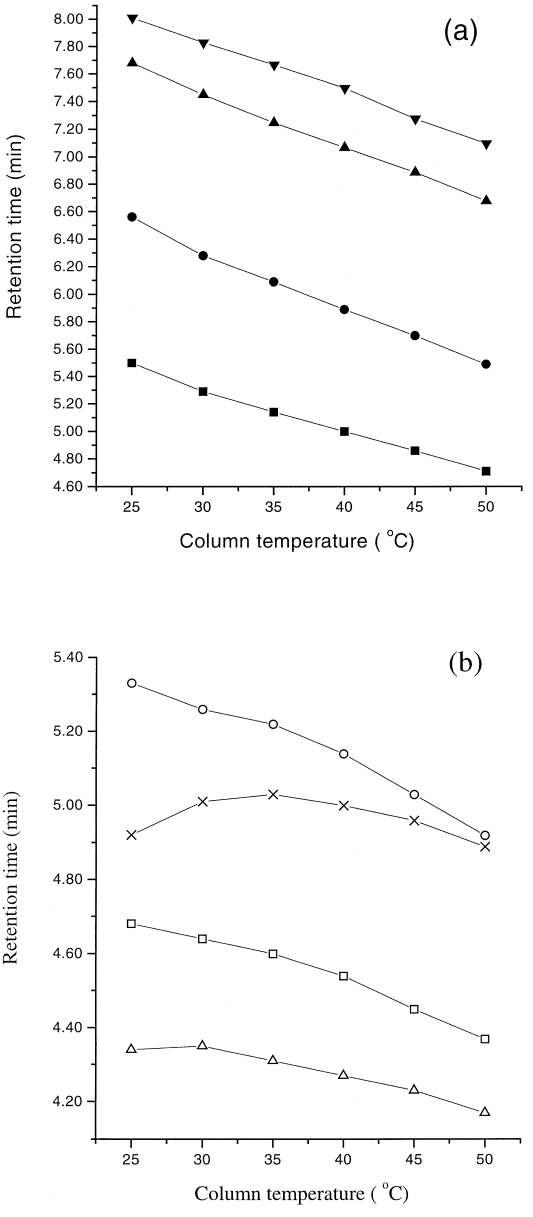

Fig. 2. Variation in the retention times of (a) the steroidal glycoalkaloids (SGAs) and (b) the steroidal glycoalkaloid aglycones (SGAAs) under isocratic conditions with column temperature. Chromatographic conditions: column, Zorbax Rx C_{18} (250× 4.6 mm I.D.), 5 μm; column temperature, 25, 30, 35, 40, 45, 50°C; flow-rate, 1 ml/min; (a) 25% and (b) 40% acetonitrile in 25 m*M* TEAP buffer (pH 3.0); UV absorbance detection, 205 nm. (■)= Dehydrotomatine, (●)=tomatine, (▲)=α-solanine, (▼)=α-chaconine, (△)=solanidine, (□)=dehydrotomatidine, (×)= solasodine, (○)=tomatidine.

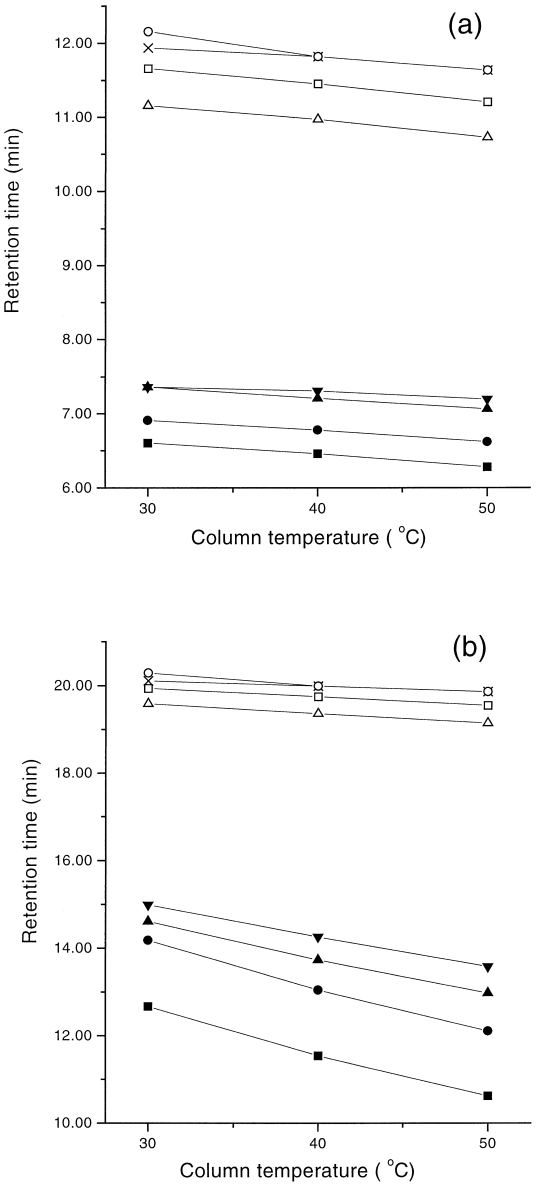

Fig. 3. Variation in the retention times of SGAs and SGAAs with column temperature under (a) linear and (b) stepwise gradient elution conditions. Chromatographic conditions: column, Zorbax Rx C_{18} (250×4.6 mm I.D.), 5 μm; column temperature, 30, 40, 50°C; flow-rate, 1 ml/min; linear acetonitrile–25 m*M* TEAP buffer (pH 3.0) gradient 20–70% acetonitrile over 20 min; stepwise acetonitrile–25 m*M* TEAP buffer (pH 3.0) gradient 20, 25, 35, 45 and 65% acetonitrile at time 0, 12, 15, 17, 25 min, respectively; UV absorbance detection, 205 nm. (■)= Dehydrotomatine, (●)=tomatine, (▲)=α-solanine, (▼)=α-chaconine, (△)=solanidine, (□)=dehydrotomatidine, (×)= solasodine, (○)=tomatidine.

mixture of commercial SGAs, tomatine, α-solanine, α-chaconine and SGAAs, solanidine, tomatidine and solasodine during a single chromatographic run analyzed under linear and stepwise gradient elution conditions using Zorbax Rx C_{18} and Zorbax SB C_{18} columns. SGAs and SGAAs are eluted in the order of dehydrotomatine, tomatine, α-solanine, α-chaconine, solanidine and dehydrotomatidine, solasodine and tomatidine. Dehydrotomatidine, solasodine and tomatidine eluted adjacent to each other and solasodine and tomatidine co-eluted in most cases, but eluted clearly separated (R_s=1.33–1.81) on Zorbax SB C_{18} column with both gradient profiles and three column temperatures. The peaks of solasodine and tomatidine approached to each other with increase in temperature from 30 to 50°C, but were still separated at 50°C (Fig. 4c and d). On the Zorbax Rx C_{18} column tomatidine was only partly separated from solasodine with both gradient profiles at column temperature of 30°C (Fig. 3a and b). Hypersil ODS and Inertsil ODS-3 columns could not resolve solasodine and tomatidine under these conditions. Another critical compound pair under gradient elution conditions was α-solanine and α-chaconine, which eluted as well-separated peaks from both Zorbax columns using stepwise gradient and column temperature of 50°C (Fig. 4b and d). Linear gradient provided only partial separation of α-solanine and α-chaconine at column temperatures of 40 and 50°C.

3.2. Purity of reference compounds

Chemical standards play an increasingly important role in assuring that analytical methods give reliable results. Purity of the standard is extremely important in any analytical calculations. Our commercial tomatine standards from Sigma (three different lots, no label purity) yielded two peaks of approximately equal size in LC (Fig. 5a). The positive FAB-MS of the two peaks collected from LC column exhibited two $[M+H]^+$ ions at *m/z* 1033 and 1035, indicating that the glycoalkaloid molecular masses are 1032 and 1034, respectively. The results are in agreement with previously reported values for dehydrotomatine and tomatine [40–42]. It must be pointed out that some reported studies on the analysis of tomatine in tomatoes assumed the presence of only a single

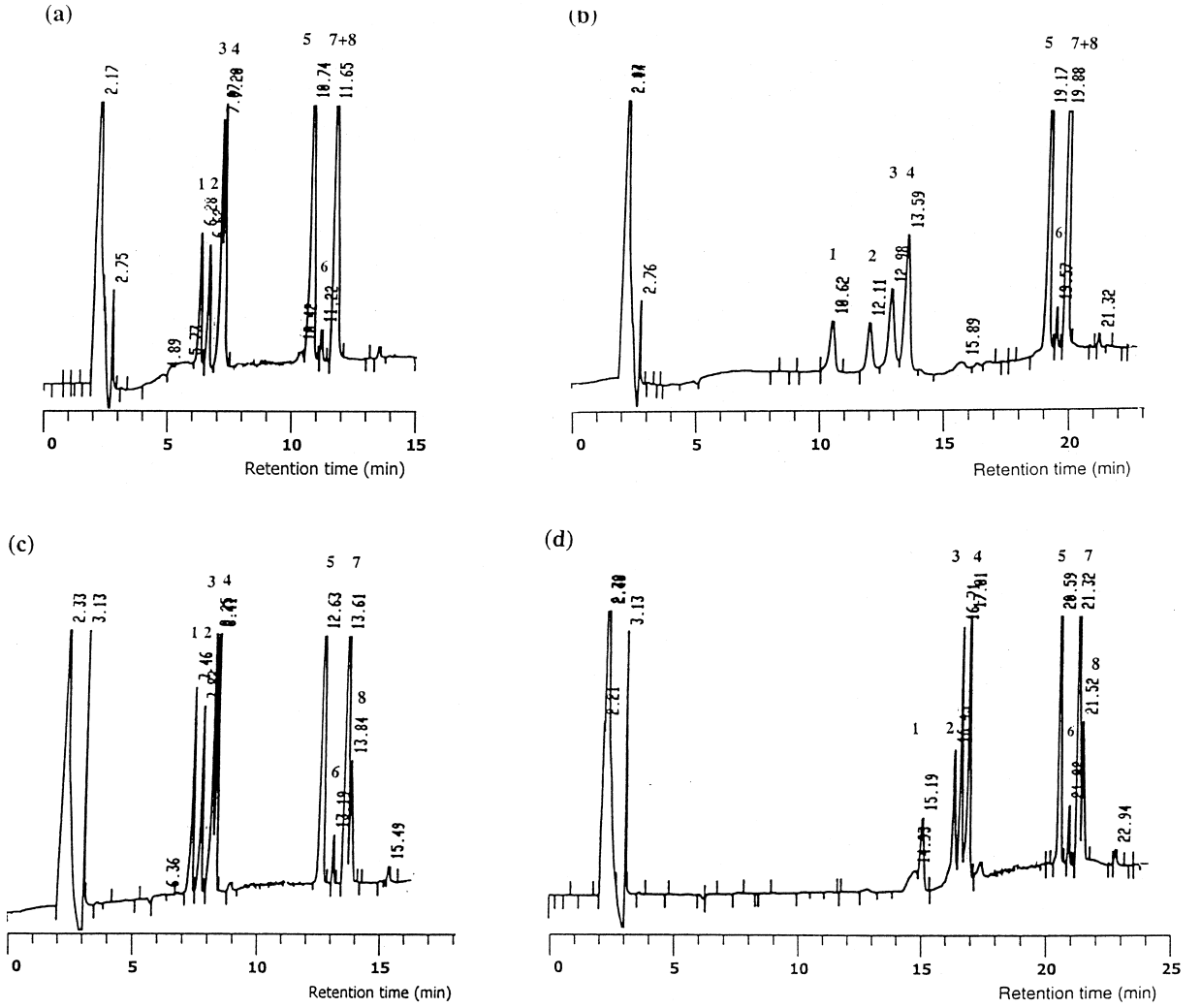

Fig. 4. LC chromatograms of the simultaneous separation of SGAs and SGAAs under linear and stepwise gradient elution conditions on two columns: (a), (b) Zorbax Rx C_{18} and (c), (d) Zorbax SB C_{18}; column temperature, 50°C; flow-rate, 1.0 ml/min; acetonitrile–TEAP buffer (pH 3.0) (a), (c) linear gradient from 20 to 70% acetonitrile in 20 min or (b), (d) stepwise gradient 20, 25, 35, 45 and 65% acetonitrile at time 0, 12, 15, 17 and 25 min, respectively; UV absorbance detection, 205 nm. Peaks: 1=dehydrotomatine, 2=α-tomatine, 3=α-solanine, 4=α-chaconine, 5=solanidine, 6=dehydrotomatidine, 7=solasodine and 8=tomatidine.

glycoalkaloid, α-tomatine, in the Sigma standard [37,43].

The commercial tomatidine also showed two peaks (size 1:5) in our LC experiments (Fig. 5b). Mass spectral analysis of the peaks collected from the LC column exhibited molecular ion peaks at m/z 413 and 415 and the ions at m/z 114 and 138, which are characteristic for the spirosolane-type alkaloids. According to Sigma, tomatidine contains solasodine, which has the same molecular mass and similar mass spectrum as compared with dehydrotomatidine. Their only structural differences are reverse configurations at C-22 and C-25 atoms: solasodine has 22*R*,25*R*-configuration and dehydrotomatidine has 22*S*,25*S*-configuration, respectively [44]. Our LC and TLC results were not, however, consistent with the results of Sigma. First, two peaks of commercial tomatidine did not co-elute with the commercial solasodine.

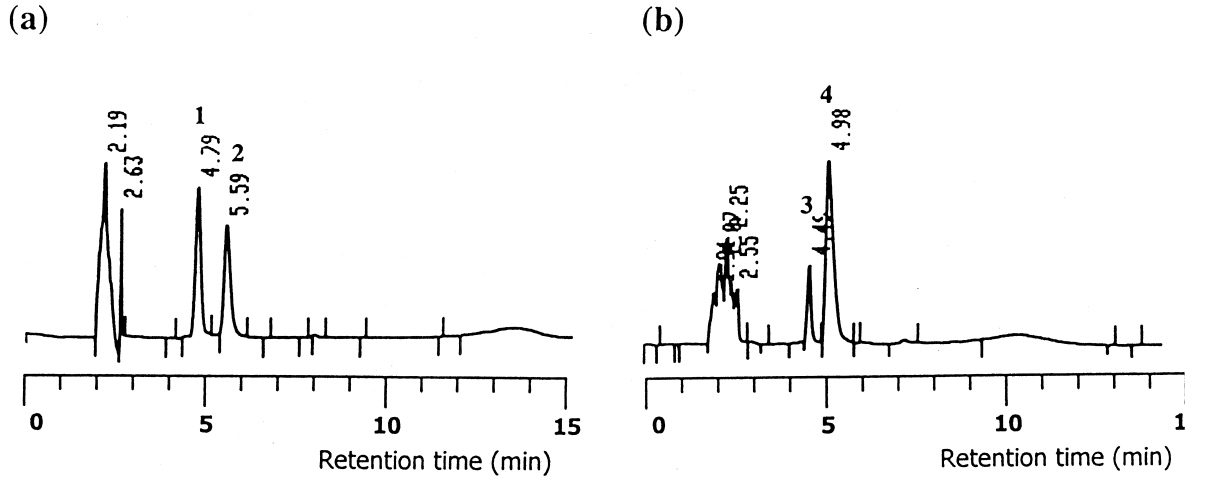

Fig. 5. LC chromatograms of commercial (a) α-tomatine and (b) tomatidine under isocratic conditions of 25 and 40% acetonitrile in 25 m*M* TEAP buffer (pH 3.0), respectively. Column, Zorbax Rx C_{18} (250×4.6 mm I.D.), 5 μm; column temperature, 50°C; flow-rate 1 ml/min; UV absorbance detection at 205 nm. Peaks: 1=dehydrotomatine (4.79 min), 2=tomatine (5.59 min), 3=dehydrotomatidine (4.49 min), 4=tomatidine (4.98 min).

Second, in our TLC experiments the difference in color between solasodine and tomatidine components after H_2SO_4–pyrolytic visualization was remarkable: solasodine showed purple color, whereas the two of tomatidine components both exhibited deep green spots. Third, the R_F values for solasodine and the two tomatidine components were 0.63, 0.58 and 0.65, respectively (Fig. 6). Thus, on the basis of our LC, MS and TLC analyses tomatidine contains dehydrotomatidine as a main impurity instead of solasodine. In addition, TLC and LC analyses of the tomatine hydrolysate indicated the presence of tomatidine and dehydrotomatidine and the absence of solasodine.

4. Conclusions

The chromatographic properties of steroidal glycoalkaloids and their aglycones were evaluated by monitoring their elution characteristics in RP-LC. The results show the importance of a careful choice of the chromatographic conditions for the successful LC separation of these compounds. The LC conditions of this study minimize problems with basic compounds and are an improvement over previous methods in terms of simplicity of mobile phase and possibility to separate both SGAs and SGAAs during a single chromatographic run under gradient elution conditions. The newer column chemistry reduces peak tailing and retention for SGAs and SGAAs. Further, it appears that isocratic and gradient retentions show no significant changes in selectivity and show unexpectedly nearly similar sensitivity to temperature changes. The stereochemical difference between solasodine and dehyrotomatidine produces minor difference in their chromatographic mobility in LC and TLC making possible their separation and identification.

In our future reports the separation will be further refined with the aid of computer simulation and the method will be applied to several wild *Solanum* species and hybride materials to profile their glycoalkaloid content. Comprehensive studies to clarify the sample preparation and clean-up of potato plant material in detail are also in progress.

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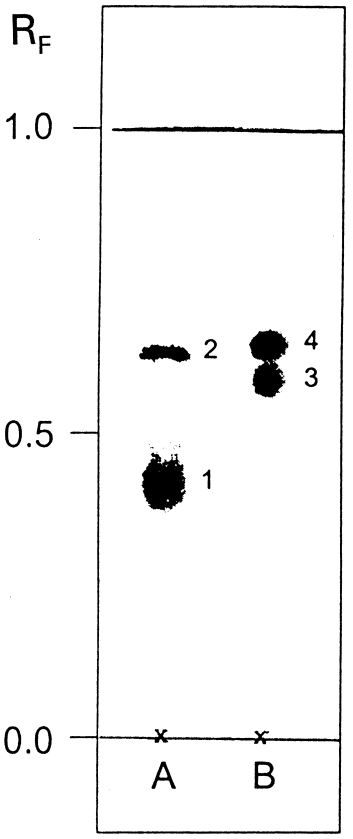

Fig. 6. TLC separation of commercial solasodine and tomatidine. Solvent system: 95% ethanol. Plate: pre-coated silica gel 60 F_{254} (Merck). Visualization: spraying with 95% H_2SO_4–methanol (1:1) and heating until spots visible. Samples: (A) solasodine; (B) tomatidine. Spot identities: (1) not identified, purple, R_F=0.41; (2) solasodine, purple, R_F=0.63; (3) dehydrotomatidine, green, R_F=0.58; (4) tomatidine, green, R_F=0.65.

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