Determination of Solanidine- and Tomatidine-Type Glycoalkaloid Aglycons by Gas Chromatography/Mass Spectrometry

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A combined derivatization method for gas chromatographic/mass spectrometric (GC/MS) analysis of steroidal glycoalkaloid (SGA) aglycons was developed using both trimethylsilylation and pentafluoropropionylation. In comparison with underivatized or only silylated aglycons, the new technique produces more specific and abundant fragmentation for compounds with a tomatidine-type structure. For example, the difference between solasodine and tomatidine, the former containing a double bond at position 5,6 in the steroidal skeleton, can be observed by their base peak fragments at *m*/*z* 417 ($C_{24}H_{41}O_2Si_2$) and *m*/*z* 419 ($C_{24}H_{43}O_2Si_2$). The method is well suited for the simultaneous determination of both solanidane- and spirosolane-type SGA aglycons from *Solanum* species and hybrids. The reproducibility of the method, including SGA extraction, hydrolysis, derivatization, and quantitative GC/MS analysis, was <6% (CV) for the principal aglycons determined from a hybrid between a wild potato species, *Solanum brevidens* Phil., and a cultivated potato, *S. tuberosum* L. A single ion monitoring technique using specific fragments *m*/*z* 419 and 417 could be applied for the determination of minor stereoisomers, which are often overlapped by large amounts of tomatidine.

Keywords: *Glycoalkaloid aglycons; gas chromatography/mass spectrometry; silylation; acylation; single ion monitoring; potato hybrids*

INTRODUCTION

Steroidal glycoalkaloids (SGA) are nitrogenous secondary plant metabolites found in all parts of *Solanum* species (Van Gelder, 1991). Because of their toxicity, reliable analytical methods are important to determine individual glycoalkaloids, especially when wild potato germplasm is used in potato breeding programs to increase biotic and abiotic stress resistance. Many wild potato species show divergent glycoalkaloid profiles and generally contain higher glycoalkaloid amounts than cultivated potato. Therefore, new cultivars, developed from interspecific potato hybrids, can contain many different glycoalkaloids derived from the wild potato species (Van Gelder, 1991; Deahl et al., 1993). In addition, significant amounts of glycoalkaloids, not present in the parental genotypes, can be detected in hybrids (Mattheij et al., 1992; Laurila et al., 1996).

High-performance liquid chromatography (HPLC) is the most frequently used method in quantitative analysis of glycoalkaloids, which are usually composed of di-, tri-, or tetrasaccharides bound glycosidically to the aglycon moiety. For some glycoalkaloids, such as tomatine, having no double bond for intensive UV absorption, a more sensitive HPLC technique using amperometric detection has been described (Friedman et al., 1994). Several mass spectrometric (MS) methods, that is, LC/MS (Bushway et al., 1994), liquid secondary ion MS (Friedman et al., 1994), tandem MS (Chen et al., 1994), or laser desorption/ionization MS (Abell and Sporns, 1996), have been applied to the identification of glycoalkaloids with sugar moieties.

Gas chromatography (GC) is well suited for the determination of glycoalkaloid aglycon patterns in potato materials (Gregory et al., 1981; Van Gelder, 1985; Van Gelder et al., 1988, 1989; Lawson et al., 1992). Using high-resolution capillary GC with a nitrogen-specific detector, a series of aglycons can be separated in a single run during a reasonable analysis time (Van Gelder et al., 1988). The aglycons can be analyzed without derivatization (Van Gelder, 1985; Van Gelder et al., 1988, 1989; Lawson et al., 1992), but relatively high injector and column temperatures are required in the GC system, which in turn can shorten the column life and cause peak tailing or aglycon decomposition. In addition, the GC method is considered to be laborious due to the preceding hydrolysis of glycoalkaloids.

Despite an increase in the molecular weight of aglycons, derivatization is a simple means for their conversion to more volatile and thermally stable derivatives. Trimethylsilylation (Juvik et al., 1982) and acylation (King, 1980) of glycoalkaloid aglycons, or permethylation of intact glycoalkaloids (Herb et al., 1975), have been introduced earlier for GC analysis. A GC/MS method using trimethylsilyl (TMS) derivatization in the aglycon analysis of potato hybrid materials has been recently described (Laurila et al., 1996). In the mass spectrometric (MS) analysis of spirosolanes the same

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main fragments (*m*/*z* 114 and 138) are obtained for underivatized tomatidine, solasodine, and tomatidenol, for example (Budzikiewicz, 1964; Van Gelder and Scheffer, 1991). The tomatidine-diTMS derivative gives one major ion (*m*/*z* 125) (Laurila et al., 1996). Most diagnostic fragments are found at higher masses having very low abundancies only, which can make the characterization of minor constituents difficult or even impossible in the case of coeluting compounds.

The aim of this study was to develop a new derivatization technique better applicable to GC/MS analysis of SGA aglycons, especially for those with a spirosolane structure. After TMS derivatization of tomatidine-type aglycons, pentafluoropropionylation was used to obtain both better resolution and specific fragments suitable for the analyses using a single ion monitoring (SIM) method. The method was tested through analyses of hybrid materials derived from the wild *Solanum* species *S. brevidens* and cultivated potato (*S. tuberosum*).

MATERIALS AND METHODS

Pure Substances. Samples of authentic glycoalkaloids (α-tomatine, α-solanine), aglycons (solanidine, solasodine), and cholesterol (internal standard, I.S.) were obtained from Sigma (St. Louis, MO). Cholesterol was dissolved in chloroform at 1 mg/mL.

Plant Materials. Wild *Solanum* species *S. brevidens* ($2n = 2x = 24$) CPC 2451 and the cultivated potato *S. tuberosum* ($2n = 2x = 24$) dihaploid line Pito 45/4 were included in this study as parental materials, and their somatic hybrids (Rokka et al., 1994) and other *tuberosum* materials were also used. In vitro plantlets were aseptically cultured on MS medium (Murashige and Skoog, 1962) with 2% (w/v) sucrose, 100 mg/L casein hydrolysate, and 0.05 mg/L α-naphthaleneacetic acid (NAA) in a 16 h photoperiod at a temperature of 23 °C. After rooting, plantlets were planted in greenhouse compost (a mixture of peat and sand 10:1 v/v) under natural daylight in summertime and in wintertime under an 18 h/day with supplementary illumination provided by sodium halide lamps. The plants were recultured periodically (at 2−3 month intervals) in vivo by taking nodal cuttings. Leaves of hybrid plants and parental materials were harvested at different stages of maturity, air-dried at 60−80 °C, and ground to fine powder.

Sample Preparation. Samples were prepared by modifying the procedure described by Laurila et al. (1996), which was originally a combination of extraction and hydrolysis methods reported by Gregory et al. (1981) and Van Gelder (1984). Samples, 100 mg of air-dried leaf powder, were extracted in 5% acetic acid (4 mL) with sonication for 15 min. Extracts were filtered, and the filtrates were washed with 4 mL of extraction medium. Cholesterol (50 μL, 1 mg/mL) was added as an internal standard. The acetic acid extracts were freeze-dried for 16−18 h and hydrolyzed.

For hydrolysis, dry samples were dissolved in 2 mL of 1 M HCl in methanol and heated for 3 h at 70 °C in a water bath. The free aglycons were liberated from the hydrolysate by adding 2 mL of 25% ammonia to the cooled tube and extracted with 2 mL of dichloromethane after a few minutes. After vigorous mixing and 5 min of centrifugation, the dichloromethane layer was removed with a Pasteur pipet. This extraction procedure was repeated with two additional 2 mL aliquots of dichloromethane, and the extracts were combined. The aglycon extracts were then evaporated to dryness under a nitrogen stream.

Derivatization of Aglycons. Dried aglycon samples (containing I.S.) obtained after hydrolysis of plant extracts or authentic SGAs were first silylated with 20 μL of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (Pierce, Rockford, IL), kept at 60 °C for 15 min, and cooled to room temperature. For acylation of diTMS derivatives of tomatidine-type aglycons, 6 μL of pentafluoropropionic acid anhydride (PFAA) (Pierce) were added. A 2 μL sample was taken for GC/MS analysis.

Mass Spectrometric and NMR Analyses. The aglycon derivatives were determined using a Hewlett-Packard (HP) 5890 gas chromatograph coupled to an HP 5970 quadrupole mass selective detector operating at an ionization voltage of 70 eV (EI mode) and an electron multiplier voltage of 1600 V. Samples were analyzed on an NB-54 fused-silica capillary column (15 m, 0.20 mm i.d., Nordion, Finland) using split sampling mode and an oven temperature of 180−285 °C heated at 7.5 °C/min. Injector and detector temperatures were 285 °C. Helium was used as the carrier gas (flow rate = 0.5 mL/min). Identification of the aglycons in the plant materials was based on the GC/MS spectra of TMS and pentafluoropropionyl derivatives of authentic compounds and on reports of GC and MS glycoalkaloid aglycon data (Van Gelder et al., 1989; Laurila et al., 1996).

The fragmentation of diTMS and diTMS-pentafluoropropionyl derivatives of tomatidine was confirmed by high-resolution MS analyses on a VG 7070E mass spectrometer operated at EI mode with an ion source temperature of 180 °C and an ion source energy of 70 eV. The temperature of the water-cooled probe was increased from 180 to 250 °C. The data were processed by the Opus program.

NMR analysis of the diTMS derivative of solasodine was performed using a Varian Gemini-200 spectrometer. NMR sample was dissolved in $CDCl_3$. Internal locking was at the deuterium resonance signal of their NMR solvent, and chemical shifts were in parts per million downfield from TMS. Two-dimensional experiments were performed according to standard pulse sequences.

Optimization of the Method. The SGA aglycon concentrations of the plant samples were calculated from peak areas (total or single ion abundancies) of aglycons to an internal standard (cholesterol). The reproducibility of the quantitative analyses was determined from the same hybrid plant material ($N = 6$), which was extracted, hydrolyzed, derivatized, and analyzed by GC/MS. To test the recovery, 100 μg of α-tomatine (corresponding 40.1 μg of tomatidine) was added to a plant extract containing 60.9 μg of tomatidine/100 mg of plant material ($N = 3$). The linearity of the method was determined for solasodine by triplicate determinations at four concentration levels in the range between 20 and 400 μg, and the calibration curve was obtained by plotting the peak area ratio (solasodine/**I.**S.) against the amount of solasodine.

RESULTS AND DISCUSSION

A GC/MS method has been recently applied to the determination of SGA aglycon composition in potato breeding materials (Laurila et al., 1996). Analyses of somatic hybrids between the wild potato species *S. brevidens* and *S. tuberosum* showed considerable variation in the concentrations of solanidine, tomatidine, and demissidine, the novel aglycon of these hybrids. In addition, tomatidine levels in the symmetric somatic hybrids were found to be closely related to the proportion of the genome derived from *S. brevidens*. The aglycons had been determined as TMS derivatives.

The GC/MS spectra of solanidine and demissidine monoTMS derivatives give the main fragments at *m*/*z* 150 and 204 and molecular ions at *m*/*z* 469 and 471, respectively (Table 1). Thus, TMS derivatization does not much affect spectra profiles, because *m*/*z* 150 ($C_{10}H_{16}N$) and 204 ($C_{14}H_{22}N$) are also found as the most abundant fragments for a number of underivatized solanidine-type aglycons (Van Gelder et al., 1989). Underivatized tomatidine (MW 415), which has the base peak at *m*/*z* 114, gives a diTMS derivative after silylation. The molecular ion at *m*/*z* 559 and the base peak at *m*/*z* 125 ($C_8H_{15}N$) (Tables 1 and 2) indicate that two TMS groups are present but neither of them in the nitrogen-containing ring. The NMR data of the diTMS derivative of solasodine [^{13}C NMR signals C-3, 72.4;

Table 1. GC/MS Fragmentation and Molecular Ions of TMS- and diTMS-pentafluoropropionyl Derivatives of Common SGA Aglycons

aglycon derivative	base peak *m/z*	other major fragments *m/z* (% abundance)	mol ion *m/z*
solanidine			
-monoTMS	150	204 (28), 151 (14), 454 (11), 73 (7)	469
demissidine			
-monoTMS	150	204 (35), 75 (16), 151 (14), 73 (7)	471
solasodine			
-diTMS	125	73 (11), 126 (10), 111 (5), 110 (4)	557
-diTMS-PFasyl	417	73 (46), 418 (38), 269 (36), 270 (21)	703
soladulcidine (?)			
-diTMS	125	111 (62), 126 (27), 73 (23), 75 (20)	559
-diTMS-PFasyl	419	269 (68), 420 (40), 73 (38), 270 (34)	705
tomatidenol			
-diTMS-PFasyl	417[a]	418 (59)[a]	703
tomatidine			
-diTMS	125	126 (10), 111 (8), 73 (6), 75 (6)	559
-diTMS-PFasyl	419	269 (48), 420 (39), 73 (38), 75 (33)	705

[a] Detected by SIM analysis.

Table 2. Elemental Composition of Characteristic Ions of diTMS and diTMS-pentafluoropropionyl Derivatives of Tomatidine Determined by High-Resolution MS

diagnostic ions of tomatidine derivatives	obsd mass *m/z*	calcd mass *m/z*	formula
diTMS			
125	125.12063	125.12045	$C_8H_{15}N$
diTMS-PFasyl[a]			
269	269.08325	269.08391	$C_{11}H_{12}NOF_5$
345	345.26068	345.26137	$C_{22}H_{37}OSi$
419	419.27905	419.28016	$C_{24}H_{43}O_2Si_2$
434	434.30441	434.30364	$C_{25}H_{46}O_2Si_2$

[a] See also GC/MS spectra in Figure 2.

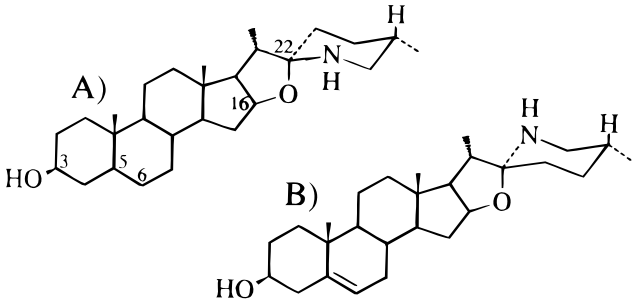

Figure 1. Structures of tomatidine (A) and solasodine (B).

C-16, 72.5; C-22, 175.4 (a quaternary carbon according to DEPT spectrum)] confirm an opening of the tetrahydrofuran ring, after which the formed hydroxyl group has been attached by a TMS group. Such a phenomenon seems to be related to the occurrence of the nitrogen ring, because the silylation of diosgenin containing oxygen instead of nitrogen gave a monoTMS derivative only. However, the fragments with two TMS groups in both tomatidine ($C_{25}H_{46}O_2Si_2$; 434) and solasodine ($C_{25}H_{44}O_2Si_2$; 432) can be clearly seen after acylation (Figure 2).

When the SGA compositions with high tomatidine concentrations, for example, are analyzed, peak broadening is common. The resolution is not always good enough for reliable quantification, especially in the case of tomatidine-type structures usually found in trace amounts in breeding materials. For this study, a derivatization technique was developed by using both silylation and acylation, not only to improve the GC resolution but also to produce more specific and abundant fragmentation for tomatidine-type aglycons. The

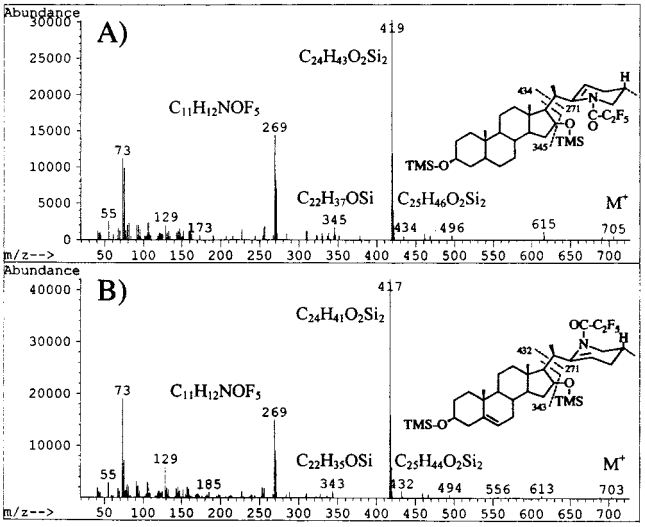

Figure 2. GC/MS spectra of trimethylsilyl/pentafluoropropionyl derivatives of tomatidine (A) and solasodine (B).

new method, which can be used through SIM, is especially suited for plant breeding purposes when a range of aglycons has to be detected at very low concentrations.

Trimethylsilylation and Pentafluoropropionylation of SGA Aglycons. The combined derivatization method, including silylation by MSTFA followed by pentafluoropropionylation using PFAA, gave a single peak for authentic solanidine. Acylation did not affect the fragmentation, and the base peak and molecular ion of solanidine showed the formation of a monoTMS derivate only (Table 1). Cholesterol was used as an I.S., and its trimethylsilylation is a traditional technique that gives a monoTMS derivative with a base peak of *m/z* 129 (Brooks et al., 1968).

When using silylation alone, one major fragment (*m/z* 125) is obtained for tomatidine and related isomers (Table 1). After their acylation, clearly different spectra profiles with abundant fragments at higher mass ranges can be seen. TMS ethers are first formed at positions 3 and 16 by silylation with MSTFA (Figures 1 and 2). After the addition of PFAA reagent, nitrogen is attached by a pentafluoropropionyl group, giving a fragment *m/z* 269 ($C_{11}H_{12}NOF_5$) (Table 2), which is typical for both solasodine and tomatidine. In contrast, the difference between them, that is, the former containing a double bond at position 5,6 (Figure 1), can be observed by their base peak fragments at *m/z* 417 ($C_{24}H_{41}O_2Si_2$) and *m/z* 419 ($C_{24}H_{43}O_2Si_2$) (Figure 2). These fragments are derived from the ions *m/z* 432 and 434, respectively, after a loss of a methyl group. In addition, a number of less abundant fragments at higher mass range show the difference of one double bond between these compounds.

Minor Tomatidine-Type Aglycons. The specific fragmentation also enabled a more reliable detection and quantification of minor isomers with tomatidine-type structures. Solasodine (Figure 1) and tomatidenol have opposite configurations at C-22 and C-25 in the nitrogen-containing ring (Friedman and McDonald, 1997) but the same molecular weight and a double bond at the 5,6 position. As indicated by the extracted iongram of authentic solasodine and tomatidine analysis (Figure 4A), solasodine and tomatidenol both showed a peak at *m/z* 417. However, they had clearly different retention times. Tomatidenol, an aglycon of dehydrotomatine commonly detected as an impurity of commercial

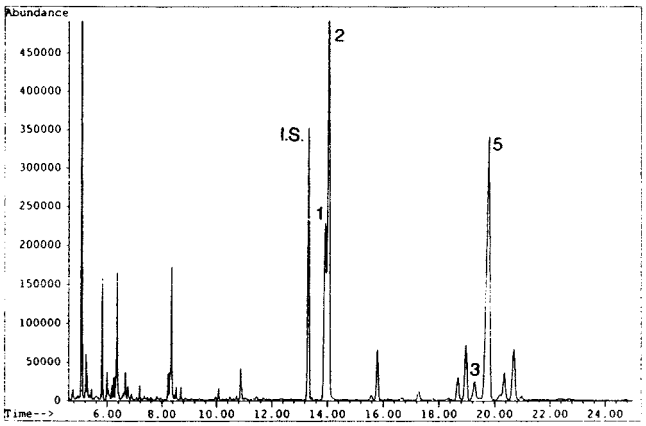

Figure 3. Total ion GC/MS analysis of derivatized SGA aglycons of a hybrid between *S. brevidens* and *S. tuberosum*. Peaks: 1, solanidine; 2, demissidine; 3, soladulcidine (?); 5, tomatidine; and **I.**S., cholesterol.

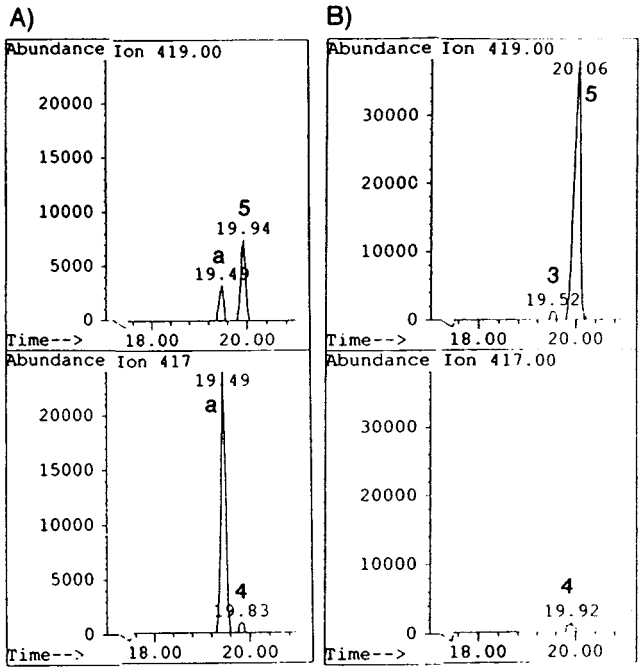

Figure 4. Extracted ion chromatograms of trimethylsilyl/pentafluoropropionyl derivatives of authentic solasodine and tomatidine (A) and spirosolane aglycons in *S. brevidens* (B) using ions *m*/*z* 419 and 417. Peaks: a, solasodine; 3, soladulcidine (?); 4, tomatidenol; and 5, tomatidine.

tomatine (Friedman et al., 1994; Bushway et al., 1994; Ono et al., 1997), was overlapped by tomatidine in total ion analysis but could be quantified by using ion *m*/*z* 417. Tomatidenol, detected here for the first time in *S. brevidens*, was found at concentrations of $\sim^1/_{25}$ (peak areas of *m*/*z* 417/419) that of tomatidine.

Compound **3** (Figures 3 and 4B) in *S. brevidens* had a base peak at *m*/*z* 419, suggesting a tomatidine-type structure. This aglycon, earlier determined to be a diTMS derivative, also showed the same molecular ion (M^+ 559) and shorter retention time than tomatidine (Laurila et al., 1996). Tomatidine and soladulcidine form another isomeric pair of tomatidine-type SGA aglycons, which also have opposite configurations at C-22 and C-25 in the nitrogen-containing ring but with no double bond at the 5,6 position. This would suggest that soladulcidine is another aglycon in *S. brevidens* having the same molecular weight as tomatidine. A group of tomatidine-type aglycons, especially soladulcidine, is

Table 3. Reproducibility of Quantitative GC/MS Analyses of SGA Aglycons in the Somatic Hybrid between *S. brevidens* and *S. tuberosum*

compound	retention time (min)	mean[a] (mg/kg)	CV (%)
1, solanidine	13.95	489.1	4.31
2, demissidine	14.09	1030.9	5.72
3, soladulcidine (?)[b]	19.29	101.3	2.39
4, tomatidenol[c]	19.63	25.6	15.14
5, tomatidine	19.83	1110.1	1.22

[a] Quantitative analysis based on total ion abundances (compounds **1**, **2**, and **5**). [b] Quantified by using ions *m*/*z* 419 and 129 (the base peak of the I.S.); identification based on retention time and GC/MS fragmentation only. [c] Quantified by using ions *m*/*z* 417 and 129.

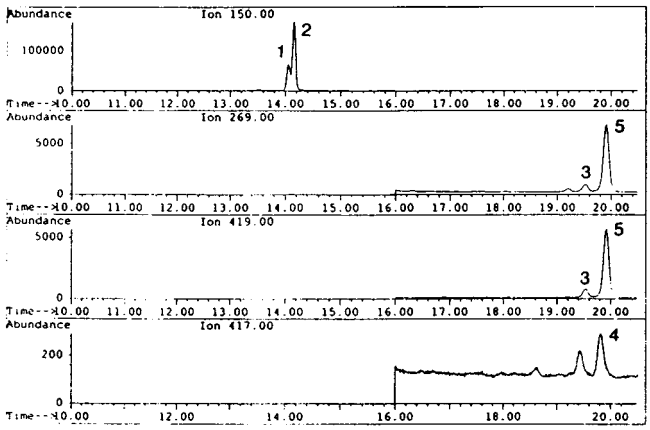

Figure 5. GC/MS-SIM analysis of SGA aglycons from a 2-week-old in vitro-grown hybrid plantlet (fresh weight = 25 mg) using *m*/*z* 150, 269, 419, and 417 as the characteristic ions. Peaks: 1, solanidine; 2, demissidine; 3, soladulcidine (?); 4, tomatidenol; and 5, tomatidine.

found in *S. dulcamara* L., which is known to form three chemovarieties containing either tomatidenol, solasodine, or soladulcidine (Ehmke and Eilert, 1986).

Analysis of Hybrid Materials. Simultaneous GC/MS analysis of solanidine- and tomatidine-type aglycons of somatic hybrids between *S. brevidens* and *S. tuberosum* took ~20 min (Figure 3). Five compounds showed typical fragments of glycoalkaloids. Using the ion *m*/*z* 150 as the specific fragment, solanthrene could not be detected. The reproducibility of the method including extraction, standard addition, hydrolysis, derivatization, and GC/MS analysis ranged from 1 to 6% (CV) for the main compounds (Table 3). The mean percent recovery of tomatidine (60.9 μg in the original plant extract), determined after hydrolysis of 100 μg of added α-tomatine, was 113% (CV 1.6%, $N = 3$) (data not shown). The calibration curve for solasodine was linear in the range of 20−400 μg ($y = 0.0080x + 0.0266$; $r = 0.998$), which is typical of SGA aglycon concentrations in 100 mg of our hybrid materials.

The GC/MS-SIM technique was applied to the analysis of SGAs from in vitro-grown plantlets (Figure 5). The ions *m*/*z* 150 and 204 were selected for solanidine-type aglycons and *m*/*z* 419 and 417 for tomatidine-type aglycons. In addition, ion *m*/*z* 125, the main fragment of the diTMS derivative of tomatidine, can be used to confirm that all tomatidine-type aglycons have been acylated. The results from these experiments show that an aglycon pattern of a hybrid already qualitatively similar to an adult one (Table 3) can be determined even from a 2-week-old plantlet. The GC/MS-SIM technique is therefore well suited for plant breeding studies, when

large numbers of samples have to be screened. In addition, the detection of minor SGA aglycons in developing hybrids is valuable for studying the biogenetic relationships among SGA aglycons.

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