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CHARACTERIZATION OF NOVEL STEROIDAL ALKALOIDS FROM TUBERS OF *SOLANUM* SPECIES BY COMBINED GAS CHROMATOGRAPHY–MASS SPECTROMETRY

IMPLICATIONS FOR POTATO BREEDING

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SUMMARY

Wild *Solanum* species are widely used in potato breeding as a source of valuable germplasm. Together with desired characteristics, toxic steroidal glycoalkaloids (SGAs), including unidentified SGAs, are sometimes transferred from the wild species to the cultivated potato. In this study, steroidal alkaloids (SAs) which originated from unidentified aglycones of SGAs found in tubers of *Solanum* species were characterized using retention indices, gas chromatography–mass spectrometry, high-resolution mass spectrometry (resolution 20 000) and hydrolysis in two-phase systems. All SAs possessed a solanidane skeleton and were probably substituted or dehydrogenated forms of solanidine. Most of these SAs have not been reported before.

Analysis of the SGAs of three wild *Solanum* species and one primitive cultivated subspecies used in potato breeding, showed that the total SGA contents varied widely (403–2228 mg kg^{-1} fresh tuber) as did the concentrations of the individual SGAs within a species. The implications of the results are discussed from the viewpoints of breeding for resistance against pathogens or insects and of food safety of household potatoes.

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INTRODUCTION

Steroidal alkaloids (SAs) are natural toxins of the Solanaceae. They occur in all parts of the plants and are in general glycosidically bound (steroidal glycoalkaloids; SGAs)[1]. Household potatoes contain the solanidine glycosides α-solanine and α-chaconine, usually in concentrations below 150 mg kg^{-1}, which are considered to present no health hazard to the consumer. The occurrence, the genetic and environmental control and the toxicology of the solanidine glycosides have been reviewed[2,3].

Wild *Solanum* species are being used in potato breeding to introduce desired traits, such as disease and pest resistance, into the cultivated potato. The tubers of some of these species contain extremely high concentrations of SGAs which may result in toxic levels in the hybrid progeny[4]. In addition to known SAs, some species contain unidentified compounds, which have been tentatively characterized as the aglycones of SGAs by their response ratios in nitrogen–phosphorus-specific and flame ionization detection (NPD–FID response ratios) after capillary gas chromatography (GC)[4,5].

This paper describes the characterization of these SAs using, amongst other techniques, gas chromatography–mass spectrometry (GC–MS) and high-resolution mass spectrometry (HRMS). A number of novel SAs were characterized. The SA compositions of tubers of three wild *Solanum* species and one primitive cultivated subspecies (ssp.), widely used in potato breeding, are presented. The implications of the results for potato breeding are discussed from the viewpoints of resistance breeding and food safety.

EXPERIMENTAL

Plant material

Tubers of *Solanum chacoense* accession number 8054, *S. leptophyes* 27208 and *S. tuberosum* ssp. *andigena* 1024 were produced by cultural techniques described elsewhere, as were the taxonomic nomenclature and the provenance of the accession numbers[4]. The analyses of *S. sparsipilum* were carried out on an extract prepared earlier from tubers of accession 8206 genotype number 7, which had been grown under different conditions[4].

Chemicals and sample preparation

SA standards, solvents, extraction of the tubers, two-phase hydrolysis, sample clean-up and capillary GC using retention indices and NPD–FID response ratios were as described previously[4–6].

GC–MS and HRMS

A Finnigan 4500 gas chromatograph–mass spectrometer coupled to an Incos data system was used to record the mass spectra of the SAs. The SAs were gas chromatographed using a fused-silica column (25 m × 0.22 mm I.D.) coated with CP-Sil 5 CB, film thickness 0.12 μm (Chrompack, Middelburg, The Netherlands). The temperature programme of the oven was 280°C (held for 20 min), increased at 8°C min^{-1} to 320°C (held for 5 min). The temperature of the injector was 300°C and that of the interface 290°C. Helium was used as carrier gas at a linear velocity of 30 cm s^{-1}.

The injection volume was 2 μl and the splitting ratio 1:50. The conditions of electron impact (EI) ionization were ion source temperature 230°C, emission current 0.25 mA and ion source energy 70 eV, and those of chemical ionization (CI) were ionization gas methane, ion source temperature 180°C, emission current 0.25 mA, ion source energy 100 eV and ionizer pressure 67 Pa. The mass range was monitored from 50 to 500 mass units in the EI mode and from 90 to 450 mass units in the CI mode. The scan rate was 1 scan per 0.5 s.

The peaks of the total ion current (TIC) profile obtained by GC–MS were compared with the peaks of the mass chromatograms recorded at *m/z* 150 and 204, which are specific for the solanidanes, and at *m/z* 114 and 138, which are specific for the spirosolanes[1]. For each tuber extract, the TIC profile and mass chromatograms were compared with the chromatogram of the N-containing compounds, obtained by capillary GC using simultaneous NPD and FID, in order to investigate whether other types of SAs were present. Coelution of components was checked by comparing the TIC profile and mass chromatograms recorded at *m/z* 150 and 204 with mass chromatograms recorded at specific *m/z* values.

HRMS was used for confirmation of the masses obtained by GC–MS, for determination of the exact masses of individual components and for calculation of their elemental compositions. A Finnigan-MAT 711 mass spectrometer coupled to a Tracor TN-1750 multichannel analyser was used. The EI conditions were ion source temperature 240°C, emission current 800 μA, ion source energy 100 eV and acceleration voltage 8 kV. The resolution was 20 000 (10% valley definition), the probe temperature was increased from 50 to 350°C in 500 s and the mass range was fixed. An amount of 1–5 μl of tuber extract was introduced into the mass spectrometer via a direct insertion probe. Only the tuber extracts of *S. chacoense* and *S. sparsipilum* were analysed by HRMS.

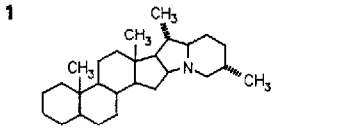
RESULTS AND DISCUSSION

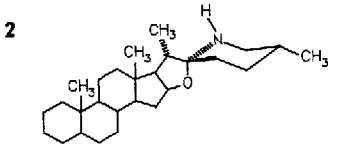
Identification and characterization of SAs

The HRMS of the tuber extracts of *S. chacoense* and *S. sparsipilum* showed abundant ions with the exact masses 150.1283 and 204.1752, for which the formulae $C_{10}H_{16}N$ and $C_{14}H_{22}N$, respectively, were calculated. These formulae agree with those of the diagnostic fragments of *m/z* 150 and 204 of the solanidanes[1] (Fig. 1). Exact masses corresponding to the base peaks of other groups of SAs, such as spirosolanes, epiminocholestanes, aminospirostanes and solanocapsines, were not measured.

All the compounds in the tuber extracts of the four *Solanum* species/ssp. that were characterized as SAs by their NPD/FID response ratios showed main ions at *m/z* 150 (base peak) and 204 in the spectra obtained by GC–MS (EI mode). This means that they were all solanidanes with unsubstituted E and F rings[1,7]. Fig. 2 shows the TIC profile (D) and the mass chromatograms at the *m/z* values of the diagnostic ions of the solanidanes (B and C). Base peaks corresponding to other groups of SAs were not detected, as is illustrated for the spirosolanes (Fig. 1) by the mass chromatogram recorded at *m/z* 114 (Fig. 2A).

Substitutions in the steroid skeleton (rings A–D) do not markedly influence the fragmentation patterns[8]. However, a comparison of the EI mass spectra of nine reference compounds showed that differentiation between the saturated SAs (demis-





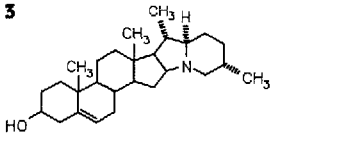


Fig. 1. Structures of the basic skeletons of the solanidanes (1) and spirosolanes (2), and structure of solanidine (solanid-5-en-3β-ol) (3).

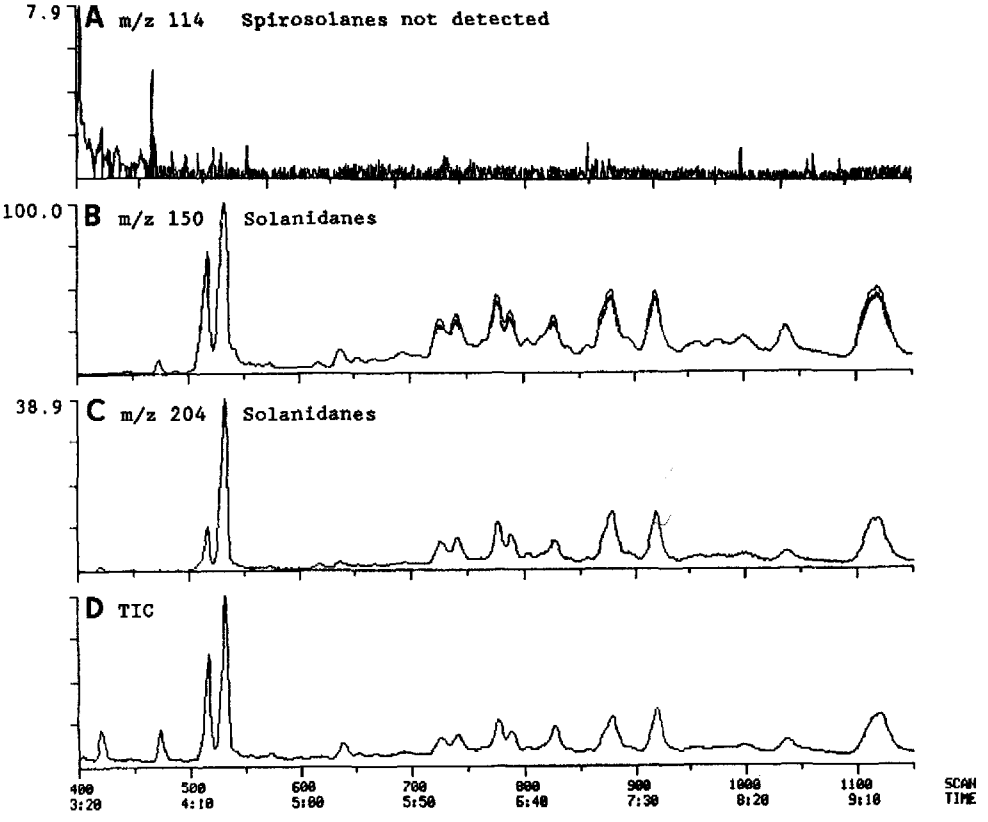


Fig. 2. Mass chromatograms recorded at the diagnostic *m/z* values of spirosolanes [(A) *m/z* 114, base peak] and solanidanes [(B) *m/z* 150, base peak; (C) *m/z* 204] and the total ion current (TIC) profile, obtained by GC–MS (EI mode) analysis of a steroidal alkaloid extract from *S. sparsipilum* tubers.

sidine, soladulcidine and tomatidine), the Δ^5-unsaturated SAs (solanidine, solasodine and tomatidenol) and their 3,5-diene dehydration products (solanthrene, solasodiene and tomatidadiene) was possible by using the ratios of the abudances of the ions at *m/z* 91 and 93, which were about 0.8 for the saturated SAs, 1.3 for the Δ^5-unsaturated compounds and 2.0 for the 3,5-dienes. The same ratios were found for the abundances at *m/z* 105 and 107 for the nine compounds tested.

GC–MS (CI mode) of the SAs showed $(M + 1)^+$ ions with a relative abundance of 30–100%. SAs containing a hydroxyl group showed in addition $(M + 1 - 18)^+$ ions with a relative abundance of 20–50%, due to the loss of water from the protonated molecules (Fig. 3).

On two-phase hydrolysis using chloroform as organic phase, the Δ^5-unsaturated SGAs produce 5-ene-3β-ol aglycones and the corresponding 3,5-diene dehydration products, but using carbon tetrachloride, they produce almost entirely 3,5-dienes[9]. This phenomenon (shift from the concentration of the 5-ene-3β-ol to the concentration of the 3,5-diene) was used for the detection of the enols and the dehydration products by capillary GC. The final identification of each 5-ene-3β-ol and 3,5-diene pair was achieved using the data from EI-mode GC–MS (ratios of the abundances at *m/z* 91:93 and *m/z* 105:107), from CI-mode GC–MS [presence of $(M + 1 - 18)^+$ ions] and from the HRMS (absence of oxygen atoms in the dehydration products and a difference of 18.0105 between the masses of the SAs and their dehydration products).

Table I shows the main physical and chemical data which enabled the SAs to be characterized. Only the naturally occurring SAs are given and not their dehydration products. Compound 1, a solanidadienol, may be dehydrosolanidine according to its retention time and that of its dehydration product $C_{27}H_{39}N$ (exact mass 377.3082), which may be dehydrosolanthrene. Compound 3 is a saturated SA, which contains two hydroxyl groups and is probably an isomer of compound 8. Compounds 4 and 5 are isomers of compound 1. Compound 7 with $M^{+\cdot}$ at *m/z* 427, showed the loss of one molecule of water on hydrolysis (difference in mass of 18.0105 with its dehydration product), an $(M + 1 - 18)^+$ peak in CI-mode GC–MS and an $(M - 31)^+$ peak in the EI mode. Therefore, compound 7 may be methoxysolanidine or hydroxymethylsolanidine. The data from HRMS revealed the presence of an SA showing the mass 427.3450, which corresponds with the formula $C_{28}H_{45}NO_2$; this supported the above hypothesis. A second compound with $M^{+\cdot}$ at *m/z* 427 was detected by GC–MS, namely compound 6, the mass spectrum of which differed from that of compound 7. HRMS also revealed the presence of a compound with a mass of 427.3086, and consequently it was assumed that this mass corresponded to compound 6, for which the formula $C_{27}H_{41}NO_3$ was calculated. Although the compound contained three oxygen atoms, it lost only one molecule of water on hydrolysis (mass difference 18.0105); it is therefore assumed to be a solanidenol containing no second enol group but more likely keto and/or hydroxy groups. Compound 10 may be methylsolanidine as it produced a dehydration product $C_{28}H_{43}N$ (mass 393.3395), which is probably a methylsolanidadiene (methylsolanthrene). One solanidane-type SA with a molecular mass of 423 was detected by EI-mode GC–MS. This compound (11) could either be $C_{27}H_{37}NO_3$ or $C_{28}H_{41}NO_2$, because exact masses corresponding to both formulae were detected by HRMS. Low intensities of the molecular ions of mass 411.3137 ($C_{27}H_{41}NO_2$) and 425.2930 ($C_{27}H_{39}NO_3$) were also measured by HRMS, but corresponding peaks were not detected by GC–MS; these masses probably correspond to trace compounds.

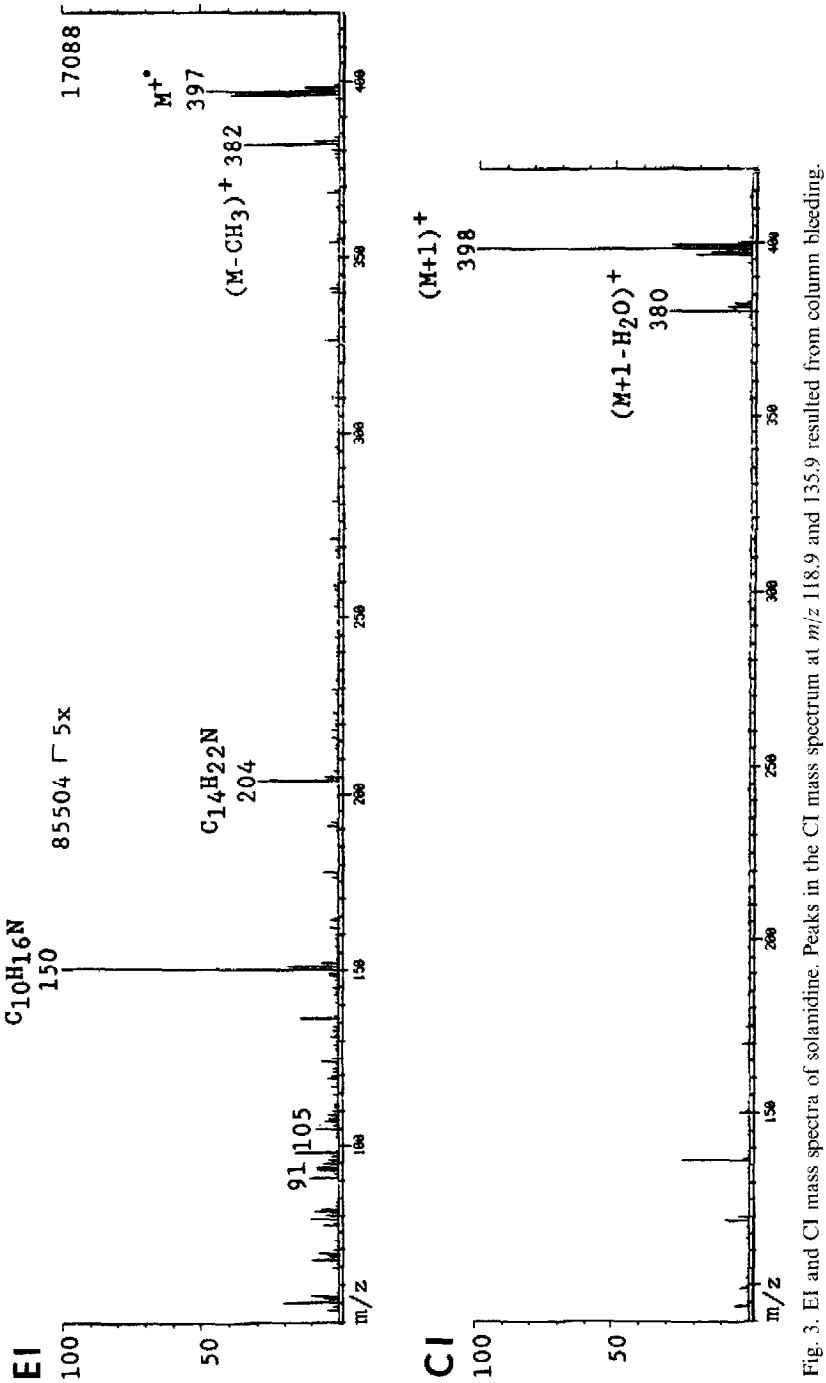


Fig. 3. EI and CI mass spectra of solanidine. Peaks in the CI mass spectrum at *m/z* 118.9 and 135.9 resulted from column bleeding.

TABLE I

STEROIDAL ALKALOIDS (SAs) FROM TUBERS OF *SOLANUM* SPECIES USED IN POTATO BREEDING, CHARACTERIZED BY RETENTION INDICES (*I*), HRMS, GC–MS AND TWO-PHASE HYDROLYSIS

No.	*SA*[a]	$I_{270}^{CP\text{-}Sil\,5\,CB}$	*HRMS*		*GC–MS diagnostic ions (% abundance)*	*Loss of H_2O*	
			Exact mass	*Formula*		*CI*[b]	*Hydrolysis*[c]
1	Solanidadienol	3040	395.3188	$C_{27}H_{41}NO$	150(100), 204(14), 380(2), 395(5)	1	1
2	Solanidine[d]	3130	397.3345	$C_{27}H_{43}NO$	150(100), 204(20), 382(4), 397(5)	1	1
3	Solanidanediol	3143	415.3450	$C_{27}H_{45}NO_2$	150(100), 204(22), 400(2), 415(3)	2	0
4	Solanidadienol	3169	395.3188	$C_{27}H_{41}NO$	150(100), 204(23), 380(5), 395(9)	1	1
5	Solanidadienol	3176	395.3188	$C_{27}H_{41}NO$	150(100), 204(22), 380(6), 395(7)	1	1
6	Substituted solanidenol	3195	427.3086	$C_{27}H_{41}NO_3$	150(100), 204(18), 380(2), 395(2), 396(2), 412(2), 427(3)	nr[e]	1
7	Hydroxymethyl- or methoxysolanidenol	3236	427.3450	$C_{28}H_{45}NO_2$	150(100), 204(28), 380(3), 395(7), 396(4), 412(1), 427(2)	1	1
8	Solanidanediol	3293	415.3450	$C_{27}H_{45}NO_2$	150(100), 204(22), 400(2), 415(5)	2	0
9	Substituted solanidenol	3319	425.3303	$C_{28}H_{43}NO_2$	150(100), 204(14), 410(5), 425(4)	nr	1
10	Methylsolanidenol	3353	411.3501	$C_{28}H_{45}NO$	150(100), 204(20), 396(16), 411(14)	nr	1
			423.2772	$C_{27}H_{37}NO_3$	150(100), 204(19), 394(2), 408(4), 409(4), 423(1)	nr	0
11	Substituted solanidanol	3357	423.3136	$C_{28}H_{41}NO_2$			

[a] Nomenclature according to ref. 1.
[b] $(M + 1 - 18)^+$ resulting from chemical ionization.
[c] Formation of dehydration product on hydrolysis; corresponds to a mass difference of 18.0105.
[d] Solanid-5-en-3β-ol.
[e] nr = CI mass spectrum not recorded.

TABLE II

CONTENTS OF STEROIDAL ALKALOIDS (SAs) OF TUBERS OF *SOLANUM* SPECIES

Steroidal alkaloids expressed as glycosides on a trisaccharide basis in mg kg^{-1} fresh tuber. All SAs possessed a solanidane skeleton.

No.	*SA (glycosidically bound)*	*Solanum chacoense*	*Solanum leptophyes*	*Solanum sparsipilum*	*Solanum tuberosum* ssp. *andigena*
1	$C_{27}H_{41}NO$	30	61	200	19
2	Solanidine	2121	199	255	450
3	$C_{27}H_{45}NO_2$	40	12	35	14
4	$C_{27}H_{41}NO$		30	125	
5	$C_{27}H_{41}NO$			100	
6	$C_{27}H_{41}NO_3$			25	
7	$C_{28}H_{45}NO_2$	5	18	100	12
8	$C_{27}H_{45}NO_2$	15			
9	$C_{28}H_{43}NO_2$		5	40	
10	$C_{28}H_{45}NO$	17	78	200	31
11	$C_{27}H_{37}NO_3$ or $C_{28}H_{41}NO_2$			75	
	Unidentified	tr[a]	tr	140	tr
	Total glycoalkaloids	2228	403	1300	526

[a] tr = Traces.

Implications for potato breeding

A large number of naturally occurring steroidal sapogenins, a well studied class of compounds closely related to the SAs, have been described in a recent review[10]. Among these were methyl-, di- and trihydroxy- and mono- and diketosapogenins, in addition to ene and diene forms. Also, although to a lesser extent, a variety of SAs have been described; up to 1981, more than 80 aglycones had been reported for the genus *Solanum* only[1]. Novel SAs and SGAs are regularly being detected in *Solanum* species, mostly in aerial plant parts[11–13], as a result of studies on pharmacologically interesting compounds. Only few studies on the SA composition of tubers have been carried out, and only occasionally have novel SAs or SGAs been found since 1981[4,14]. So far, six SAs of the solanidane group have been described for *Solanum* species, of which only two, solanidine and demissidine, have been detected in tubers[6].

Table II shows that a variety of minor SAs occurred in the tubers of the *Solanum* species/ssp. used in this study. The detection of these SAs was achieved by the application of capillary GC using simultaneous FID and NPD[5]. In addition to solanidine, at least four of these SAs may be regarded as common minor compounds, as they occurred in species which belong to different series of the genus *Solanum*, namely the series *Commersoniana* (*S. chacoense*) and *Tuberosa*.

The nature of the novel SAs of the *Solanum* species/ssp. studied could be revealed, as was described above. They all belonged to the solanidane group and were probably substituted, dehydrogenated or substituted saturated forms of solanidine (Fig. 1), in which, for instance, a hydrogen has been replaced with a hydroxyl or methyl group. However, their origin remains unclear. In the *S. sparsipilum* sample studied, elevated levels of novel SAs were present. This may have been (partly) the result of the different growing conditions of these tubers[4], as it has been shown that environmental

conditions may affect the biosynthesis of SAs in *Solanum* species in many ways[15]. In *Veratrum* species, solanidine accumulated, or was converted to jervine and veratramine, depending on the illumination[16]. Solanidine, solasodine and tomatidenol, common end-products in the SA biogenesis in *Solanum* species, seemed to be precursors in the biogenesis of camtschatcanidine, hapepunine, anrakorinine and 27-hydroxyspirosolane, which are SAs of *Fritillaria* species[7]. Hence the SAs found in the wild and primitive potato tubers studied might be intermediates in SA metabolism and/or stress metabolites.

Studies on the relationship between (glycosidic-bound) SAs and resistance against pathogens and insects have been reviewed[17,18]. Differences in biological activity between SAs are associated with the structural differences of these SAs, and the sugar moieties may also influence the biological activities. Therefore, in resistance studies, the SGAs or their aglycones should be quantified individually. Preferably, the composition of their sugar moieties should also be determined. Consequently, when the solanidane glycosides that we detected are present in plant samples, they may not be disregarded as being a result of imperfections in the analytical techniques used.

For a complete structure elucidation, the SAs and their glycosides need to be isolated and costly and time-consuming studies by MS, infrared and nuclear magnetic resonance spectroscopy must be carried out. Once the entire structure of the individual compounds is known, information on their toxicity will still be lacking. It is therefore more efficient to investigate whether these compounds either occur normally or are induced by environmental (stress) conditions during cultivation and storage of the tubers. If these compounds do not occur in the current potato cultivars considered safe for consumption, their introduction into future household cultivars should be prevented.

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