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Intestinal Metabolism of Selected Steroidal Glycoalkaloids in the Pig Cecum Model

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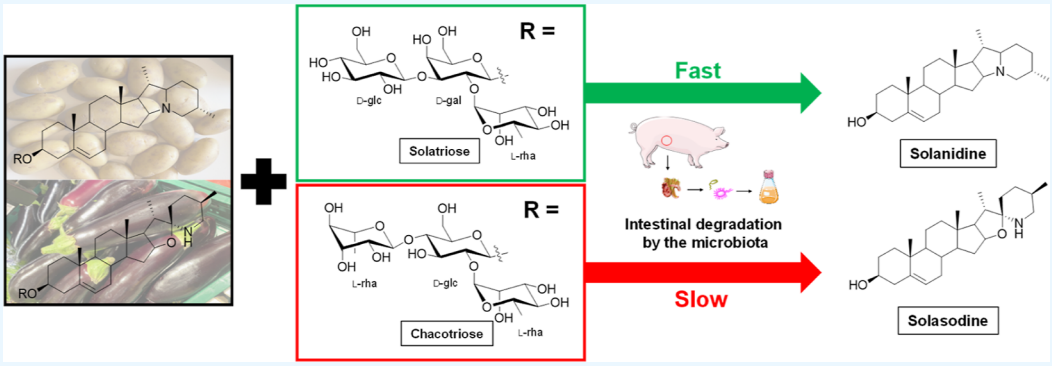

ABSTRACT: Due to the presence of the steroidal glycoalkaloid solanine, the potato was chosen as Germany's poisonous plant of the year 2022. Steroidal glycoalkaloids are secondary plant metabolites which have been reported to induce toxic as well as beneficial health effects. Nevertheless, data regarding occurrence, toxicokinetics, and metabolism of steroidal glycoalkaloids is scarce, and substantially more research is required for a proper risk assessment. Therefore, the intestinal metabolism of solanine, chaconine, solasonine, solamargine, and tomatine was investigated using the ex vivo pig cecum model. All steroidal glycoalkaloids were degraded by the porcine intestinal microbiota, releasing the respective aglycon. Furthermore, the hydrolysis rate was strongly dependent on the linked carbohydrate side chain. Solanine and solasonine, which are linked to a solatriose, were metabolized significantly faster than the chaconine and solamargin, which are linked to a chacotriose. In addition, stepwise cleavage of the carbohydrate side chain and the formation of β- and γ-intermediates were detected by HPLC−HRMS. The results provide valuable insights into the intestinal metabolism of selected steroidal glycoalkaloids and help to reduce uncertainties and improve risk assessment.

INTRODUCTION

Potatoes and potato products have been an integral part of the human diet for centuries. With 11.72 million tons in 2020, Germany is the biggest producer of potatoes in Europe.[1] The potato (*Solanum tuberosum*) is an important source of carbohydrates, resistant starch, valuable proteins, vitamins C and B_6, as well as potassium,[2] and has shown promising health-promoting properties in human cell culture and experimental animal and human clinical studies.[3] However, in November 2015, illness in the form of vomiting and stomach-ache occurred in a German family after consumption of potato-based meals. Investigations identified the steroidal glycoalkaloids (SGA) α-solanine (solanine) and α-chaconine (chaconine) to be responsible for this intoxication.[4]

SGA are secondary plant metabolites which are present in numerous species of the nightshade family (Solanaceae). So far, over 90 different SGAs from more than 300 species have been identified and isolated.[5] Structurally, SGA are composed of an oligosaccharide sidechain connected to the 3β-hydroxyl group of a steroidal aglycone. Predominantly, a trisaccharide or tetrasaccharide is linked to the steroidal backbones of which solanidine (solanidane group) or solasodine (spirosolane group) are two of the most relevant aglycons.[6] The major SGAs in commercial potatoes are solanine and chaconine, which are composed of solanidine as the steroidal backbone and a solatriose or chacotriose as the sugar moiety, respectively (Figure 1). α-Solasonine (solasonine) and α-solamargine (solamargine) with solasodine glycosidically linked to solatriose and chacotriose (Figure 1), respectively, are primarily found in eggplants (*Solanum melongena*), whereas α-tomatine (tomatine, see Figure S2) and α-dehydrotomatine (tomatidine/tomatidenol backbone coupled to the tetrasaccharide lycotetraose) are major compounds in the tomato fruit (*Solanum lycopersicum*).[6,7] The content of SGA varies greatly in the different parts of the

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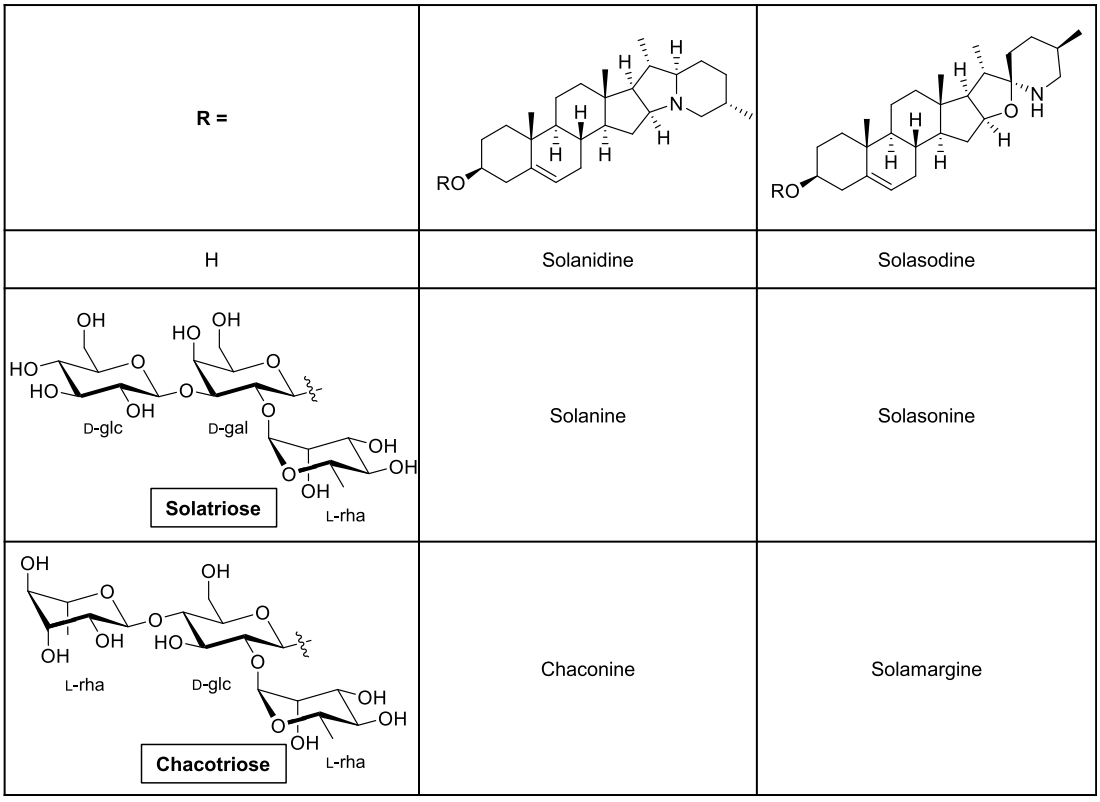

Figure 1. Chemical structures of the steroidal glycoalkaloids solanine, chaconine, solasonine, solamargine, their aglycons solanidine and solasodine and their carbohydrate sidechains.

plants and heavily depends on climate conditions,[8] such as temperatures[9] or exposure to sunlight in the case of potatoes.[10] High levels of SGA are especially found in potato tuber sprouts[8,11] and an increasing concentration during storage is often associated with greening of the potato.[12] Many toxic effects, such as vomiting, diarrhea, headache, or even death, are reported in humans after consumption of high SGA levels.[8,13] The mode of action regarding their biological activity and adverse effects is mainly based on two factors: the inhibition of acetylcholinesterase[14] or butyrylcholinesterase[15] and a disruption or a loss of cell membrane integrity due to their ability to complex with membrane 3β-hydroxy sterols.[16] Besides the toxic effects of SGA, also beneficial effects such as anticancer,[17] antimicrobial,[18] or anti-inflammatory[19] properties have been attributed to different SGAs.

The German Federal Institute for Risk Assessment (BfR) recommended that the content of SGA in potatoes not to exceed 100 mg/kg fresh weight[4] and a lowest-observed-adverse-effect level of 1 mg total potato SGA/kg body weight per day were identified by the European Food Safety Authority (EFSA) Panel on Contaminants in the Food Chain (CONTAM).[20] Nevertheless, due to insufficient data on occurrence, toxicokinetics, or metabolism, a proper risk assessment is not possible, as the EFSA stated in 2020.[20] Therefore, the aim of this study is to contribute more data focusing on the intestinal metabolism of the SGA. The literature attributes a relatively low oral bioavailability to solanine and chaconine in rats and hamsters and a partly excretion via the feces or urine as solanidine.[21−23] Moreover, an in vitro study in ruminants revealed an extensive conversion of solanine and chaconine to solanidine and further metabolism of the aglycon, assuming the SGA to be degraded during digestion.[24] Studies with human volunteers showed long serum half-lives after oral uptake for solanine and chaconine[25,26] and an increase in solanidine concentrations after a decrease of the parent compounds.[25]

To further investigate the potential degradation of SGA, the intestinal metabolism of solanine, chaconine, solasonine, and solamargine, as well as their aglycons solanidine and solasodine (Figure 1), were analyzed in this study using the ex vivo pig cecum model. Tomatine, which is connected to a lycotetraose, was also included in the study to gain more data about the relevance of the glycosidic bond configuration for intestinal degradation. The pig cecum model is a powerful tool to elucidate the human intestinal metabolism, as specifically the microbiota in the gastrointestinal tract of humans and pigs is comparable, which was demonstrated by the characterization of the cecal microbiota using fluorescence in situ hybridization with 16S rRNA-based oligonucleotide probes and terminal-restriction fragment length polymorphisms of human and porcine stool samples.[27,28] It was successfully applied in several previous studies which investigated the intestinal metabolism of polyphenols[29−32] modified mycotoxins,[33] imidazole alkaloids[34] or lignin.[35] Obtained results should provide further information on the metabolism of selected SGA and reduce uncertainties on the risk assessment.

MATERIALS AND METHODS

Chemicals and Reagents. Analytical standards of solanine, chaconine, solasonine, solamargine, tomatine and their aglycons solanidine, solasodine, and tomatidine were purchased from Sigma-Aldrich (Steinheim, Germany), Carl Roth (Karlsruhe, Germany), and Extrasynthese (Genay, France). The standards were solved in methanol, obtaining stock solutions of 1 mM for storage at −18 °C, respectively.

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The solvents used for high-performance liquid chromatography coupled to quadrupole time-of-flight mass spectrometery (HPLC−QToF−MS) and high-performance liquid chromatography coupled to tandem mass spectrometery (HPLC−MS/MS) analysis (HPLC−MS grade) and all other chemicals used for incubation experiments were purchased from Fisher Scientific (Schwerte, Germany), Merck (Darmstadt, Germany), or Sigma-Aldrich (Steinheim, Germany) in gradient or reagent quality. Purified water was obtained by a Purelab flex 2 system (ELGA LabWater Veolia Water Technologies, Celle, Germany).

Preparation of Fecal Suspensions. The pigs used for the preparation of fecal suspensions (Bentheim Black Pied, age: 12−18 months, weight: 90−120 kg) were raised by biodynamic farming following regulation (EU) 2018/848 to maintain the natural composition of the microbiota. Directly after the slaughtering process, the ceca were obtained by separation from the gastrointestinal tract and further handled under anaerobic conditions. Briefly, the content of the ceca is removed, a reducing buffer is added, and the resulting suspension is filtered. A part of the suspension is inactivated using a laboratory autoclave to obtain the deactivated cecal suspension (DCS). The untreated part of the suspension with healthy microbiota is used as active cecal suspension (ACS). Detailed information about the process to prepare active and DCS from the ceca for the incubation experiments is described in the literature.[33]

Incubation Experiments. The incubations of steroidal glycoalkaloids in ACS and DCS were performed for incubation periods from 0 to 24 h (0, 15, 30, 60, 120, 240, 480, 1200, and 1440 min). The bacterial activity of each cecum was tested by incubation of quercetin and fluorescein diacetate in ACS as indicators for metabolically active microbiota.[34,35] Incubation of the analytes in DCS was used as a control to exclude matrix effects and chemical degradation of the compounds. For incubation experiments, the 1 mM stock solutions were diluted in methanol to obtain 10 μM incubation solutions. Fifty microliters of the incubation solutions were added to 450 μL of the cecal suspensions in separate 2 mL reaction tubes, resulting in final analyte incubation concentrations of 1 μM, respectively. The tightly sealed reaction tubes were stored in an incubator at 37 °C under continuous shaking for the respective incubation time. The metabolic reaction was stopped at the end of each incubation period by adding 1 mL of ice-cold acetonitrile (ACN, −20 °C) to the solutions, and samples were stored at −80 °C until sample preparation. For recovery correction of the analytes, a sub-sample of the incubation experiments at the beginning of the incubation process (0 min) was inactivated immediately by adding 1 mL ice-cold ACN, and quantified analyte concentrations at this point were set to 100% (1 μM). All incubation experiments were performed in duplicate with three different ceca ($n = 3 \times 2$).

Sample Preparation. The frozen samples were thawed in a water bath at 37 °C, vortexed and centrifugated with 10,000g at 4 °C for 15 min. A supernatant of 200 μL was transferred in a well of a 96-well microtiter plate, and the solvent was evaporated in a vacuum concentrator. The residue was dissolved with 200 μL of ACN/H_2O (1 + 4, v/v) + 0.1% formic acid, transferred into a 1.5 mL glass vial equipped with an insert and used for analysis.

Due to the strong matrix effects of the cecal suspensions, a matrix-matched calibration was chosen for quantitation of the analytes. Therefore, a matrix solution was prepared by adding 1 mL of ice-cold ACN to 500 μL of DCS in a 2 mL reaction tube, followed by a centrifugation step with 10,000g at 4 °C for 15 min. The supernatant was used to prepare the calibration points for quantification. To do so, 50 μL of each 10 μM incubation solution were transferred into one 1.5 mL glass vial, and the solvent was evaporated under nitrogen at 40 °C. The residue was diluted with 500 μL of the matrix solution to obtain a mix standard solution of 1 μM. Calibration points ranging from 5 to 1000 nM were prepared by diluting the 1 μM mix standard solution with the matrix solution to a total volume of 200 μL in a 1.5 mL glass vial equipped with an insert. Subsequently, the solvent was evaporated under nitrogen at 40 °C, and the residue was diluted with 200 μL ACN/H_2O (1 + 4, v/v) + 0.1% formic acid to match HPLC starting conditions.

HPLC−MS/MS Settings. A 1260 Infinity HPLC system (Agilent, Waldbronn, Germany) and a 100 × 2 mm Nucleodur C_{18} HTec (3 μm) column equipped with a 4 × 2 mm guard column of the same material (Macherey-Nagel, Düren, Germany) were used for the chromatographic separation of the analytes. A binary gradient consisting of ACN (A) and H_2O (B), both containing 0.1% formic acid, was applied with initial conditions of the gradient of 20% A at a flow rate of 0.5 mL/min. After an increase of the percentage of A to 25% at 2.5 min, the percentage of A slowly increased to 30% at 3.5 min, followed by an increase to 55% of A at 5 min. Subsequently, the ratio of A was set to 100% after 7 min and held for 2 min. The percentage of A was then decreased to initial conditions at 9.1 min until 11 min to re-equilibrate the column. The integrated column oven was set to a temperature of 45 °C, and the sample rack cooled at 8 °C. Five microliter of each sample was injected into the system for analysis.

MS/MS analysis was achieved by using a QTRAP 6500 mass spectrometer with an IonDrive Turbo V electrospray ionization (ESI) source, which was operated with the Analyst software version 1.6.2 for data acquisition and processing (SCIEX, Darmstadt, Germany) and coupled to the HPLC system. The mass spectrometer was operated using the parameters summarized in Table 1 and the quantifier transitions are highlighted in gray.

Table 1. MS/MS Parameters for the Analysis of Selected Steroidal Glycoalkaloids

instrument	Sciex QTRAP® 6500 - IonDrive Turbo V source	
ionization mode	electrospray ionization (positive)	
ion spray voltage	4500 V	
source temperature	450 °C	
nebulizer gas (GS 1)	45 psi	
heating gas (GS 2)	50 psi	
curtain gas	30 psi	
collision gas	high	
valve	time [min]	position
	0.00	waste
	2.00	MS

MRM experiments (scheduled, cycle time = 0.7 sec)

ion $[M+H]^+$	Q1 [m/z]	Q3 [m/z]	time [min]	window [sec]	DP [V]	CE [V]	CXP [V]
solanine	868	722	3.0	90	200	90	10
	868	398	3.0	90	200	97	10
chaconine	852	706	3.0	90	130	95	10
	852	398	3.0	90	130	40	10
solamargine	868	850	3.0	90	150	70	10
	868	253	3.0	90	150	65	10
solasonine	884	866	3.0	90	200	70	10
	884	253	3.0	90	200	65	10
tomatine	1034	1016	3.0	90	220	85	10
	1034	416	3.0	90	220	55	10
solanidine	398	382	4.5	90	100	65	10
	398	206	3.0	90	100	70	10
solasodine	414	396	5.0	60	70	40	10
	414	253	3.0	90	70	45	10
tomatidine	416	398	5.0	60	100	45	10
	416	161	5.0	60	100	50	10

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HPLC−QToF−MS Settings. The analysis of potential intermediate metabolites of the SGA was performed using a targeted metabolomic approach via HPLC−QToF−MS. Therefore, m/z ratios of SGA with a loss of a variable amount of sugar moieties were calculated, and the samples from incubation experiments were screened for calculated m/z ratios. The separation of the metabolites was performed on a Bruker Elute HPLC system using the same column, binary gradient and autosampler settings as described in the HPLC−MS/MS settings chapter. The mass spectrometer was operated using the parameters listed in Table 2. At the beginning of each run (0.5−1.5 min), the mass spectrometer was calibrated with a sodium formate calibration solution at a flow rate of 3 μL/min introduced via the syringe pump. Data were acquired in profile and centroid mode using Bruker Hystar 4.1 software.

Table 2. QToF-MS Parameters for the Analysis of Selected Steroidal Glycoalkaloids

instrument	Bruker impact II - Apollo II ESI source		
ionization mode	electrospray ionization (positive)		
capillary voltage	4500 V	**dry gas temperature**	220 °C
end plate offset	500 V	**spectra rate**	4 hz
nebulizer pressure	5.0 bar	**mass scan range**	m/z 50-1500
dry gas flow	12.0 L/min	**mode**	Auto MS/MS
precursor acquisition control		on	
1. segment	1.0-3.0 min	**2. segment**	3.0-9.0 min
transfer&quadrupole settings			
funnel 1 RF	150|150 Vpp	**low mass**	m/z 322|90
funnel 2 RF	200|200 Vpp	**collision energy**	15|13 eV
isCID energy	0|0 eV	**collision RF**	1500|650 Vpp
hexapole RF	50|50 Vpp	**transfer time**	75|40 µs
ion energy	7|7 eV	**pre-pulse storage**	20|13 µs
auto MS/MS settings			
cycle time	3 sec	**smart exclusion**	2×
active exclusion		enabled, precursors excluded after 3 spectra and released after 0.2 min	

RESULTS AND DISCUSSION

The human intestinal metabolism of the SGA solanine, chaconine, solasonine, and solamargine was mimicked in this study using the ex vivo pig cecum model. Before the incubation experiments with cecal suspensions, the stability of the analytes in slightly modified artificial gastric juice was checked to mimic conditions in the stomach.[36] No degradation of the analytes was observed after 4 h (240 min) of incubation time, indicating the SGA to be stable during the simulated gastric conditions. Even though the artificial gastric juice does not fully represent the conditions in the stomach, especially due to the lack of muscular contraction of the chymus, it is assumed that the SGA reaches the gut mostly unmodified (Supporting Information, Figure S1). For the studies in the pig cecum model, the analytes solanine, chaconine, solasonine, and solamargine were incubated with cecal suspensions prepared from ceca of freshly slaughtered pigs. The obtained results after incubation of the SGA in ACS and DCS are displayed in Figure 2. Incubation with DCS was performed to validate the chemical stability of the analytes under incubation conditions and to compensate for potential matrix effects. The black lines around 1000 nM in all panels of Figure 2 reveal that the analytes are not degraded after incubation with DCS and are stable over the incubation time of 24 h (1440 min), as all analytes were almost entirely recovered. A possible degradation of the SGA after incubation with ACS is therefore exclusively attributed to microbial metabolism. Incubation of solanine with ACS resulted in a rapid cleavage of the solatriose sugar moiety and a release of the aglycon solanidine (Figure 2A). Already after an incubation period of 10 min, no more solanine was detected, and solanidine was quantified with a concentration of 312 ± 103 nM. The concentration of the aglycon slightly increased to around 450 nM after 4 h (240 min), after which it did not change significantly until the end of the maximum incubation period, suggesting solanidine to be a stable metabolic product. Furthermore, the difference between the released aglycon solanidine and the initial concentration of 1000 nM solanine points toward further metabolite formation. In contrast, the steroidal glycoalkaloid chaconine, which structurally consists of the same aglycon solanidine but is linked to a chacotriose instead of a solatriose (Figure 1), was metabolized significantly slower than solanine (Figure 2B). Only after an incubation period of 1 h (60 min), a degradation of chaconine and a slow release of the aglycon was detected. In the course of time, the concentration of chaconine decreased further until it reaches a plateau after 8 h (480 min) of incubation period with a concentration of around 200 nM. The concentration of the released solanidine increases at the same time until it reaches a plateau after 4 h (240 min) with a concentration of 384 ± 157 nM, supporting the hypothesis of a stable metabolic product. Solasonine was also rapidly metabolized by the microbiota to its aglycon solasodine (Figure 2C), similar to the results obtained for solanine shown in Figure 2A. Both SGA share solatriose as a sugar moiety but consist of different steroidal backbones, suggesting the sugar sidechain to have a stronger impact on the rate of degradation than the type of steroidal aglycon. After an incubation period of 10 min, solasonine was already fully metabolized, and the aglycone solasodine was released and quantified with a concentration of around 800 nM. Hints for further microbial metabolism of solasodine were received as its concentration decreased in the course of time until a concentration of 196 ± 252 nM was reached after 24 h (1440 min). The degradation of solamargine (Figure 2D) is comparable to that of chaconine, as both SGAs were metabolized slower compared to the solatriose-conjugated SGA. Here, too, both analytes share the same chacotriose carbohydrate sidechain. After the start of the incubation period, the concentration of solamargine gently decreased until 4 h (240 min) with a quantified amount of only 21 ± 30 nM.

Thereafter and until the end of the incubation period, solamargine was only detected in negligible amounts. The corresponding aglycon solasodine was detected after 1 h (60 min), after which the concentration increased to a maximum of 610 ± 331 nM after 2 h (120 min). Subsequently, the concentration of solasodine continuously decreased until the end of the incubation period, with a quantified amount of 150 ± 171 nM after 24 h (1440 min). This result supports the assumption that the aglycon solasodine is further metabolized, as already observed during the incubation study with solasonine.

To further investigate this assumption and to clarify the intestinal stability, the aglycons solanidine and solasodine were separately incubated with ACS and DCS for 0 and 24 h (Figure 3). Both aglycons were stable when incubated with DCS, as they were almost fully recovered (1000 nM) after 24 h. In contrast, incubation of solanidine and solasodine with ACS revealed degradation of both compounds after 24 h. Solanidine was only partly degraded with 601 ± 149 nM still quantified after 24 h, whereas solasodine was almost fully metabolized, and only 63 ± 87 nM were detectable. These results fit to observations made in the incubation experiments with the respective glycoalkaloids solasonine and solamargine. These studies revealed the released aglycon solasodine to be subject to further metabolism, respectively. In case of the incubation experiments with solanine

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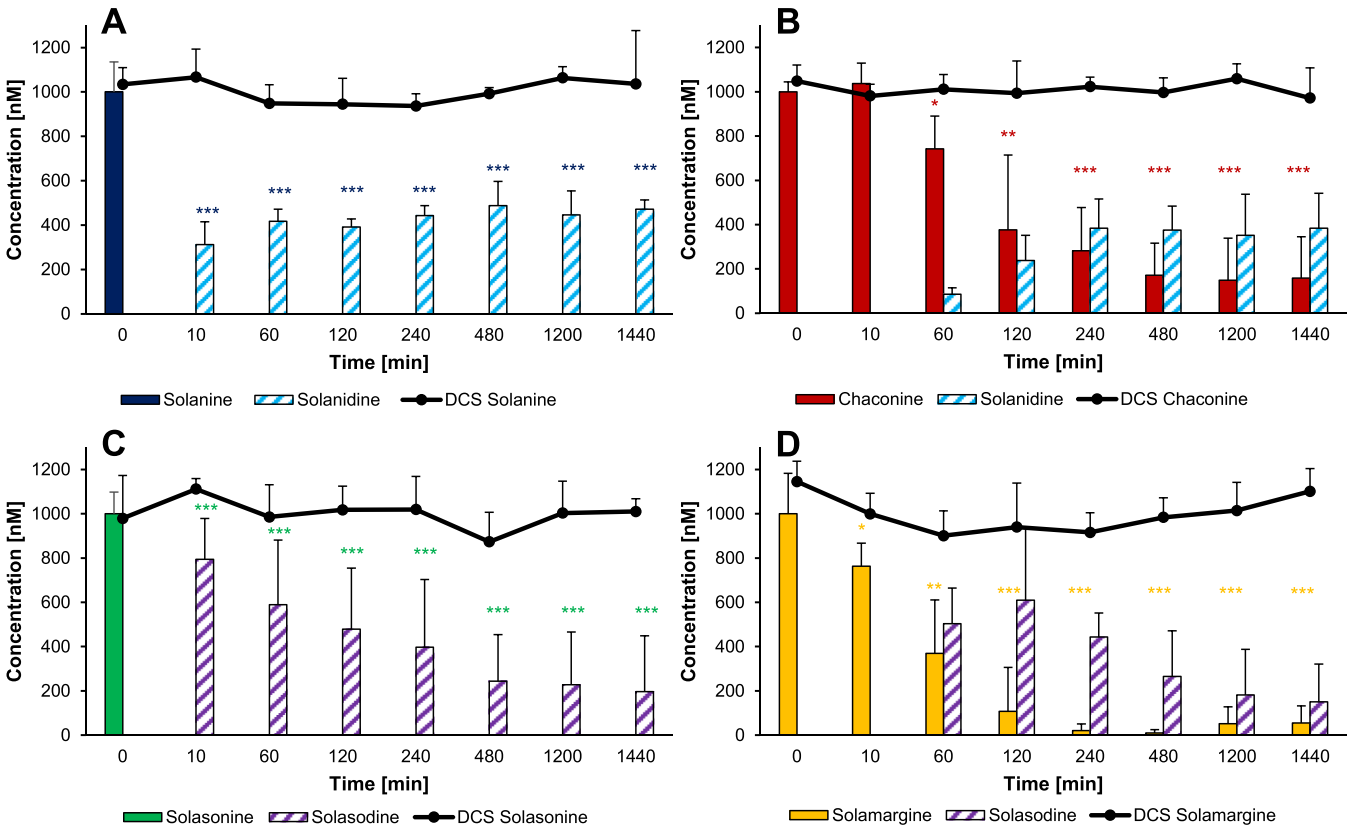

Figure 2. Time-dependent degradation profiles of 1000 nM solanine (A), chaconine (B), solasonine (C), and solamargine (D) after incubation with cecal suspensions in the pig cecum model ($n = 3 \times 2$, mean ± standard deviation). Colored bars represent the incubation of the steroidal glycoalkaloids with ACS and the bacterial degradation to the respective aglycon, which is displayed as dashed colored bars. The black lines display the incubation of the analytes with DCS to verify the stability of the compounds in the matrix. Statistically significant differences between parent SGA concentrations of the respective incubation periods compared to the beginning of the incubation experiment (0 h) are marked by asterisks (*: $p \leq 0.05$; **: $p \leq 0.01$; and ***: $p \leq 0.001$).

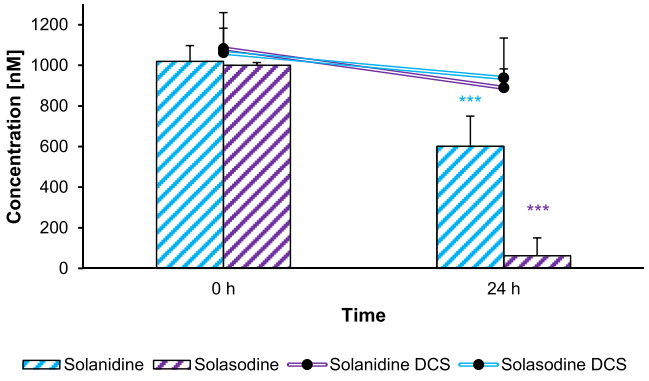

Figure 3. Time-dependent degradation profile of 1000 nM solanidine and solasodine after incubation with cecal suspensions in the pig cecum model for 0 and 24 h ($n = 3 \times 2$, mean ± standard deviation). Dashed colored bars represent the incubation of the aglycons with ACS, and the dashed colored lines display the incubation of the analytes with DCS to verify the stability of the compounds in the matrix. Statistically significant differences between solanidine and solasodine concentrations of the respective incubation periods compared to the beginning of the incubation experiment (0 h) are marked by asterisks (*: $p \leq 0.05$; **: $p \leq 0.01$; and ***: $p \leq 0.001$).

and chaconine, no further degradation of released solanidine was observed, even though solanidine was partially metabolized when incubated as a single compound. The temporal availability and concentration of solanidine in the different experiments (400 nM released after 4 h vs 1 μM incubated at 0 h) could be an explanation for this.

To study the detailed metabolism of the SGA and to answer the question if the sugar moiety is cleaved in one step or stepwise, the samples used for incubation experiments were also analyzed via HPLC−QToF−MS. Therefore, m/z ratios of potential intermediate metabolites with cleaved sugar moieties were calculated, and a targeted analysis of intermediate products was performed. A list of detected m/z ratios of potential metabolites is attached in Table S1 in the Supporting Information. Figure 4 shows the observed time-dependent relative peak area profiles of the SGA (Figure 4A_1,B_1) and their detected intermediate metabolites (Figure 4A_2,B_2). As analytical standards for potential intermediate metabolites are commercially not available, statements about the metabolism pathways are only based on the calculated m/z ratios and relative peak areas. In general, for each of the incubated SGA, an intermediate β-metabolite with a lack of the outer sugar moiety (D-glc, L-rha, Figure 1) was detected (Table S1, Supporting Information). An intermediate γ-metabolite with a loss of two sugar moieties was not identified for any SGA. For the SGA solanine and solasonine, which are linked to a solatriose and are rapidly metabolized (Figure 4A_1), the loss of D-glucose via the cleavage of the $\beta(1 \rightarrow 3)$ bond was observed. The intermediate products β-solanine and β-solasonine were detected with its maximum relative peak areas of about 35% after an incubation time of 10 min, after which both peak areas decreased to a value of about 10% after 24 h (1440 min) (Figure 4A_2). These results indicate a

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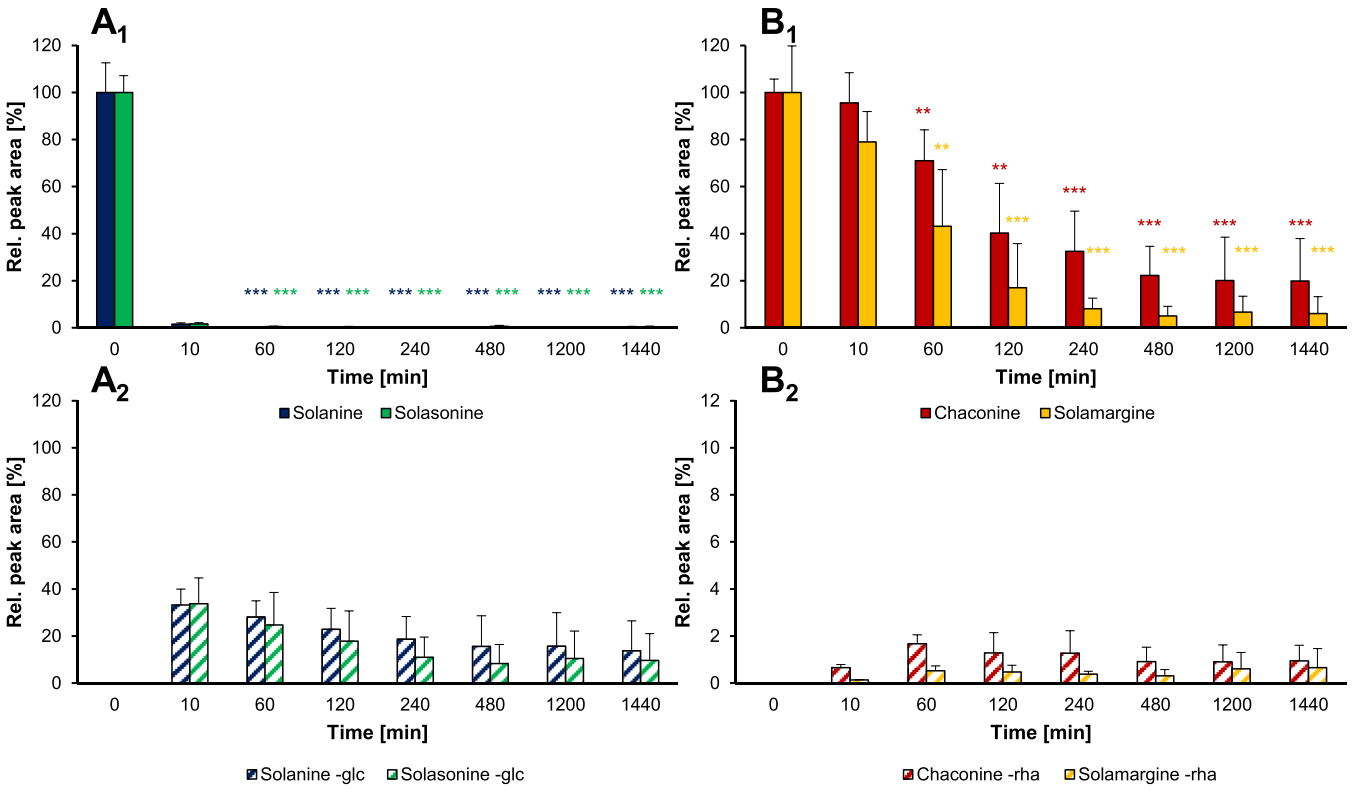

Figure 4. Time-dependent degradation profile of solanine and solasonine (A_1), chaconine and solamargine (B_1), and the formation of their intermediate β-metabolites with a loss of glucose (-glc, A_2) or a loss of rhamnose (-rha, B_2) after incubation with ACS in the pig cecum model. Displayed are to the corresponding highest values normalized peak areas after analyte incubation obtained via HPLC−QToF−MS ($n = 3 \times 2$, mean ± standard deviation). For overview reasons, the y-axis of panel B_2 was adjusted due to the low values. Statistically significant differences between parent SGA relative peak areas of the respective incubation periods compared to the beginning of the incubation experiment (0 h) are marked by asterisks (*: $p \leq 0.05$; **: $p \leq 0.01$; and ***: $p \leq 0.001$).

fast cleavage of the D-glucose at both SGAs after the start of the incubation periods and further metabolism of the intermediate metabolites. An intermediate product with a lack of L-rhamnose was detected for chaconine and solamargine, which both are linked to a chacotriose and are slowly metabolized (Figure 4B_1). After the start of the incubation period, both intermediate metabolites, β-chaconine and β-solamargine, are slowly released via the cleavage of the $\alpha(1 \rightarrow 4)$ or $\alpha(1 \rightarrow 2)$ bond and reach maximum relative peak areas of only up to 2% after 1 h (60 min), after which the peak areas did not change significantly (Figure 4B_2). As the relative peak areas only reached a maximum of 2%, the proportion of the intermediate metabolites with the loss of one L-rhamnose moiety is supposed to be very low. In comparison, the intermediate metabolites β-solanine and β-solasonine with a lack of one D-glucose and maximum relative peak areas of about 35% are expected to occur at significantly higher levels. Furthermore, the results of HPLC−QToF−MS analysis support the data obtained by HPLC−MS/MS analysis of the incubation experiments, as the degradation profiles of the SGA are highly comparable (Figures 4A_1,B_1 and 2).

The above presented results clearly show that the type of carbohydrate bound to the alkaloid is crucial for the overall rate of SGA degradation. To provide more comprehensive insight into the degradation rate of different saccharides, tomatine was incubated in the same manner as the previously mentioned SGA. Aside from a different aglycon structure, the main difference compared to the other described SGA is the connected lycotetraose, which is a tetrasaccharide compared to the trisaccharides solatriose and chacotriose. Due to the highly differing data of one cecum, only two incubation experiments were considered and displayed in the Supporting Information. Figure S2 displays the time-dependent concentration profile of tomatine (A, HPLC−MS/MS) as well as the time-dependent relative peak areas and the detected intermediate metabolites during stepwise metabolism of the SGA (B, HPLC−QToF−MS). Results of HPLC−MS/MS analysis demonstrate tomatine to be stable after incubation in DCS and a slow degradation of tomatine to 60% of the initial incubation concentration after 24 h (1440 min) of incubation in ACS. The aglycon tomatidine was first detected after 2 h (120 min) and increased in its concentration to comparable small amounts of 41 ± 16 nM after 24 h (1440 min) (Figure S2A). Analysis by HPLC−QToF−MS confirmed tomatine to be metabolized comparatively slow but constantly to a remaining relative peak area of 60% after 48 h (2880 min), which fits to the degradation profile in Figure S2A. In the course of time, the β-metabolites with either a loss of D-glucose or D-xylose were detected (Figure S2B). The β-tomatine with a loss of D-xylose via a cleavage of the $\beta(1 \rightarrow 4)$ bond was formed at a higher rate than the metabolite with a loss of D-glucose via the cleavage of the $\beta(1 \rightarrow 2)$ bond. The highest relative peak areas were detected after 1200 min, after which both β-metabolite peak areas decreased again. In addition to the β-metabolites, γ-tomatine with a loss of D-glucose together with D-xylose was detected after 1 h (60 min). Its relative peak area further increases to a maximum value of about 60% after 24 h (1440 min). An intermediate metabolite with a loss of three sugar moieties was not detected, whereas the aglycon tomatidine with a loss of the complete lycotetraose was identified in the

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samples via HPLC−HRMS as well. In comparison to the SGA connected to a trisaccharide, the degradation rate of tomatine is slower. The more complex structure as well as the different type and configuration of glycosidic bonds in the lycotetraose might affect the bacterial degradation. To investigate the major reason for delayed tetrasaccharide SGA degradation, further tetrasaccharide SGA with different carbohydrates bound to the steroidal alkaloid backbone should be analyzed in the pig cecum model in the future.

To sum up, our results demonstrate the degradation of the SGA solanine, chaconine, solasonine, and solamargine by the porcine cecal microbiota and the release of the corresponding aglycons solanidine and solasodine. In addition, the results of two incubation experiments revealed the degradation of tomatine to tomatidine. As the composition of the microbiota of the gastrointestinal tract between pigs and humans is comparable,[27,28] similar metabolic reactions are assumed during the human intestinal metabolism. An enzymatic cleavage of glycosides is already described in the literature as a common metabolic reaction by the human gut microbiota.[37] Furthermore, recent studies in the pig cecum model demonstrated the cleavage of glycosidic bonds in case of the modified mycotoxins HT-2 and T-2 toxin glucosides,[33] as well as the imidazole alkaloid *N*-caprylhistamine-β-glucoside.[34] Differences in the rate of degradation were demonstrated in both studies, as the *O*-glycosidic bond was cleaved faster than the *N*-glycosidic bond. In the current study, the microbiota rapidly metabolized solanine and solasonine when incubated with active cecum suspensions, releasing the aglycons solanidine and solasodine, respectively. By contrast, chaconine and solamargine were degraded much slower, and at the end of the incubation period, the parent compound was still detectable in low concentrations. The results reveal a preferred cleavage of the solatriose by the pig cecal microbiota compared to chacotriose. A property of the microbiota to only cleave specific sugar linkages or an increased expression of specific enzymes like galactosidases or glucosidases compared to rhamnosidases might explain these observations. Moreover, by high-resolution mass spectrometry, it was shown that besides the respective aglycons, intermediate metabolites in the form of the β-compounds with a lack of D-glucose, L-rhamnose, or D-xylose were formed, proofing a stepwise cleavage of the glycoside side chain. This analysis provided a more detailed insight into the stability of different glycosidic bonds and therefore enables easier estimation regarding the stability of other SGAs not tested in the present study. Based on the relative peak areas, the occurrence of intermediate metabolites and the rate of degradation, it is assumed that the cleavage of the D-glucose at the $\beta(1 \rightarrow 3)$ bond is preferred by the bacteria compared to the L-rhamnose moiety via a cleavage of the $\alpha\ (1 \rightarrow 4)$ or $\alpha\ (1 \rightarrow 2)$ bond. A more rapid degradation was also observed for the $\beta(1 \rightarrow 3)$ glycosidic bond, connecting D-xylose to D-glucose, whereas the $\beta(1 \rightarrow 2)$ bound D-glucose was cleaved slower. A cleavage of β-bound D-galactose was observed for solanine, solasodine, and tomatine, resulting in the release of the respective aglycones. However, it is also important to note that D-galactose is the sterically most hindered monosaccharide and might therefore require the cleavage of most other terminal monosaccharides until the enzymes are finally capable of cleaving the β-bound D-galactose.

In the case of solanine and solasonine with a solatriose sidechain, only the formation of β-solanine and β-solasonine with a loss of D-glucose was detected, whereas the β-compounds with a lack of L-rhamnose were not detected. In comparison, the cleavage of the L-rhamnose to the intermediate β-compounds was observed for chaconine and solamargine. Specificity for the hydrolysis of carbohydrates units in the case of chaconine and solanine has already been described in the literature during fungal degradation of the compounds.[38,39] A stepwise degradation of chaconine and solanine via the β-metabolites was observed by groundwater microorganisms as well. In the study of Jensen et al., the microorganisms solely removed the D-glucose unit releasing β-solanine. After the carbohydrate unit was fully cleaved, solanidine was further degraded like in our study.[40] The degradation of SGA was also reported in vitro in rumen with a release of solanidine after incubation of a solanine and chaconine mixture. Further metabolism of the aglycon to a dihydro derivative was observed in this study.[24] In rats, 65% of radioactivity was excreted as solanidine in feces after oral exposure to [^{3}H]-solanine. Moreover, an increased blood level of solanidine after oral exposure to solanine and chaconine in human volunteers suggested hydrolysis of the SGA.[25] Even though data about the metabolism of SGA, especially for solasonine and solamargine, is scarce or even unavailable, these previous observations support the results of the SGA metabolism obtained in our study.

Transferring the obtained metabolism data to glycoalkaloid-induced toxicity data in animals, certain correlations can be identified. Blankemeyer et al. concluded that the glycosidic sidechain is required for bioactivity and membrane-disrupting activity. In their studies investigating the embryotoxicity of the SGA, solamargine was by factor 2−3 more potent than solasonine,[41] and chaconine was 4 times more potent than solanine,[42] when administered parentally into mice. In addition, Friedman et al. reported chaconine to be more toxic than solanine by factor 3 applying the frog embryo teratogensis assay-*Xenopus*.[43] All mentioned studies demonstrated the SGA containing a chacotriose (solamargine and chaconine) to be more potent than those with a solatriose (solasonine and solanine). In our study, the SGA linked to a chacotriose was degraded significantly slower than the SGA with a solatriose moiety and was consequently longer available in the gastrointestinal tract to potentially be absorbed and exhibit toxic effects mainly caused by the carbohydrate sidechain. With this assumption, a fast and full degradation of SGA by the microbiota relates to a detoxification of the compounds. In the same study mentioned above, Friedman et al. revealed the aglycones solanidine and solasodine to be much less toxic to frog embryos, supporting the assumption of detoxification.[43]

In conclusion, our data demonstrated the degradation of solanine, chaconine, solasonine, solamargine, and tomatine to their respective aglycons solanidine, solasodine, and tomatidine by the intestinal pig cecal microbiota with a dependence of the degradation rate on the linked sugar sidechain. Solanine and solasonine linked to a solatriose were significantly faster metabolized than chaconine and solamargine connected to a chacotriose. Moreover, a stepwise metabolism of the carbohydrate sidechain and the formation of intermediate metabolites were shown for all analytes, with a preferred cleavage of sugar moieties dependent on the configuration of the glycosidic linkage. Our data contributes valuable data about the intestinal metabolism of different SGAs and help to improve risk assessment for humans as demanded by the EFSA. Nevertheless, due to still very limited data about the metabolism, toxicokinetics, and toxicity of SGA and their aglycons in humans, more research is needed to further improve risk assessment and reduce uncertainties.

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■ ASSOCIATED CONTENT

Supporting Information

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time-dependent degradation profiles of the steroidal glycoalkaloids with modified artificial gastric juice; time-dependent degradation profiles of tomatine in cecum suspension; and calculated m/z ratios of intermediate metabolites after incubation of the steroidal glycoalkaloids in ACS (PDF)

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Notes

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■ ABBREVIATIONS

α-chaconine, chaconine; α-solamargine, solamargine; α-solanine, solanine; α-solasonine, solasonine; α-tomatine, tomatine; ACN, acetonitrile; ACS, active cecum suspension; CE, collision energy; CONTAM, European Food Safety Authority Panel on Contaminants in the Food Chain; CXP, collision cell exit potential; DCS, deactivated cecum suspensions; DP, declustering potential; EFSA, European Food Safety Authority; EP, entrance potential; ESI, electrospray ionization; Glc, glucoside; HPLC, high-performance liquid chromatography; MeOH, Methanol; MS/MS, tandem mass spectrometry; QToF, quadrupole time-of-flight mass spectrometer; Rha, rhamnose; RT, retention time; SG1, segment 1; SG2, segment 2; SGA, steroidal glycoalkaloids; TW, time window

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