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Isolation, Characterization, and Surfactant Properties of the Major Triterpenoid Glycosides from Unripe Tomato Fruits

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Various triterpenoid glycosides were extracted from whole unripe tomato fruits (*Lycopersicon esculentum* cv. Cedrico), using aqueous 70% (v/v) ethanol to study their surfactant properties. Cation-exchange chromatography using a Source 15S column and subsequent semipreparative HPLC using an XTerra RP18 were employed to purify individual triterpenoid glycosides from the extract. The structure of the purified compounds was established by mass spectrometry and nuclear magnetic resonance spectroscopy. The furostanol glycoside tomatoside A (749 mg/kg of DW) and the glycoalkaloids α-tomatine (196 mg/kg of DW) and esculeoside A (427 mg/kg of DW) were the major triterpenoid glycosides present. Furthermore, minor amounts of a new dehydrofurostanol glycoside, dehydrotomatoside, were found. The critical micelle concentrations of the major triterpenoid glycosides, α-tomatine, tomatoside A, and esculeoside A, were determined as 0.099, 0.144, and 0.412 g/L, respectively. The results show that tomatoside A, and not the more well-known α-tomatine, is the predominant triterpenoidal surfactant in unripe tomato fruits.

KEYWORDS: Glycoalkaloid; tomato; chromatography; mass spectrometry; surfactant properties; triterpenoid glycoside

INTRODUCTION

Tomato (*Lycopersicon esculentum*) is a vegetable widely consumed by humans, not least because it is rich in several health-promoting compounds such as vitamin C, vitamin E, flavonoids, β-carotene, and lycopene (*1*). Worldwide, about 125 million tons of tomato fruits are produced annually (*2*). In Europe, nearly 18% of the tomato harvest is used for consumption as such, whereas the majority is processed into juice, puree, paste, ketchup, and canned products (*3*).

Upon processing of tomatoes, a number of byproducts are obtained, and already in the 1970s and 1980s investigations were carried out to valorize them (*4*). In more recent years, processes for the enrichment of valuable compounds have been developed, which are (or might be) used as food ingredients or dietary supplements. Besides seeds and skins, also immature tomatoes (green and breaker stage) constitute an important side-stream in tomato processing, as they can compromise the quality of the end product. It is known from the literature that unripe tomatoes are a rich source of the steroidal glycoalkaloid α-tomatine (*5*). Properties of α-tomatine, which might be of interest for future product development, include its reduction of the plasma LDL cholesterol level (*5*), inhibition of the growth of cancer cells (*6, 7*) and micoorganisms (*5*), and stimulation of the immune system (*5, 8*). Moreover, α-tomatine might serve as a precursor or scaffold for the synthesis of various steroid hormones (*7, 9*).

α-Tomatine is a triterpenoid glycoside of the steroid-type (*5*), the structure of which is shown in **Figure 1**, with the characteristic nitrogen atom in the F-ring. Dehydrotomatine, the unsaturated Δ5 analogue of α-tomatine, is also found in green tomato fruit, but at a ∼10-fold lower level than α-tomatine (*5*). It should be noted that several other glycoalkaloids have been found in tomato as well, the majority of which have been associated with red (or overripe) tomatoes (*7, 10*−*12*). These glycoalkaloids differ from α-tomatine in stereochemistry and substitution of the F-ring. To this end, data on the presence of glycoalkaloids other than α-tomatine are lacking.

Many of the properties of triterpenoid glycosides are thought to be related to the amphiphilic character of the molecules, by which they can act as surfactants. To determine their surface-active properties, triterpenoid glycosides were extracted from unripe tomato fruits, using aqueous ethanol. The main triter-

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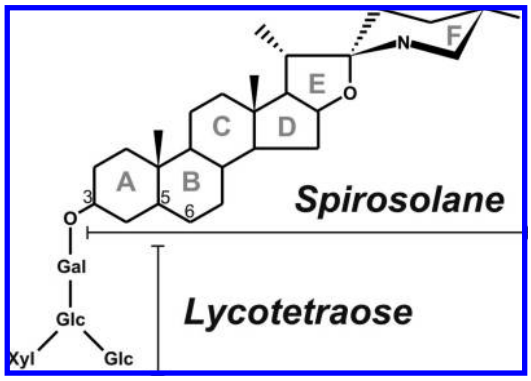

Figure 1. Structure of α-tomatine.

penoid glycosides were purified by using a combination of cation-exchange and reversed-phase chromatography, structurally characterized by spectroscopic techniques, and investigated with respect to their surfactant properties.

MATERIALS AND METHODS

Materials. Tomato fruits in green mature and breaker stage (cv. Cedrico) were provided by Rijk Zwaan (De Lier, The Netherlands). Tween 80, α-tomatine, and 2,4,6-trihydroxyacetophenone were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Sodium cholate was purchased from Merck (Darmstadt, Germany).

Extraction of Triterpenoid Glycosides from Tomato Fruits. Whole tomato fruits were cut and homogenized using a blender (Braun GmbH, Kronberg, Germany), prior to freeze-drying. Next, the freeze-dried material was ground into a fine powder with a particle size of ~0.7 mm using a type A70 grinder (Framo, Eisenbach, Germany), cooled with liquid nitrogen. This powder was stored at −20 °C until use.

The extraction protocol was obtained through optimization of a number of parameters, performed in a series of small-scale experiments. First, the ratio of tomato powder to extractant was varied: 20 and 80 mL of 0.5% (v/v) of acetic acid in water per gram of freeze-dried tomato powder were used. It appeared that 80 mL of extractant/g of tomato powder was more effective than 20 mL/g. Subsequently, the residue of the 80 mL/g extraction was re-extracted with 20 mL/g of the extractant. This procedure was repeated once. The first re-extraction yielded an additional 5% of α-tomatine. The composition of this extract differed from the previous one in that it contained relatively less α-tomatine and more of a compound later identified as esculeoside A. No more glycoalkaloids were obtained with the second re-extraction. Finally, other extraction parameters were investigated: extraction time (20 min, 2 h, and 12 h), extraction temperature (25 and 50 °C), and the concentration of acetic acid (0, 0.5, and 5% v/v). For the 20 min extraction time, the recovery of α-tomatine was less (−33%) than that for the 2 h extraction. With 0% acetic acid concentration, the recovery of extracted tomatine was decreased by 21%, compared to using 0.5% acetic acid. An extraction time of >2 h and an acetic acid concentration >0.5% did not increase the extraction yield of α-tomatine. At 50 °C extraction temperature, less α-tomatine (−34%) was analyzed than at 25 °C. In the RP-HPLC elution profiles, the ratio of α-tomatine to other triterpenoid glycosides was constant, and degradation of specific triterpenoid glycosides was not observed, even after prolonged extraction times (12 h) or exposure to high acetic acid concentrations (5%).

The following, optimized extraction protocol was used throughout this study. Freeze-dried tomato powder (100 g) was extracted [2 h, constant stirring at 200 rpm, room temperature (RT)] with 8 L of 70% (v/v) aqueous ethanol, containing 0.5% (v/v) acetic acid. After filtration through no. 589/2 filter paper (Schleicher & Schuell, Dassel, Germany), the residue was collected and re-extracted (2 h, constant stirring at 200 rpm, RT) with 1.5 L of the same extractant. After paper filtration, the residue was rinsed with 500 mL of the same extractant on the filter paper. The filtrates were combined and concentrated under reduced pressure with a rotary evaporator (below 40 °C), until the ethanol was completely removed. *n*-Hexane was added to the residual aqueous phase in a ratio of 1:1 to remove pigments from the extract. Subsequently, the aqueous phase was adjusted to pH 8.0 with 0.1 M ammonia. Low molecular weight polar compounds were removed from the extract using solid phase extraction with Sep-Pak tC18 vac 35 cm^3/10 g cartridges (Waters, Etten-Leur, The Netherlands). The cartridge was preconditioned with 35 mL of methanol, followed by 35 mL of water. After sample loading (35 mL of extract), the cartridge was washed with 35 mL of water and eluted with 50 mL of methanol. The eluate was evaporated until dryness under reduced pressure. The extract obtained (980 mg from 100 g of freeze-dried tomato powder) was suspended in 10 mL of 0.1% (v/v) aqueous acetic acid and centrifuged (14000*g*, 5 min, RT) prior to reversed-phase high-performance liquid chromatography (RP-HPLC) analysis. A small amount of white pellet was formed after centrifugation. It contained, on a weight basis, approximately 5% of the total of extracted triterpenoid glycosides from tomato material, identical in composition to the soluble portion, and was discarded in this experiment.

Analysis of Triterpenoid Glycosides by RP-HPLC-MS. RP-HPLC, in combination with evaporative light-scattering (ELS) and UV detection, was used for analysis of tomato triterpenoid glycosides. Separation was performed on a 150 mm × 4.6 mm i.d., 3.5 μm, XTerra RP18 column (Waters, Milford, MA) with a 10 mm × 4.6 mm i.d., 3.5 μm, XTerra RP18 guard column run on a Spectra System HPLC (Thermo Separation Products, Fremont, CA). The solvents used were water/acetic acid (100:0.1, v/v) (eluent A) and acetonitrile/acetic acid (100:0.1, v/v) (eluent B). The following elution program was used: 0 → 5 min, 0% B (isocratic); 5 → 35 min, 0 → 30% B (linear gradient); 35 → 40 min, 30 → 40% B (linear); 40 → 45 min, 40 → 100% B (linear); 45 → 50 min, 100% B (isocratic); 50 → 52 min, 100 → 0% B (linear); 52 → 60 min, 0% B (isocratic). Samples (20 μL) were injected. The flow rate (1 mL/min) was split into three directions: 250 μL/min to the Alltech ELSD 2000 detector (Deerfield, IL) in series with the Thermo UV3000 detector (Thermo Separation Product Inc., San Jose, CA), 50 μL/min to the LCQ Deca XP max MS (Thermo Finnigan, San Jose, CA), and 700 μL/min to the waste. The evaporator temperature of the ELSD was set at 115 °C with a nebulizing gas flow rate of 3.2 L/min. The molecular mass of the triterpenoid glycosides was identified by mass spectrometry. MS analysis was performed using electrospray ionization (ESI) and detection in the positive ion mode, with a spray voltage of 5.5 kV, a capillary voltage of 15 V, and a capillary temperature of 200 °C, according to the method of Cataldi and co-workers (*13*). The instrument was tuned to optimize the ionization process and sensitivity using commercial α-tomatine. Total ion current was used to record the abundances of the protonated triterpenoid glycosides and their fragments. A full-scan mass spectrum over a range of *m*/*z* values of 150−1500 was recorded. The control of the instrument and data processing were done with Xcalibur software (Thermo Finnigan). The triterpenoid glycosides were quantified using commercial α-tomatine as a reference compound. A calibration curve was made with α-tomatine (0.25−2.00 mg/mL; R^2 with ELSD = 0.997), from which the amounts of the various triterpenoid glycosides were calculated, assuming that their response factors were similar.

Fractionation of Triterpenoid Glycosides by Cation-Exchange Chromatography. Preparative cation-exchange chromatography was performed with a 140 mm × 26 mm, ~74 mL, Source 15S column (GE Healthcare BioScience, Uppsala, Sweden) run on an Äkta Explorer System (GE Healthcare BioScience). The column was equilibrated with 5 column volumes of 10 mM sodium acetate buffer (pH 5.5). The tomato extract (up to 200 mg DW) was suspended in the same buffer at a concentration of 100 mg/mL and centrifuged (14000*g*, 2 min, RT) prior to application (at 10 mL/min) onto the column. After washing with 5 column volumes of this buffer, a linear gradient of 5 column volumes from 0 to 1 M sodium chloride in 10 mM sodium acetate buffer (pH 5.5) was applied at a flow rate of 10 mL/min. The eluate was monitored at 205 nm and by the analysis using matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF-MS). Fractions (130; 10 mL each) were collected and pooled according to their MALDI-TOF mass spectra.

Purification of Triterpenoid Glycosides by Semipreparative RP-HPLC. The pools obtained by cation-exchange chromatography were further fractionated on a Waters preparative HPLC system, using a

semipreparative 150 mm × 19 mm, 5 μm, XTerra RP18 column (Waters, Milford, MA) with a 19 × 10 mm i.d., 5 μm, XTerra RP18 guard column. The solvents used were water/acetic acid (100:0.1, v/v) (eluent A) and acetonitrile/acetic acid (100:0.1, v/v) (eluent B). The following elution program was used: 0 → 5 min, 0% B (isocratic); 5 → 35 min, 0 → 30% B (linear gradient); 35 → 40 min, 30 → 40% B (linear); 40 → 45 min, 40 → 100% B (linear); 45 → 50 min, 100% B (isocratic); 50 → 52 min, 100 → 0% B (linear); 52 → 60 min, 0% B (isocratic). Pools 1 and 2 obtained by cation-exchange chromatography were dissolved in eluent A at a concentration of 30 or 10 mg/mL, respectively, and filtered through a 0.2 μm cellulose acetate filter (Schleicher & Schuell), prior to loading it onto the system. The flow rate was 17 mL/min. The elution profile was monitored at UV 205 nm, and the eluent was collected in glass tubes every 15 s in the time span of 20−40 min of each run. Appropriate fractions were pooled on the basis of MALDI-TOF-MS and analytical RP-HPLC-MS. The purity of the pools obtained was verified by analytical RP-HPLC. Finally, the pools were lyophilized after removal of acetonitrile.

Screening of Fractions by MALDI-TOF-MS. To qualify the components of the chromatographic fractions collected, MALDI-TOF-MS analysis was carried out using an Ultraflex workstation (Bruker Daltonics, Bremen, Germany), equipped with a nitrogen laser of 337 nm. For the analysis of fractions obtained with cation-exchange chromatography, saturated α-cyano-4-hydroxycinnamic acid in acetone/0.1% (v/v) TFA (97:3, v/v) was used as a matrix. One microliter of matrix solution was spotted on an AnchorChip target (Bruker Daltonics), and the remaining liquid was immediately removed with a pipet tip. Subsequently, 2 μL of the fraction was applied onto the same spot and kept for 3 min. After that, 4 μL of 10 mM $NH_4H_2PO_4$ in 0.1% (v/v) TFA was added to the spot and removed immediately (desalting step). Finally, 1 μL of 0.1 mg/mL of α-cyano-4-hydroxycinnamic acid in ethanol/acetone/0.1% (v/v) TFA (6:3:1, v/v) was added to the spot to recrystallize the compounds of the fraction, and the spot was dried. For the analysis of fractions obtained with semipreparative RP-HPLC, 2,4,6-trihydroxyacetophenone in methanol (10 mg/mL) was used as matrix. One microliter of fraction and 1 μL of matrix solution were spotted on a target gold plate, dried, and analyzed. The analysis was performed in the positive-ion mode.

Analysis of Triterpenoid Glycosides with Nuclear Magnetic Resonance. Samples were dissolved in pyridine-d_5 (99 atom % D, Bio-Rad). NMR spectra were recorded at a probe temperature of 25 °C on a Bruker AMX-500 spectrometer located at the Wageningen NMR Centre. Chemical shifts are expressed in parts per million relative to TMS at 0.00 ppm. 1D and 2D-dqf (double quantum filtered) COSY, TOCSY, ROESY, and HMQC spectra were acquired using standard pulse sequences delivered by Bruker. For the 2D HMBC spectrum, a standard gradient-enhanced 2D HMQC pulse sequence, delivered by Bruker, was changed into a HMBC sequence by setting the delay between the first proton and carbon pulse to 53 ms. For the ^{1}H COSY and TOCSY spectra, 800 experiments of 16 and 8 scans (tomatoside A) or 32 and 32 scans (dehydrotomatoside) were recorded, resulting in measuring times of 8.5 and 4 h (tomatoside A) or 16.5 and 17 h (dehydrotomatoside), respectively. The mixing time for the TOCSY spectra was 80 ms. For the 2D-ROESY spectrum (dehydrotomatoside), 800 experiments of 32 scans were recorded with a mixing time of 200 ms, resulting in a measuring time of 17.5 h. For the [^{1}H,^{13}C] HMBC and HMQC spectra (tomatoside A), 1024 experiments of 128 and 64 scans, respectively, were recorded, resulting in measuring times of 68.5 and 37 h, respectively.

Determination of the Critical Micelle Concentration (CMC). Surface tension of the purified triterpenoid glycosides was measured using an automated drop tensiometer (IT Concept, Longessaigne, France). Before measurement, all glassware was rinsed successively with chromosulfuric acid and deionized water and then dried. Each experiment started with a clean interface of a newly formed air bubble (5 μL) in a cuvette containing the sample solution. Surface tension was determined by bubble shape analysis. The procedure is described in detail elsewhere (*14*). To verify the performance of the equipment, the surface tension of deionized water was verified between measurements.

For each tomato triterpenoid glycoside, samples were freshly prepared prior to analysis, by dissolving the purified compounds in 50 mM sodium acetate buffer (pH 4.0) in concentrations ranging from 10^{-3} to 10^{0} mg/mL. The temperature was controlled at 20 °C for all measurements. The decrease in surface tension was monitored for a period of 1−2 h, until the surface tension reached a constant value. The CMC was deduced from the plot of the surface tension against the logarithm of the sample concentration, in which the inflection point corresponds to the CMC value. The equations describing the two linear parts in the plot were established by linear regression. The density of the surfactants (Γ_{max}) was calculated from the slope of the decreasing line in the plot of surface tension versus concentration of the compounds. The minimal attainable surface tension (γ_{CMC}) was determined as the surface tension at the CMC.

The surface tensions of two reference compounds, Tween 80 and sodium cholate, were measured at different concentrations to validate our method for the determination of the CMC. The CMCs of Tween 80 and sodium cholate were determined to be 0.018 and 4.460 g/L, respectively. These values were in accordance with data reported earlier using a different method for monitoring surface tension as a function of surfactant concentration: 0.015−0.036 and 2.3−5.6 g/L for Tween 80 and sodium cholate, respectively (*15*).

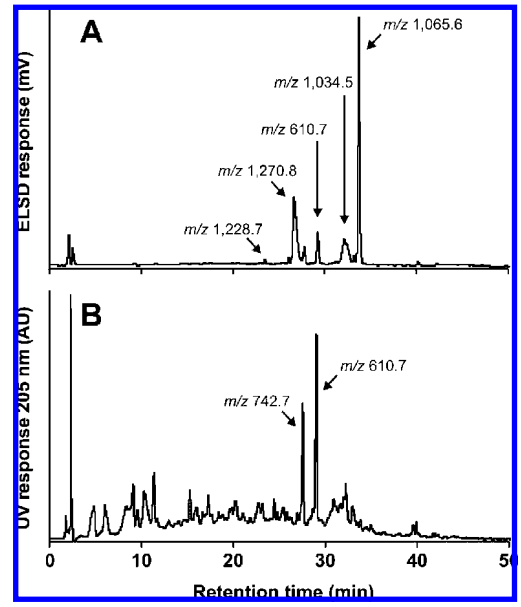

Figure 2. RP-HPLC elution profile of glycoalkaloids extracted from tomato material detected on ELS detector and UV detector.

RESULTS AND DISCUSSION

Analysis of the Extract of Unripe Tomatoes by RP-HPLC. The extract from unripe tomatoes was analyzed by RP-HPLC with triple detection: UV 205 nm, ELS, and MS. It was observed that certain compounds were better detected by ELSD, whereas others were better detected with UV 205 nm (**Figure 2**). Most of the compounds with a pronounced ELSD signal had an *m/z* ratio exceeding 1000 (**Table 1**). The extraction protocol employed and the high ELSD to UV signal ratio suggested that they might represent glycoalkaloids or, more generally, triterpenoid glycosides.

Glycoalkaloids. The commercial α-tomatine reference eluted at a retention time of 31.2 min, with the characteristic *m/z* 1034.5. In the unripe tomato extract, dehydrotomatine seemed to coelute with α-tomatine, as *m/z* 1032.5 was evident at the same retention time (data not shown). No attempts were made to separate these compounds. The amount of α-tomatine was calculated to be 196 mg/kg of DW. Two other peaks in the ELSD trace also seemed to correspond to glycoalkaloids. The compound with *m/z* 1228.7 at a retention time 23.1 min was tentatively annotated as esculeoside B. The compound with *m/z*

Table 1. Tentative Annotation of Compounds Found in Extracts from Unripe Tomato Fruit, Based on Mass Spectrometric Analysis (ESI-MS, Positive Mode)

RT (min)	m/z $[M + H]^+$	fragments (MS mode)	annotation	refs
Glycoalkaloids				
23.1	1228.7	934.9, 772.6, 610.6	esculeoside B	*10, 12*
27.0	1270.8	1,138.7, 1,108.7, 814.7, 652.4	esculeosides A, F, G	*10, 12*
27.8	1050.5	918.5, 756.5, 594.5	lycoperoside H, singly hydroxylated α-tomatine	*11, 12*
28.4	1108.7	814.4, 652.4, 490.4	esculeoside A lacking O27 glucosyl residue	
31.2	1032.5	900.5, 738.5, 576.5, 414.4	dehydrotomatine	*16*
31.2	1034.5	902.5, 740.5, 578.5, 416.4	α-tomatine	*5*
Putative Other Triterpenoid Glycosides				
32.9	1063.6	901.5, 739.3, 577.4, 415.2	unknown compound	
33.4	1065.6	903.7, 741.7, 579.4, 417,3	unknown compound	
Flavonoid Glycosides				
27.5	742.7	610.7, 465.0, 303.3	quercetin 3-trisaccharide	*17*
29.0	610.7	465.3, 303.3	quercetin 3-rutinoside	*17*

1270.8 at a retention time 27.0 min might correspond to esculeoside A or lycoperoside F or G, which cannot be distinguished by mass spectrometry. The amount of the compound with m/z 1270.8 was estimated to be 427 mg/kg of DW.

Upon MS^2, these glycoalkaloids showed fragment ions formed by elimination of pentose and/or hexose moieties from their lycotetraose side chain. For example, for α-tomatine, the m/z values of 902.5, 740.5, 578.5, and 416.5 corresponded to $[\text{tomatidine} + \text{Gal} + 2\text{Glc} + \text{H}]^+$, $[\text{tomatidine} + \text{Gal} + \text{Glc} + \text{H}]^+$, $[\text{tomatidine} + \text{Gal} + \text{H}]^+$, and $[\text{tomatidine} + \text{H}]^+$, respectively. Furthermore, m/z 1016.5 was observed in the MS^2 spectrum, which might be produced by a cleavage and rearrangement process in the E- and F-rings (loss of a water molecule), as suggested by Cataldi and co-workers (*13*). Also, molecular ions with m/z 273.5 and 255.5 were found, the presence of which might be explained by cleavage of the E- and F-rings of the aglycone tomatidine. Such cleavage mechanisms have been proposed before for tomatine (*13*) and saponins (*18*).

Putative Other Triterpenoid Glycosides. The major peak at a retention time 33.2 min had m/z 1065.6. For this compound, mass losses corresponding to a multiple of 162 were found at m/z 903.5, 741.5, 579.5, and 417.5. This indicated that this compound contained four hexose moieties, but no pentose moieties. Therefore, it is unlikely that this compound represented a glycoalkaloid, as it did not contain the characteristic lycotetraose side chain. However, its mass of >1000, its pronounced ELSD, and its negligible UV signal suggested that this compound might be a triterpenoid glycoside. The amount of the compound with m/z 1065.6 was estimated to be 749 mg/kg of DW.

Flavonoid Glycosides. The m/z values of 610.7 and 742.7 correspond to those of quercetin 3-rutinoside and quercetin 3-trisaccharide, respectively, which were reported before to be present in tomato (*17*). They were tentatively annotated as such, on the basis of the mass number of the molecular (parent) ion and its fragments. No further attempts were made to characterize these components.

Our results show that for the analysis of triterpenoid glycosides, ELS detection is superior to UV 205 nm detection, a method employed by other researchers (*19, 20*). Similar results were previously obtained by Decroos and co-workers (*21*) for saponins, also a type of triterpenoid glycoside. ELSD might be an alternative to pulsed amperometric detection (*22*).

Fractionation of Triterpenoid Glycosides by Cation-Exchange Chromatography. To establish the structure of the compounds with m/z 1065.6 and 1270.8, the extract of unripe tomatoes was fractionated by cation-exchange chromatography. Glycoalkaloids are positively charged at a low pH, because of protonation of the nitrogen atom in their aglycone. It has been reported that the pK_a value of solasodine, the C-22 isomer of dehydrotomatidine, is 7.7 (*23*). By performing cation-exchange chromatography at pH 5.5, the tomato glycoalkaloids might be separated from other (uncharged) constituents of the extract, thereby enabling an effective isolation.

All fractions obtained upon cation-exchange chromatography were subjected to MALDI-TOF-MS analysis to annotate the compounds contained in each fraction. The compound with m/z 1065.6 was observed in fractions 4−14, and the compound with m/z 1270.8 was predominantly present in fractions 18−26. α-Tomatine (m/z 1034.5) was observed in a wide range of fractions, that is, from 48 to 130. On the basis of the composition of the fractions, four pools were made. The RP-HPLC elution profiles of the four pools are shown in **Figure 3**. The total recovery of triterpenoid glycosides after cation-exchange fractionation was ∼75%, on the basis of the comparison of the sum of all peak areas in the RP-HPLC-ELSD profiles of the four pools with that of the sample applied.

Quercetin 3-rutinoside and quercetin 3-trisaccharide were mainly observed in pool 2. It was unexpected that these compounds were retarded on the cation-exchange column. Interestingly, pool 3 contained two minor triterpenoid glycosides with m/z 1050.5 and 1108.7. Further purification of these compounds was not attempted. Their masses might correspond

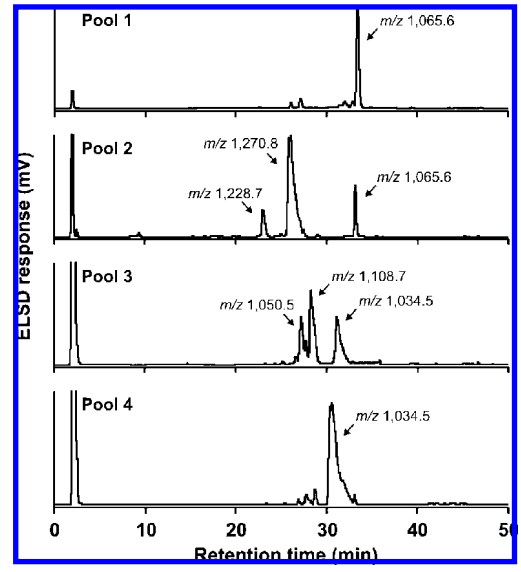

Figure 3. RP-HPLC-ELSD profiles of the pools obtained with cation-exchange chromatography.

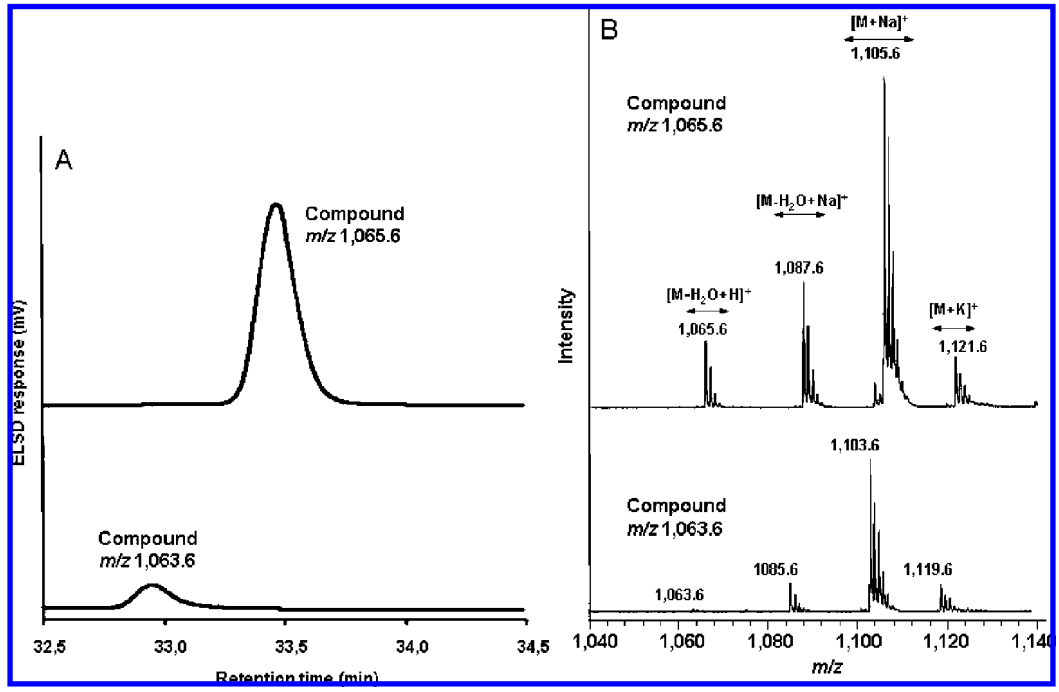

Figure 4. RP-HPLC-MS profiles (**A**) and MALDI-TOF-MS spectra (**B**) of the purified compounds with *m/z* 1063.6 and 1065.6.

to lycoperoside H (or singly hydroxylated α-tomatine) and esculeoside A lacking the *O*-27 glucosyl residue, respectively (**Table 1**).

Fractionation by cation-exchange chromatography facilitated a separate recovery of the different putative triterpenoid glycosides. The affinity for the column decreased in the order α-tomatine > 1050.5 (singly hydroxylated α-tomatine) ≈ 1108.7 (esculeoside A lacking the glucosyl residue) > esculeoside B (1228.7) > esculeoside A > 1065.6. It is hypothesized that substitution of the F-ring of α-tomatine might shield the positive charge and prevent binding to the cation-exchange resin. However, this needs to be established further.

Purification of Triterpenoid Glycosides by Semipreparative RP-HPLC. Pools 1 and 2 were applied onto a semipreparative RP-HPLC column to purify the unknown compounds with *m/z* 1065.6 and 1270.8, respectively. The fractions were analyzed with MALDI-TOF-MS for the presence of these components and pooled accordingly.

The compound with *m/z* 1065.6 was recovered individually, as can be seen from **Figure 4A**, which shows the analysis of the isolated component by RP-HPLC. From 100 g of freeze-dried powder from unripe tomatoes, ~45 mg of this compound was obtained. Moreover, a small amount (about 3 mg) of a compound with *m/z* 1063.6 was recovered, which eluted just before the compound with *m/z* 1065.6. This compound might represent the dehydro analogue of compound *m/z* 1065.6, because of the difference of 2 amu. A more thorough analysis by MALDI-TOF-MS of these two compounds showed that they had a very similar patterns of signals (**Figure 4B**). Interestingly, *m/z* 1063.6 and 1065.6 were not the main peaks observed in the mass spectrum, but rather *m/z* 1103.6 and 1105.6. These signals were not observed with electrospray ionization (ESI), which is known to be less gentle than MALDI. These signals might represent sodium adducts of the compounds (more common with MALDI than with ESI), suggesting that the actual masses of the compounds are 1080.6 and 1082.6. This was corroborated by the observations that *m/z* 1121.6 might be the potassium adduct of the compound with a mass of 1082.6 and that *m/z* 1087.6 might represent the sodium adduct ($[M + Na]^+$) of *m/z* 1065.6 ($[M + H]^+$). Taken together, our data suggested that *m/z* 1065.6 might actually be a fragment obtained after water loss of a compound with a mass of 1082.6, which is not uncommon with triterpenoid glycosides (*13*).

The compound with *m/z* 1270.8 was successfully separated from the quercetin saccharides in pool 2, as judged by analytical RP-HPLC and mass spectrometry of the purified compound (data not shown). From 100 g of freeze-dried powder from unripe tomatoes, ~20 mg of this compound was obtained.

Identification of Triterpenoid Glycosides Purified from Unripe Tomato Fruits. The three compounds with *m/z* 1065.6, 1063.6, and 1270.8, obtained from semipreparative RP-HPLC, were analyzed with ^{1}H NMR and ^{13}C NMR.

m/z 1065.6. Careful assignment of all resonances and cross peaks in the 1D and 2D NMR spectra of the compound with *m/z* 1065.6, resulting in full assignment of the ^{1}H NMR and ^{13}C NMR spectra (**Table 2**), confirmed the identity of this compound as tomatoside A (**Figure 5A**). This compound is a furostanol glycoside, which was identified in tomato seeds previously (*24, 25*). The calculated molecular mass of tomatoside A is 1082, which is not consistent with the molecular ion of 1065.6 predicted by MS. This discrepancy might be explained by the loss of a water molecule.

m/z 1063.6. The assignment of the furostan moiety in the ^{13}C spectrum of the compound with *m/z* 1063.6 was in complete agreement with the ^{13}C NMR data of compounds**3** and **4** reported previously by Kang and co-workers (*26*). The assignment of the sugar residues in the ^{13}C spectrum of compound *m/z* 1063.6 is identical to that of the sugar residues of tomatoside A. The amount of material was insufficient for 2D-HMQC or -HMBC spectroscopy, but a complete ^{1}H assignment of the sugar residues and an almost complete ^{1}H assignment of the furostanol moiety was possible based on the 2D-COSY, -TOCSY, and -ROESY spectra. The compound will be referred to as dehydrotomatoside (**Figure 5B**) and was found earlier in *Solanum lyratum* by Murakami and co-workers (*27*).

As no complete assignments of the NMR spectra for tomatoside A and dehydrotomatoside have been reported in the literature before, we present them in **Table 2**. The C-25

Table 2. ^{1}H NMR and ^{13}C NMR Spectral Data of Tomatoside A and Dehydrotomatoside[a]

	tomatoside A		dehydrotomatoside	
position	δ_C	δ_H, multiplicity, J	δ_C	δ_H, multiplicity, J
furostan				
1	37.22	1.54, pn; 0.81, pn	37.49	1.71, pn; 0.99, pn
2	30.02	2.07, pn; 1.67, pn	30.25	2.13, pn; 1.77, pn
3	77.49	3.95, pn	78.22	3.91, pn
4	34.84	1.810, bd, 13.0	39.27	2.697, bd, 12.7; 2.469, m
5	44.71	0.91, pn	141.01	
6	28.93	1.15, pn; 1.09, pn	121.73	5.31, pn
7	32.44	1.52, pn; 0.79, pn	32.30	1.86, pn; 1.49, pn
8	35.24	1.41, pn	31.64	1.42, pn
9	54.45	0.510, ddd, 11.8, 10.9, 3.8	50.33	0.89. pn
10	35.82		37.05	
11	21.29	1.41, pn; 1.218, m	21.46	nd
12	40.21	1.71, pn; 1.07, pn	39.94	nd
13	41.12		40.69	
14	56.37	1.03, pn	56.57	1.05, pn
15	32.36	2.02, pn; 1.42, pn	32.44	2.01, pn; 1.44, pn
16	81.16	4.961, ddd, 7.4, 7.4, 7.4	81.14	4.966, ddd, 7.4, 7.4, 7.4
17	63.86	1.95, pn	63.72	1.95, pn
18	16.74	0.882, s	16.49[b]	0.895, s
19	12.33	0.665, s	19.41	0.906, s
20	40.69	2.242, dq, 6.8, 6.7	40.80	2.245, dq, 6.3, 5.6
21	16.45	1.331, d, 6.9	16.46[b]	1.338, d, 6.8
22	110.73		110.78	
23	37.11	2.11, pn; 1.99, pn	37.12	2.01, pn; 2.01, pn
24	28.32	2.06, pn; 1.70, pn	28.32	2.09, pn; 1.70, pn
25	34.43	1.94, pn	34.44	1.94, pn
26	75.40	4.10, pn; 3.501, dd, 9.3, 7.0	75.41	4.10, pn; 3.507, dd, 9.5, 7.0
27	17.46	1.040, d, 6.7	17.46	1.042, d, 6.7
3-Gal′				
1	102.38	4.921, d, 7.7	102.67	4.928, d, 7.0
2	73.27	4.491, dd, 9.2, 8.2	73.25	4.502, dd, 7.5, 7.2
3	75.57	4.14, pn	75.54	4.13, pn
4	80.93	4.591, bs	80.88	4.59, pn
5	75.12	4.07, pn	75.09	4.02, pn
6	60.58	4.779, bdd, 10.1, 10.1; 4.27, pn	60.49	4.761, bdd, 10.1, 10.0; 4.22, pn
4′-Glc″				
1	105.12	5.132, d, 7.8	105.13	5.128, d, 7.9
2	85.94	4.15, pn	85.93	4.15, pn
3	78.37	4.28, pn	78.37	4.28, pn
4	71.80	3.98, pn	71.81	3.98, pn
5	78.15	3.98, pn	78.15	3.98, pn
6	63.19	4.650, bd, 10.6; 4.11, pn	63.19	4.653, bd, 10.9; 4.12, pn
2″-Glc‴				
1	106.85	5.201, d, 7.6	106.86	5.208, d, 7.6
2	76.69	4.07, pn	76.70	4.08, pn
3	77.57	4.14, pn	77.58	4.14, pn
4	70.35	4.23, pn	70.36	4.24, pn
5	78.92	3.809, ddd, 9.6, 2.6, 2.6	78.92	3.820, ddd, 9.6, 2.8, 2.8
6	61.61	4.60, pn; 4.38, pn	61.61	4.61, pn; 4.38, pn
26-Glc⁗				
1	105.07	4.819, d, 7.8	105.08	4.824, d, 7.8
2	75.20	4.04, pn	75.21	4.04, pn
3	78.55	4.27, pn	78.57	4.26, pn
4	71.70	4.23, pn	71.71	4.26, pn
5	78.43	3.96, pn	78.45	3.97, pn
6	62.79	4.562, bdd, 11.8, 2.2; 4.38, pn	62.79	4.57, pn; 4.39, pn

[a] ^{13}C and ^{1}H NMR chemical shifts in ppm, multiplicity, and J in hertz, Recorded in pyridine at 25 °C. pn, peak splitting not assigned due to overlap; nd, not determined. [b] Values might be interchangeable.

configuration for both compounds was confirmed to be *S*, on the basis of the chemical shift differences of 0.60 ppm (tomatoside A) and 0.59 ppm (dehydrotomatoside) between the two protons at position 26 (*28*). The linkages of the sugar residues were confirmed by long-range correlations in the HMBC spectrum of tomatoside A and by correlations in the ROESY spectrum of dehydrotomatoside.

m/z 1270.8. The identity of the compound with *m/z* 1270.8 was confirmed as esculeoside A (**Figure 5C**) on the basis of the chemical shifts in the 1D ^{1}H NMR and ^{13}C NMR spectra of this compound (data not shown). Esculeoside A has also been identified before in ripe fruits of cherry tomato and pink color-type tomato (*10*).

Facilitated Fragmentation of Tomatoside A upon Positive Mode ESI-MS. NMR analysis of the compounds initially annotated as *m/z* 1065.6 and 1063.6 showed that their actual $[M + H]^+$ molecular ions should have masses of 1083.6 and 1081.6, respectively. This is consistent with the water loss of the compounds, as suggested earlier. Similar observations have been done for α-tomatine (*13*). It has been described that the

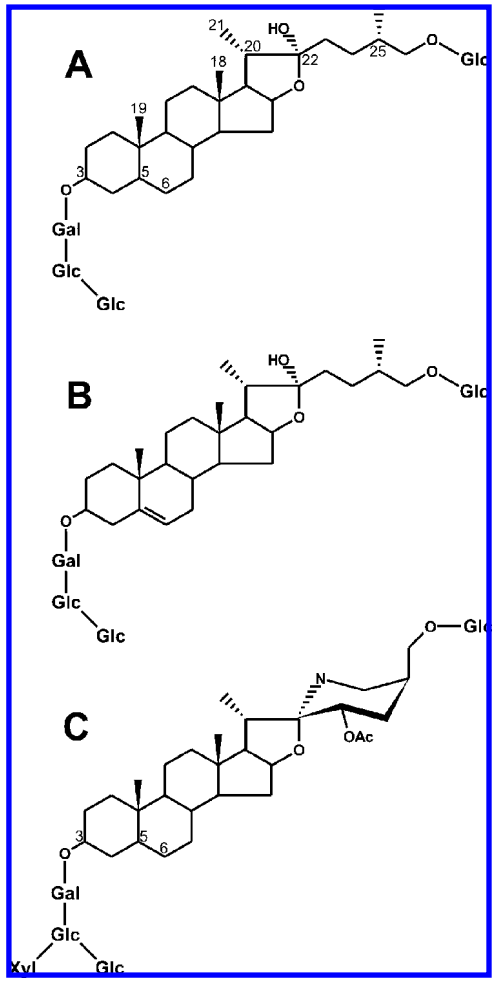

Figure 5. Structures of triterpenoid glycosides extracted from unripe tomato fruits identified in this study: tomatoside A (**A**), dehydrotomatoside (**B**), and esculeoside A (**C**). Carbohydrate chain of **A** and **B**: β-D-Glc*p*-(1→2)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→3)-triterpenoid skeleton. Carbohydrate chain of **C**: β-D-Glc*p*-(1→2)-[β-D-Xyl*p*-(1→3)-]β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→3)-triterpenoid skeleton.

most prominent product ion of α-tomatine upon MS^2 corresponds to $[M + H - H_2O]^+$, which was suggested to be formed by dehydration through a rearrangement process in the E-ring (**Figure 6A**). When LC-MS is performed under acidic conditions, dehydration of tomatoside A and dehydrotomatoside already occurs during MS^1, suggesting that these compounds are much more sensitive to water loss than α-tomatine. Upon protonation of the hydroxyl group attached to C-22, a water molecule is expelled (**Figure 6B**), and the resultant C-22 carbocation is resonance-stabilized by the lone pair of the vicinal oxygen.

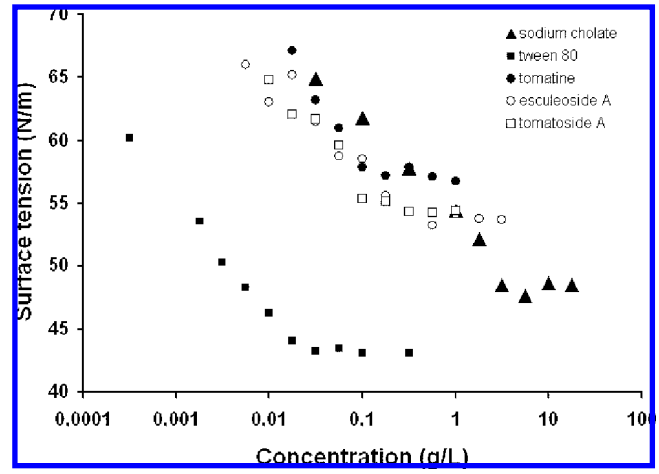

Figure 7. Surface tension as a function of the concentration of the purified compounds. Tween 80 and sodium cholate were used as reference compounds.

Surface Tension, CMC of Purified Tomato Triterpenoid Glycosides. Recent studies have shown that glycoalkaloids from tomatoes and potatoes have antifungal and cholesterol-lowering potential (*5*). This potential might be related to the amphiphilic character of the molecules and, consequently, their ability to lower the surface tension of water and form micelles. The surface tensions of α-tomatine, esculeoside A, and tomatoside A were measured at various concentrations (**Figure 7**). The surface tension decreased linearly ($R^2 > 0.96$) with increasing concentrations of the three triterpenoid glycosides, until a constant value was reached. From the intersection of the two lines, the CMC values for α-tomatine, esculeoside A, and tomatoside A were calculated as 0.094, 0.412, and 0.144 g/L, respectively (**Table 3**).

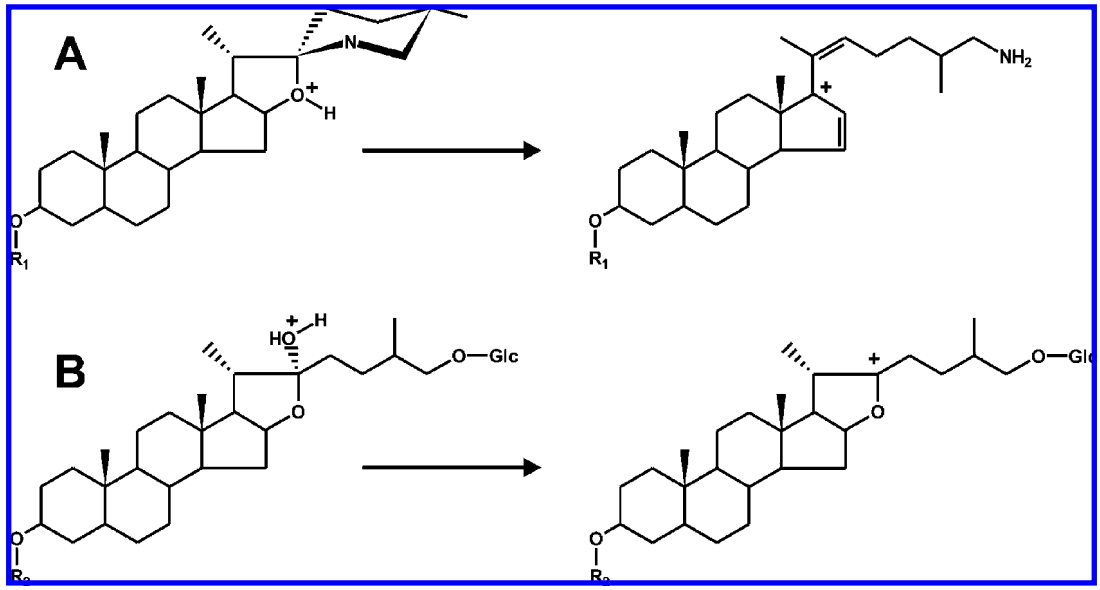

Figure 6. Putative fragmentation schemes of α-tomatine (**A**) and tomatoside A (**B**) using ESI-MS in the positive mode.

Table 3. Critical Micelle Concentrations (CMC), Surface Densities (Γ_{max}), and Minimal Attainable Surface Tensions (γ_{CMC}) of Purified Triterpenoid Glycosides and Reference Surfactants (Sodium Cholate, Tween 80), Determined by Automated Drop Tensiometer

compound	CMC (g/L)	Γ_{max} (mg/m^2)	γ_{CMC} (mN/m)
α-tomatine	0.099	5.2	56.7−57.8
esculeoside A	0.412	3.3	53.2−54.1
tomatoside A	0.144	3.7	54.3−55.4
sodium cholate	4.460	3.6	47.6−48.6
Tween 80	0.018	4.1	43.0−44.0

General rules for deriving the CMC from the molecular structure of a compound are not established, but it has been suggested that the larger the hydrophobic proportion of a molecule, the lower the CMC value (*15*). The highest CMC value of esculeoside A might be explained by the glucosyl residue on the F-ring. Consequently, the polarity of this side of the molecule increases and the hydrophobic proportion decreases, resulting in a higher CMC value. Similarly, the CMC of soyasaponin Ab (a representative of group A saponins, having two glycosyl chains) is significantly higher than that of soyasaponin Bb (a group B saponin, having only one glycosyl chain) (*15*). The observed CMC values of esculeoside A (0.412 g/L) and α-tomatine (0.094 g/L) are similar to those of soyasaponin Ab (0.56 g/L) and Bb (0.085 g/L), respectively. Thus, the presence of an extra glycosyl residue (or chain) influences the surfactant properties of triterpenoid glycosides. The intermediate CMC of tomatoside A (0.144 g/L) might be explained as follows. Tomatoside A has an extra glucosyl residue attached to the skeleton, like esculeoside A, resulting in a higher CMC value compared to α-tomatine. The flexible alkyl chain in the molecule may allow the glycosyl chains to come closer together, resulting in a more obvious amphiphilic character of tomatoside A (and a lower CMC value) than of esculeoside A.

The surfactant surface density (Γ_{max}) was ranked as α-tomatine > tomatoside A > esculeoside A, whereas the minimal attainable surface tensions (γ_{CMC}) were similar for the three triterpenoid glycosides tested (**Table 3**). The surface density (Γ_{max}) indicates the arrangement of surfactant molecules at the water−air interface. Less steric hindrance between molecules might facilitate closer packing of the molecules, resulting in a high surface density. The surface densities on a molar basis of α-tomatine, esculeoside A, and tomatoside A are calculated as 5.03×10^{-6}, 2.60×10^{-6}, and 3.41×10^{-6} mol/m^2, respectively. The observed surface density of α-tomatine is significantly higher than that of the other two compounds. The extra glycosyl chain of esculeoside A and tomatoside A might prevent a dense packing. The flexible alkyl chain of tomatoside A might enable a closer packing at the water−air surface in comparison with the more rigid F-ring of esculeoside A, which might explain the slightly higher surface density found for the former.

Whereas α-tomatine is commonly regarded as the main triterpenoid glycoside in unripe tomato fruits, it is now shown that tomatoside A is actually almost 4 times more abundant: 196 versus 746 mg/kg of DW, respectively. Given their similar critical micelle concentration, it was concluded that tomatoside A is the most important triterpenoidal surfactant in unripe tomato fruits. This does not mean that tomatoside A is more important than α-tomatine with respect to antimicrobial activity or disease resistance, as it is known that the two compounds are differently localized in tomato tissue (*25, 29*). To our knowledge, dehydrotomatoside has not been detected in tomato before. This, and the recent discovery of dehydroglycoalkaloids other than the known dehydrotomatine (*30, 31*), suggests that Δ5 unsaturation is a common feature to all tomato triterpenoid glycosides, although these compounds seem to be less abundant than their saturated analogues.

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