α-Tomatine Determination in Tomatoes by HPLC Using Pulsed Amperometric Detection†

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As part of a program to control the biosynthesis of glycoalkaloids, we used an improved HPLC method with pulsed amperometric detection (PAD) to measure the α-tomatine content of store-bought and field-grown, including transgenic, red, and green tomatoes. The HPLC method responded linearly to α-tomatine in the range 0.125–12.5 μg, suggesting a lower limit of detection of about 125 ng of alkaloid. Recoveries from tomato extracts spiked with α-tomatine ranged from 97 to 107%. α-Tomatine and a new glycoalkaloid tentatively identified as dehydrotomatine, whose molecular mass determined by mass spectrometry is 2 Da less than that of α-tomatine, separated well on the HPLC column. The ratio of α-tomatine to dehydrotomatine in commercially available standards was approximately 10:1. The α-tomatine content of ripe red tomatoes ranged from 0.03 to 0.6 mg/100 g of fresh weight. The corresponding values for unripe green tomatoes ranged from 4 to 17 mg/100 g of fresh weight. These results show that the ratio of α-tomatine content for the highest concentration in green tomatoes to the lowest value in red tomatoes is more than 500. The difference in α-tomatine content of transgenic and nontransgenic tomatoes, at the same level of ripeness, was negligible. Possible applications of the HPLC-PAD method are discussed.

Keywords: *Dehydrotomatine; HPLC; pulsed amperometric detection; tomatidenol; tomatidine; α-tomatine; transgenic tomatoes*

INTRODUCTION

α-Tomatine, the major glycoalkaloid present in the leaves, stems, and immature fruit of tomato plants, is reported to be potentially toxic (Friedman et al., 1992; Ripperger and Schreiber, 1981; Roddick, 1974; Wilson et al., 1961). The glycoalkaloid is also reported to exert antifungal activity (Jiratko, 1993) and to inhibit growth of fruitworm and spiny bollworm larvae (Elliger et al., 1981; Weissenberg et al., 1986) and moth eggs (Lu and Chu, 1992). However, its role in host-plant resistance to the Colorado potato beetle (Sinden et al., 1978) may need clarification (Barbour and Kennedy, 1991).

Although ripe red tomatoes contain low levels of α-tomatine, this is not the case for unripe green tomatoes (Eltayeb and Roddick, 1984). We are interested in comparing the α-tomatine content of red and green store-bought and field-grown tomatoes to the α-tomatine content of transgenic tomatoes, genetically altered for improved quality characteristics (Comai, 1993; Kramer et al., 1992; Redenbaugh et al., 1992). This is a challenging analytical problem because α-tomatine contains no chromophore and thus cannot be easily measured by spectrophotometric detection.

The literature reports that the α-tomatine content of tomatoes can be measured by the following techniques: (a) precipitation with ^{14}C-labeled cholesterol (Elliger, 1988; Heftmann and Schwimmer, 1973); (b) gas chromatography, following hydrolysis of the glycoside to the aglycon, tomatidine, and derivatization (Juvik et al., 1982); and (c) HPLC using refractive index detection (Voldřich et al., 1991). The inherent disadvantages of both gas chromatography and cholesterol precipitation are that they measure α-tomatine indirectly and they require multistep procedures which may not always be quantitative. HPLC seemed ideal for measuring α-tomatine. However, we had difficulties applying refractive index detection to the chromatography methods we had previously developed for the closely related glycoalkaloids in potatoes (Bushway et al., 1986; Carman et al., 1986; Friedman and Dao, 1992; Friedman and Levin, 1992). Sensitivity was poor and there was considerable drift. This study describes an improved HPLC method for direct measurement of α-tomatine using pulsed amperometric electrochemical detection.

MATERIALS AND METHODS

Materials. Solvents were of HPLC grade. Reagents were of ACS grade. α-Tomatine, α-solanine, α-chaconine, tomatidine, solanidine, solasodine, and solasonine were obtained from Sigma (St. Louis, MO). An additional sample of α-tomatine was obtained from Research Plus, Inc. (Bayonne, NJ). Tomatidenol was a gift of Prof. H. Ripperger. Calgene (Davis, CA) donated field-grown red and green tomatoes of the genetically altered variety (Manteca) and its parent (control) strain and a series of field-grown nontransgenic control tomatoes at various stages of ripeness (immature green, mature green, breaker, turning, pink, light red, and very red). These terms define the level of ripeness as described by Kramer et al. (1992). Other fresh tomatoes were obtained from a local market (beefsteak, roma, standard, and cherry).

The HPLC eluent for α-tomatine analysis was prepared by combining 100 mL of concentrated buffer with 550 mL of polished water, 200 mL of acetonitrile, and 150 mL of methanol. The eluent for tomatidine analysis consisted of 35% acetonitrile, 100 mM NaH_2PO_4, and 20 mM dibutylamine and was adjusted to pH 3 with phosphoric acid. Water was polished by passing it through a C_{18} solid phase extraction

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(SPE) device (Supelclean Envi 18 SPE tube). The concentrated buffer was prepared by combining 28.97 g of disodium phosphate and 93.72 g of citric acid in 1 L of water. This buffer was filtered through a 0.45 μm nylon membrane from Schleicher and Schuell (Keene, NH), passed through a 3 × 1 cm bed of Chelex 100 (to remove any heavy metals), and then passed through a C_{18} SPE device. These precautions were taken to decrease the eluent's contribution to background current in the electrochemical cell.

Instrumentation. A Dionex Series 4500i gradient liquid chromatography system with a Dionex Ionchrom/pulsed amperometric detector and a Spectra-Physics ChromJet integrator were used. The original cell was replaced with a newer cell designed for use with organic/aqueous eluents (Dionex part no. 42867). The working electrode was gold, the counter electrode was stailness steel, and the reference electrode was a silver/silver chloride combination. The cell design was thin layer. Applied potentials and their durations were as follows: $E_1 = 0.6$ V, $t_1 = 120$ ms; $E_2 = 1.0$, $t_2 = 420$ ms; $E_3 = -0.35$ V, $t_3 = 420$ ms ($E_1 = 0.4$ for tomatidine analysis). Response time was set to 1 s. The sampling period was 16.67 ms. The integrator attenuation was set to 1024. Sensitivity was controlled by the output range setting and was set at 100 or 300 nA.

The chromatography column for α-tomatine analysis was a 4.6 × 250 mm, 5 μm, Supelcosil LC-ABZ with a 2 cm guard of the same material (Supelco Inc., Bellefonte, PA). The chromatography column for tomatidine analysis was a 4.6 × 150 mm, 3 μm, Supelcosil C_{18}-DB with a 2 cm guard of the same material.

Chart speed was set to 0.5 cm/min. Flow rate was set to 1.0 mL/min, and eluent was recycled. Recycling eluent was necessary to avoid the long daily wait for electrode stabilization. The electrode was left on and eluent was continuously recirculated from a 2 L vessel. This procedure made the detector much more reliable and the baseline more stable. Presumably, recycling causes oxidation of the impurities present in the eluent and thus actually decreases the background over time (private communication, ESA, Inc., Bedford, MA). Upon installing the fresh eluent, we saw a steady decrease and stabilization of the background over a period of several days. We changed the eluent for a fresh solution after 2 months of use.

Mass spectrometry was carried out as described previously for potato alkaloids (Evans et al., 1993; Friedman et al., 1993).

Methods. Fresh tomatoes were cubed and immediately frozen in liquid nitrogen. Samples were then lyophilized. Samples were weighed before and after lyophilization for moisture determination. The dried tomatoes were then ground in an Omnimixer (Ivan Sorvall Inc., Newtown, CT) so they passed through a 0.5 mm screen.

Tomatoes were extracted by stirring 1 g in 20 mL of 1% acetic acid for 2 h. An aqueous system was chosen to avoid formation of a gel by precipitation of pectic substances present in the extract. The suspension was then centrifuged for 10 min at 13 300 relative centrifugal force (RCF) and the supernatant filtered through a Whatman GF/C filter. The pellet was resuspended in 10 mL of 1% acetic acid, centrifuged, and filtered, and the two extracts were combined. This extract was further purified using solid phase extraction (SPE). A C_{18} SPE tube, equipped with a 60 mL reservoir (Supelco), was conditioned with 5 mL of methanol followed by 5 mL of water. The aqueous extract (now about 30 mL) was applied and allowed to gravity drip. When the sample was fully absorbed onto the packing, the tube was washed with about 10 mL of water, followed by 5 mL of 30:70 acetonitrile–1% NH_4OH, and then 5 mL of water. The alkaloids were eluted with 10 mL of 70:30 acetonitrile–pH 3 citric acid/disodium phosphate buffer (as used in the eluent). The organic solvent was then evaporated off. The aqueous residue was basified with ammonia water extracted twice into water-saturated butanol, using a separatory funnel. This sample was then dried on a rotovapor. The residue was taken up to 1 mL of 50% methanol–0.1% acetic acid and filtered through a 0.45 μm HV membrane obtained from Millipore (Bedford, MA). This filtrate was ready for HPLC injection. The combination of liquid–liquid extraction and solid phase extraction was necessary to obtain a chromatogram that was free of interferences.

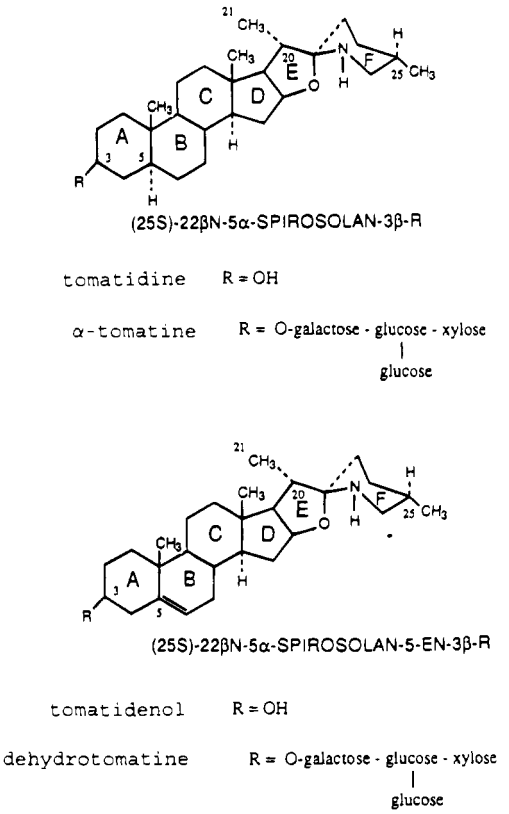

Figure 1. Structures of tomato alkaloids measured by HPLC.

Because the red field-grown tomatoes contained significant peaks other than α-tomatine, an additional extraction method employing precipitation was applied to these samples. Tomato powder (50 g) was extracted into 500 mL of 1% acetic acid for 2 h. The sample was centrifuged at 13 200 RCF for 15 min. The supernatant was filtered through Whatman (England) No. 50 filter paper. The pellet was resuspended in 250 mL of 1% acetic acid. The sample was centrifuged and filtered as above. The supernatants were combined and extracted with 200 mL of ethyl acetate. The acid layer was collected, basified with ammonium hydroxide, and twice extracted into 200 mL of water-saturated butanol. The butanol fraction was taken to dryness on a rotovapor and then taken up to 20 mL of 1% acetic acid and filtered through a 0.45 μm membrane. The filtrate was basified with ammonium hydroxide and heated for 30 min at 70 °C. This was left to cool overnight at 40 °C. The sample was centrifuged at 13 200 RCF for 15 min and the supernatant discarded. The sample was again taken up to 20 mL of 1% acetic acid and the precipitation step repeated. The pellet was then taken up in 30 mL of 50% methanol–0.1% acetic acid for HPLC.

Dehydrated tomato samples, 1 g of standard tomatoes, were spiked with 0.1 and 0.5 mg of commercial α-tomatine in duplicate. These samples were extracted as usual to determine percent recovery. To determine repeatability, cherry tomato powder was extracted in duplicate on three separate occasions.

RESULTS AND DISCUSSION

α-Tomatine Contents of Store-Bought and Field-Grown Tomatoes. Figure 1 shows the structures of the alkaloids evaluated in this study. Figure 2 depicts HPLC chromatograms for commercially available α-tomatine using (a) UV detection and (b) PAD detection. Figure 3 shows the PAD detector's linear relationship between the concentration of α-tomatine in the range

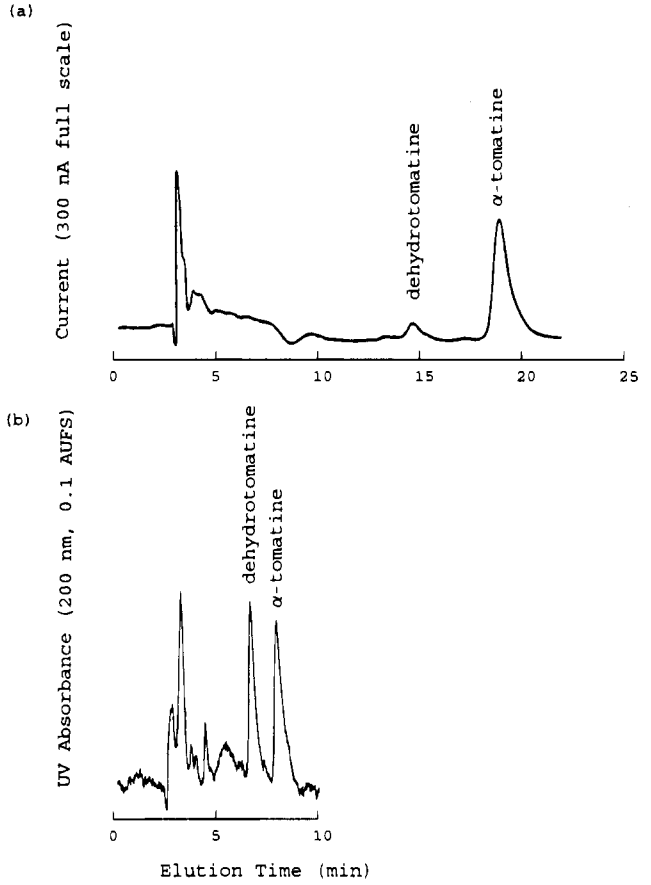

Figure 2. HPLC chromatogram of commercial α-tomatine by (a) PAD detection (2 μg) and (b) UV detection (40 μg). Column for (a) and (b): Supelcosil LC-ABZ, 5 μm, 4.6 × 250 mm. Flow rate: 1 mL/min. Eluents: (a) 20% acetonitrile, 15% methanol, citric acid/disodium phosphate buffer, pH 3; (b) 25% acetonitrile, 15% methanol, 100 mM sodium phosphate (monobasic), pH 3.

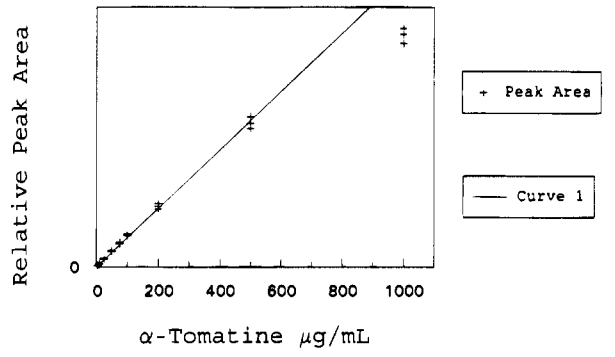

Figure 3. α-Tomatine peak area responses.

10−500 μg/mL and the peak area. Figure 4 illustrates the separation of glycoalkaloids in control and transgenic tomato extracts. Figure 5 depicts the mass spectra for α-tomatine and dehydrotomatine. Figure 6 is an HPLC chromatogram of a mixture of the commercial aglycons: solanidine, solasodine, and tomatidine. Tables 1 and 2 list the α-tomatine content of dehydrated and fresh tomatoes of field-grown nontransgenic and transgenic and store-bought tomatoes, respectively.

The two purification techniques (precipitation and SPE/liquid extraction) produced similar chromatograms except precipitation yields were significantly reduced. Since the chromatograms produced the same peaks with

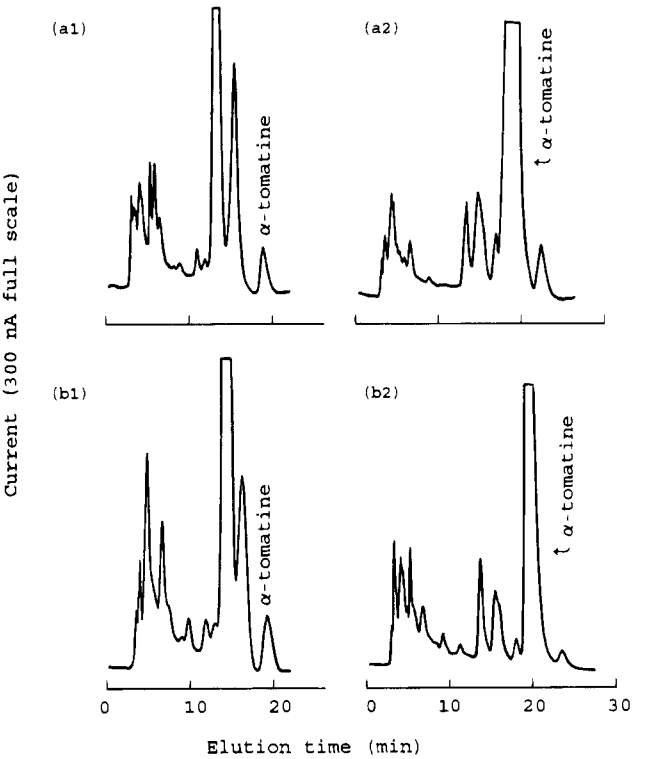

Figure 4. HPLC chromatograms of α-tomatine in field-grown (a) control tomatoes and (b) transgenic tomatoes for (1) ripe red tomatoes and (2) unripe green tomatoes. Conditions were the same as in Figure 2b. Unlabeled peaks are unknown compound.

the same ratios (except the solvent front), the peaks preceding α-tomatine may not be unrelated impurities. They merit further study. It is interesting that most of the peaks were common to both the red and green tomatoes but present in different ratios; some increased upon ripening, while others decreased. These major unknown peaks appear to be the same in the control and transgenic samples of the same ripeness.

This study shows that despite its producing cleaner chromatograms, cleanup by precipitation should not be used to purify α-tomatine due to poor recovery. This is probably due to the greater solubility of α-tomatine compared to the potato glycoalkaloids, α-solanine and α-chaconine, both of which are usually precipitated during purification. The SPE cleanup procedure produced chromatograms that were free of interferences in the region of interest, despite occasionally large solvent fronts.

Our results show that (a) use of the PAD detector allowed direct measurement of the α-tomatine peak; (b) spiking experiments of tomato extracts with authentic α-tomatine gave recoveries from 97 to 107% of added alkaloids; (c) the α-tomatine peak produced a linear response between 125 and 12 500 ng, with linearity dropping off at greater levels (Figure 3); (d) the α-tomatine content for green tomatoes ranged from 4 to 17 mg/100 g of fresh weight and for red tomatoes from 0.03 to 0.6 mg/100 g of fresh weight; and (e) the α-tomatine contents of transgenic and nontransgenic red tomatoes were the same. The corresponding green tomatoes did, however, differ in tomatine content, with the transgenic variety having the lower level. This difference may have been caused by environmental stress or slight differences in maturity between the samples, factors that can greatly affect tomatine levels.

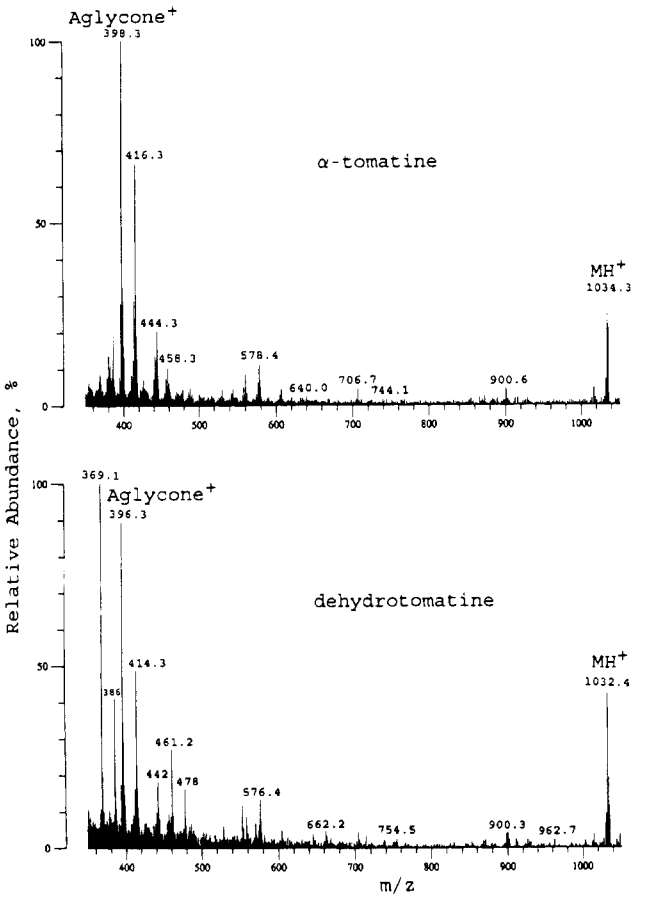

Figure 5. Positive ion liquid secondary ion mass spectrometry (LSIMS) of α-tomatine (upper plot) and dehydrotomatine (lower plot).

Table 1. α-Tomatine Content of Field-Grown Transgenic (Manteca) and Nontransgenic (Control) Tomatoes

	duplicate tomatine determinations, mg of α-tomatine/100 g of tomato			
	dry wt		fresh wt	
tomato sample	A	B	A	B
Manteca and control, ripe and unripe				
Manteca[a] green, unripe	97	98	5.8	5.8
control[b] green, unripe	225	223	16.7	16.6
Manteca red, ripe	6.3	6.4	0.38	0.38
control red, ripe	5.8	6.2	0.39	0.42
nontransgenic at various stages of ripeness[c]				
immature green	110	117	6.7	7.1
mature green	77	75	4.5	4.4
breaker	28	31	1.6	1.7
turning	13	11	0.67	0.59
pink	4.7	4.8	0.25	0.25
light red	6.9	7.0	0.38	0.39
very red	3.1	2.9	0.16	0.15

[a] Manteca is the transgenic variety. [b] Control tomatoes are the same variety, before genetic manipulation, grown under the same conditions as the Manteca. [c] The tomatoes in this grouping are listed in order of increasing ripeness (Kramer et al., 1992).

It is striking that, whereas postharvest greening of fresh potatoes results in up to a 5-fold increase in glycoalkaloid content (Dale et al., 1993; Kaaber, 1993), the difference for the lowest value (0.03 mg/100 g) found for red tomatoes and the highest value (17 mg/100 g) found for green tomatoes (not of the same variety) was more than 500-fold. Although the specific mechanisms for the changes in potatoes are not well-understood (Dao and Friedman, 1994), the changes in tomatoes appear to be due to enzymatic degradation of α-tomatine during ripening of tomatoes (Heftmann and Schwimmer, 1972, 1973).

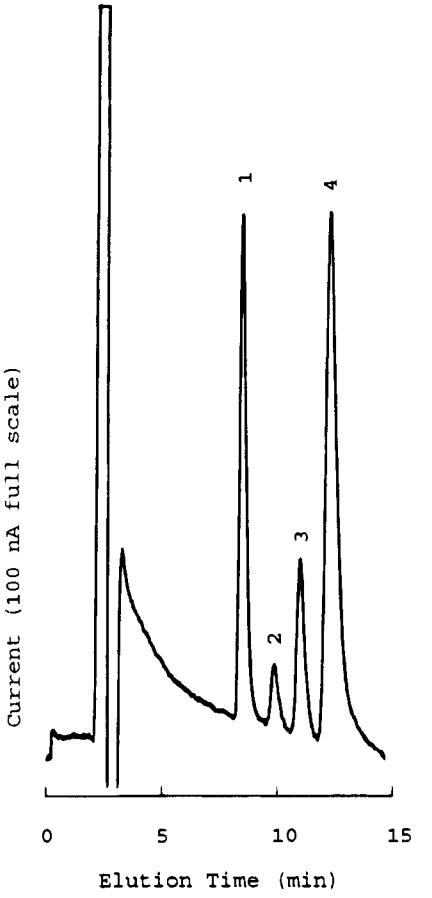

Figure 6. HPLC chromatogram of a mixture of commercial solanidine, solasodine, and tomatidine, 125 ng each. See text for chromatographic conditions. Peak 1 is solanidine, peak 2 is the first of the two peaks present in tomatidine (tomatidenol), peak 3 is solasodine, and peak 4 is the second of the two peaks present in tomatidine (pure tomatidine).

Table 2. α-Tomatine Content of Store-Bought Tomatoes[a]

	duplicate tomatine determinations, mg of α-tomatine/100 g of tomato			
	dry wt		fresh wt	
tomato sample	A	B	A	B
beefsteak	1.6	1.4	0.09	0.08
roma	0.7	0.6	0.04	0.03
standard tomato	0.4	0.4	0.03	0.03
cherry, first run[b]	4.0	4.0	0.27	0.27
cherry, second run[b]	4.2	4.4	0.28	0.29
cherry, third run[b]	4.2	3.9	0.28	0.26

[a] Store-bought tomatoes were purchased at a local supermarket. [b] The cherry sample was run in duplicate on three separate occasions to determine method reproducibility.

Dehydrotomatine. Chromatography of commercial α-tomatine revealed two peaks, the smaller being about 10% the size of the larger one (Figure 2a). The elution time of this smaller peak did not coincide with any of our standards such as α-, β-, or γ-solanines or -chaconines, α-tomatine, or solasonine. The two peaks in commercial α-tomatine were separated and collected off the HPLC column. Mass spectrometry results of the collected HPLC samples are consistent with the assignment of the larger peak to α-tomatine (Figure 5). Mass spectral analysis of the smaller HPLC peak shows a

mass spectrum peak with a molecular mass of 1032.2 Da, which is 2 Da less than the molecular mass of α-tomatine. This result suggests that the new glycoalkaloid may be similar in structure to α-tomatine, except that it has a double bond, possibly in the 5,6-position of ring B, in analogy with potato glycoalkaloids α-chaconine and α-solanine.

While applying chromatography previously described for potato glycoalkaloids (Friedman and Levin, 1992), we observed that the UV absorbance at 200 nm of the dehydrotomatine peak is about 10 times greater than that of the α-tomatine peak (Figure 2b). The greater absorbance, presumably due to the presence of a UV-absorbing double bond, is also consistent with the proposed structure for dehydrotomatine. Note that the tentative structure has the same carbohydrate residues as α-tomatine attached to the known aglycon, tomatidenol.

Chromatography of commercial tomatidine, the aglycon of α-tomatine, revealed a similar small peak eluting before tomatidine with area about 10% of that of the tomatidine peak (Figure 6). Collection and subsequent mass spectrometry of this peak suggest that it is likely tomatidenol. The label on commercial tomatidine states that it contains approximately 10% solasodine. Solasodine and tomatidenol elute adjacent to each other and could coelute in a less efficient chromatography system. Also, they have the same molecular weight and similar mass spectra. We found no peaks in commercial tomatidine coeluting with the commercial solasodine peak. A tomatidenol sample was found to coelute with our unknown peak and produce matched mass spectra. We, therefore, believe that tomatidenol is probably the aglycon of the dehydrotomatine present in commercial α-tomatine. However, more definitive studies are needed on the nature of the carbohydrates and the structure of the aglycon.

We were unable to confirm the presence of dehydrotomatine in extracts of tomato fruits. There were large peaks eluting close to the elution position of dehydrotomatine, which would have obscured a small dehydrotomatine peak. HPLC with UV detection showed a small peak with the retention time of dehydrotomatine present in some of the tomato extracts. Further work needs to be done to confirm this as dehydrotomatine, as well as to determine the nature of the other unknown peaks.

Conclusions. The conditions developed in this study for tomato alkaloids are an improvement over previous methods in terms of sensitivity, simplicity, and efficiency. The PAD is considered to be more selective and sensitive than refractive index detectors (Snyder and Kirkland, 1979; Kissinger, 1977, 1983). The extraction procedure avoids both the precipitation step (responsible for significant losses when using small samples) and hydrolysis to the aglycon tomatidine, thus simplifying the procedure and reducing possible errors. Extraction with acetic acid has additional benefits because reducing organic solvent use improves safety and lowers operating expenses. The practice of recycling HPLC eluent also keeps organic solvent use to a minimum.

Our studies also revealed the presence of a second tomato glycoalkaloid in commercial α-tomatine, tentatively named dehydrotomatine. This glycoalkaloid's structure is being further elucidated.

Finally, the applicability of the HPLC-PAD methods to the analysis of tomatine and dehydrotomatine metabolites and biosynthetic intermediates, in analogy with previously described measurements of potato glycoalkaloid hydrolysates (Friedman and Levin, 1992; Friedman et al., 1993), also merits exploration.

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