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Dose Formulation and Analysis of Diapocynin

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Procedures based on high-performance liquid chromatography (HPLC) with ultraviolet (UV) detection and liquid chromatography−mass spectrometry (LC−MS) are described for analyzing diapocynin. Diapocynin was synthesized by oxidative coupling of two apocynin monomers, through the *in situ* generation of sulfate radicals. It was purified by washing 3 times each with boiling water, followed by boiling methanol. HPLC was used to determine the concentration of unreacted apocynin and other impurities and the purity of the diapocynin that had been synthesized. Negative-ion, atmospheric pressure chemical ionization (APCI) LC−MS was used to determine the molecular weights of impurities. The method using HPLC with UV detection provided a calibration curve that was linear from 0.16 to 24 μg/mL. The LC−MS method was linear from 0.005 to 2 μg/mL. It was found that diapocynin has low solubility in deionized water and corn oil but is soluble in dimethylsulfoxide (DMSO) and alkaline aqueous solutions. Also, diapocynin is 13 times more lipophilic than apocynin, even though both compounds have the same pK_a of 7.4. The log of the octanol/water partition coefficient (log *P*) was 1.01 for apocynin and 1.82 for diapocynin. A solution of 5.5 mg/mL (16.7 mM) diapocynin in DMSO was found to be stable for at least 30 days when stored at room temperature.

KEYWORDS: Diapocynin; triapocynin; HPLC; LC−MS

INTRODUCTION

Diapocynin is a metabolite of apocynin, which has antioxidative and anti-inflammatory properties (*1*–*6*). Apocynin (4-hydroxy-3-methoxyacetophenone) was identified during activity-guided isolation of immunomodulatory constituents from *Picrorhiza kurroa*, a native plant grown in the mountains of India, Nepal, Tibet, and Pakistan. This compound specifically blocks the activity of NADPH oxidase, a cell-membrane enzyme known to protect against reactive oxygen species (ROS) (*7*). It has also been suggested that apocynin is metabolized to diapocynin, which may be more active than apocynin (*6*).

The synthesis of diapocynin was described in a dissertation that was published on the Internet (*8*). It was based on the synthesis of a similar compound, dehydrodivanillin (*9*), except that it started with apocynin, used the potassium salt of persulfate instead of the sodium persulfate, and reported a lower yield (*8*). Persulfate is a powerful oxidizing agent, with a standard oxidation–reduction potential of 2.1 V, compared to 1.8 V for hydrogen peroxide.

$$S_2O_8^{2-} + 2H^+ + 2e^- \rightarrow 2HSO_4^- \quad (1)$$

Sodium persulfate can also be converted to a sulfate radical, $SO_4\bullet^-$, with a standard oxidation–reduction potential of 2.6 V (*10*). Ferrous sulfate is a common initiator.

$$S_2O_8^{2-} + \text{initiator} \rightarrow SO_4\bullet^- + (SO_4\bullet^- + SO_4^{2-}) \quad (2)$$

However, it has been reported that this often results in the cleavage of side chains to form compounds with lower molecular weight (*11*), not coupling to form a dimer.

In the original paper, the sulfate radical was the reactive species in the oxidative coupling of two vanillin molecules through the carbon that was *ortho* to the phenolic hydroxyl of each vanillin and it was converted to dehydrodivanillin (*9*). In the dissertation (*8*), apocynin was used. It was soluble in hot water, but diapocynin was not. When diapocynin was produced, it precipitated out of solution. However, some iron, persulfate, and sulfate were probably coprecipitated and were present as contaminants. The precipitate was redissolved in aqueous NH_4OH and reprecipitated by adding 6 N HCl, to remove iron, persulfate, and sulfate contaminants (*8*). The structures of apocynin and diapocynin are in **Figure 1**.

Diapocynin has also been synthesized in smaller quantities using the enzymes horseradish peroxidase and myeloperoxidase

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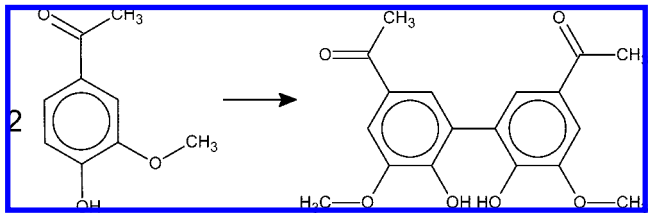

Figure 1. Conversion of apocynin to diapocynin. This is an oxidation–reduction reaction, requiring the *in situ* generation of sulfate radicals, which remove one hydrogen from each molecule of apocynin, producing diapocynin.

(*12, 13*). Diapocynin was separated from apocynin using isocratic, reverse-phase high-performance liquid chromatography (HPLC) (*14*). The mobile phase was strong enough to cause diapocynin to elute before apocynin (*14*). Still, diapocynin is not readily available from commercial sources, and no methods have been reported for its analysis.

In preparation for possible cell culture or animal studies, the objectives of this study were to develop methods based on reverse-phase HPLC with ultraviolet (UV) detection and liquid chromatography−mass spectrometry (LC−MS) to determine the purity of diapocynin, to establish ways to optimize the synthesis and purification procedures, and to measure the log *P* and pK_a of apocynin and diapocynin. These methods were used to analyze diapocynin in different stages of its synthesis and purification, to measure its solubility in octanol and water, and to determine the suitability of dimethylsulfoxide (DMSO) as a vehicle or solvent for preparing doses of diapocynin.

MATERIALS AND METHODS

HPLC-grade methanol was from Burdick and Jackson (Muskegon, MI). All other chemicals were from Aldrich-Sigma (St. Louis, MO). Apocynin (acetovallinone) was recrystallized from water and dried in a desiccator before use. Diapocynin was synthesized by dissolving 2 g of recrystallized apocynin in 200 mL of deionized water with stirring and heating until the solution was boiling gently. To this was added 0.15 g of ferrous sulfate heptahydrate and 1.6 g of sodium persulfate. A brown precipitate formed. After 5 min, the solution was cooled and filtered. The precipitate was dissolved in 3 N NH_4OH and then reprecipitated by adding 6 N HCl. The precipitate was filtered and washed 3 times with 100 mL of boiling water. The diapocynin precipitate was further purified by washing 3 times with 100 mL of boiling methanol. The water and methanol washings were analyzed for impurities by HPLC and LC−MS. The purified product was dried in a desiccator before being used in measurements of solubility, log *P*, pK_a, and stability in DMSO.

^{1}H nuclear magnetic resonance (NMR) and ^{1}H decoupled ^{13}C NMR spectra of diapocynin in DMSO-d_6 were obtained using a Bruker ARX 500 MHz NMR. A 30° pulse width was used for the ^{1}H NMR, with a 1 s pulse delay. A 30° pulse width was used for the ^{13}C NMR spectra, with a 2 s pulse delay. The hydrogen and carbon chemical shifts were referenced to the DMSO peaks, which were set to 2.50 ppm for hydrogen and 39.50 ppm for carbon, respectively. The attached proton test (APT) was used to distinguish between two groups of signals, methyl/methine and methylene/quaternary.

The IR spectrum was obtained using a Varian 800 Fourier transform infrared spectroscopy (FTIR, Palo Alto, CA) with an attenuated total reflectance (ATR) accessory, from PIKE Technologies (Madison, WI).

Reverse-phase HPLC was performed using a Shimadzu LC-10 HPLC (Kyoto, Japan), equipped with an autosampler and a LC-10 AV UV–Vis detector, set at 276 nm. An Alltech Platinum EPS C_{18} column, with a 5 μm particle diameter and 100 Å pore size, was used with an isocratic mobile phase consisting of 48:52:0.31 methanol/water/ammonium acetate (v/v), flowing at 1.0 mL/min. The injection volume was 20 μL. For quantitative analysis, diapocynin standards (0.16–25 μg/mL or 1.8–76 μM) were prepared in 1 mM NaOH. Diapocynin standards were prepared as described (*12*). Data were fit to a straight line by linear regression analysis using Microsoft Excel.

Negative-ion, atmospheric pressure chemical ionization (APCI) LC−MS analysis was performed using a Finnigan MAT LCQ Duo ion trap (West Palm Beach, FL), equipped with a Spectra System P4000 pump, UV6000 LP UV diode array detector, and AP3000 auto sampler. A C_{18} Ace analytical column from MacMod Analytical, Inc. (Chadds Ford, PA), 10 cm × 2.1 mm i.d. and 100 Å pore size, was used for separation. To determine the molecular weight of impurities, such as triapocynin, a full-scan mass spectrum over a mass range of 100–750 amu was obtained and an isocratic mobile phase of 35:65:0.38 methanol/water/ammonium acetate (v/v/w) at pH 7 was used. For the determination of the concentration of unreacted apocynin, a gradient elution was used. Solvent A was 480:20:0.38 methanol/water/ammonium acetate (v/v/w) at pH 7, and solvent B was 20:480:0.38 H_2O/CH_3OH/ammonium acetate (v/v/w) at pH 7. The mobile phase started with 100% A for the first min, followed by a linear increase to 100% B from 1 to 16 min. This was followed by 100% B from 16 to 31 min and then a linear decrease to 100% A from 31 to 40 min. The injection volume was 20 μL, and the eluent flow rate was 0.25 mL/min. The analysis used selected ion monitoring (SIM) of ions with *m*/*z* 163.5–166.5 and 327.5–330.5, corresponding to the $[M - H]^-$ ions produced by apocynin and diapocynin, respectively. Apocynin standards (0.05–8 μg/mL or 0.3–48 μM) were prepared in 1 mM NaOH. Data were fit to a straight line by linear regression analysis using Microsoft Excel.

The pK_a of apocynin was determined by titrating it with an aqueous solution with NaOH and observing the pH and midpoint. An Orion 520A pH meter was used to measure the pH. The pK_a of diapocynin was determined by measuring the apparent log *P* at different pH values (2.0, 7.4, and 7.9). The diapocynin was at least 99% pure, on the basis of the HPLC and LC−MS analyses. That is, diapocynin accounted for at least 99% of the total UV peak area by HPLC, and there was less than 1% unreacted apocynin, as determined by LC−MS analysis. The log *P* was determined by the shake flask method (*15*). Diapocynin in the aqueous phase was determined using the HPLC method with UV detection. The concentration of diapocynin in the octanol phase was determined by extracting the octanol with 0.1 N NaOH, followed by dilution with deionized water and analysis by HPLC. Five different measurements of log *P* were made at each pH; therefore, results could be reported as an average ± standard deviation.

The concentrations of diapocynin in aqueous suspensions in 10 mM phosphate buffer at pH 7.0–8.5 were determined by filtering mixtures of excess diapocynin and buffer that had been sonicated 10 min and diluting it into 10 mM NaOH until the diapocynin concentration was within the range of concentrations of standards used in the calibration curve (1.8–92 μM).

Solutions of diapocynin in DMSO were prepared by sonicating mixtures of diapocynin and solvent for 10 min. The DMSO solutions did not need filtering. DMSO solutions were diluted $^1/_{10}$ in 4 mM NaOH, followed by another $^1/_{50}$ dilution in water.

The diluted samples were analyzed by HPLC with UV detection. The concentrations of diapocynin in samples that had been stored for 30 days in closed vials at room temperature were determined by analyzing them by HPLC, using freshly prepared diapocynin standards.

RESULTS AND DISCUSSION

The ^{1}H NMR spectrum of diapocynin (*16*) contained peaks with the following chemical shifts, in ppm, and assignments, in parentheses: 2.494 (−CH_3), 3.895 (−OCH_3), 7.451 and 7.463 (aromatic CH), and 9.465 (−OH). The ^{13}C NMR spectrum contained peaks with the following chemical shifts, in ppm, and assignments by comparing with the APT spectrum, in parentheses: 26.25 (−CH_3), 55.98 (−OCH_3), 109.66 (aromatic C-2, CH), 124.46 (C-5, C), 125.25 (C-6, CH), 127.86 (C-1, C), 147.44 (C-3, C), 149.11 (C-4, C), and 196.15 (C═O). The FTIR spectrum contained the following peaks, λ_{max} cm^{-1}: 3318 (OH), 1666 (C═O), 1588 (aryl C═C), 1286, 1204, 1127, 1083, and 910.

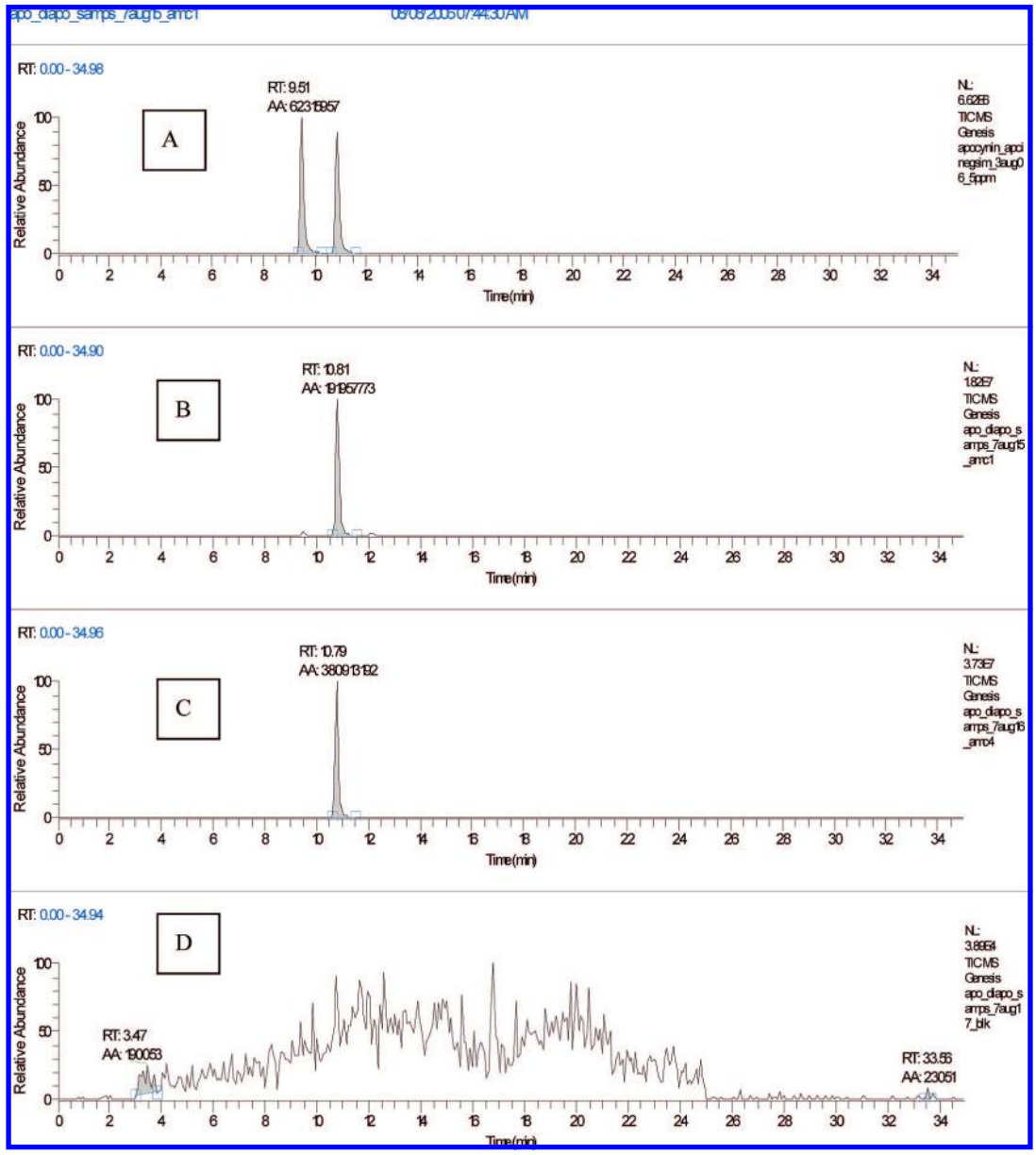

Figure 2. LC−MS analysis of diapocynin, showing the removal of apocynin by washing 3 times with boiling water. (**A**) Mixture of 0.5 μg/mL apocynin and 0.5 μg/mL diapocynin standards, (**B**) sample before washing with water, (**C**) sample after washing, and (**D**) water blank.

When the Platinum EPS C_{18} column was used with 48% methanol in the HPLC analysis, diapocynin eluted before apocynin and the analysis time was 4 min. This might be unexpected, because diapocynin is more hydrophobic than apocynin. This was apparent in the synthesis of diapocynin. Apocynin was soluble in boiling water, but diapocynin was not. To measure hydrophobicity or lipophilicity, the octanol–water partition coefficients of apocynin and diapocynin were measured. Apocynin is more soluble than diapocynin in both octanol and water at pH 2, and its octanol–water partition coefficient was 10.3 ± 1.5 ($\log P = 1.01 \pm 0.06$). However, the solubility of diapocynin in octanol−saturated water at pH 2 was much lower (0.24 μg/mL or 3.4 μM) than the its solubility in octanol−saturated water, and its octanol–water partition coefficient was 66 ± 13 ($\log P = 1.82 \pm 0.08$, $n = 5$). The apparent log P of diapocynin increased as the pH of the octanol−saturated water was increased. The pK_a of diapocynin was found to be 7.4, which is also the pK_a of apocynin, which was obtained by titrating an aqueous solution of apocynin. The octanol–water partition coefficient at pH 7.4 was 0.924 ± 0.019 ($\log P = -0.034 \pm 0.009$, $n = 5$). The log P is used to predict the bioavailability of a compound, its ability to pass through a cell membrane, permeate the skin, partition between soil and water, and persist in the environment (*17*). The standard method for measuring log P specifies that the pH of the aqueous phase should be adjusted so that the compound being tested is nonionic (*15*). For this reason, log P was measured first at pH 2. However, physiological pH is close to 7.4; therefore, the apparent log P at pH 7.4 might be a better indication of the bioavailability of any dose of diapocynin that might reach the blood or other organs, except the stomach, which is below pH 2. Thus, estimates of the bioavailability of diapocynin should depend upon the route of administration. Also, even though diapocynin has a higher log P than apocynin, it is not as soluble in either *n*-octanol or water. The ratio of its solubility in these solvents is higher than the ratio for apocynin, but its absolute solubility is less.

The HPLC method produced a linear calibration curve ($r^2 = 0.9999$) for diapocynin at concentrations from 0.16–25 μg/mL or 1.8–76 μM. It was used to analyze diapocynin produced after

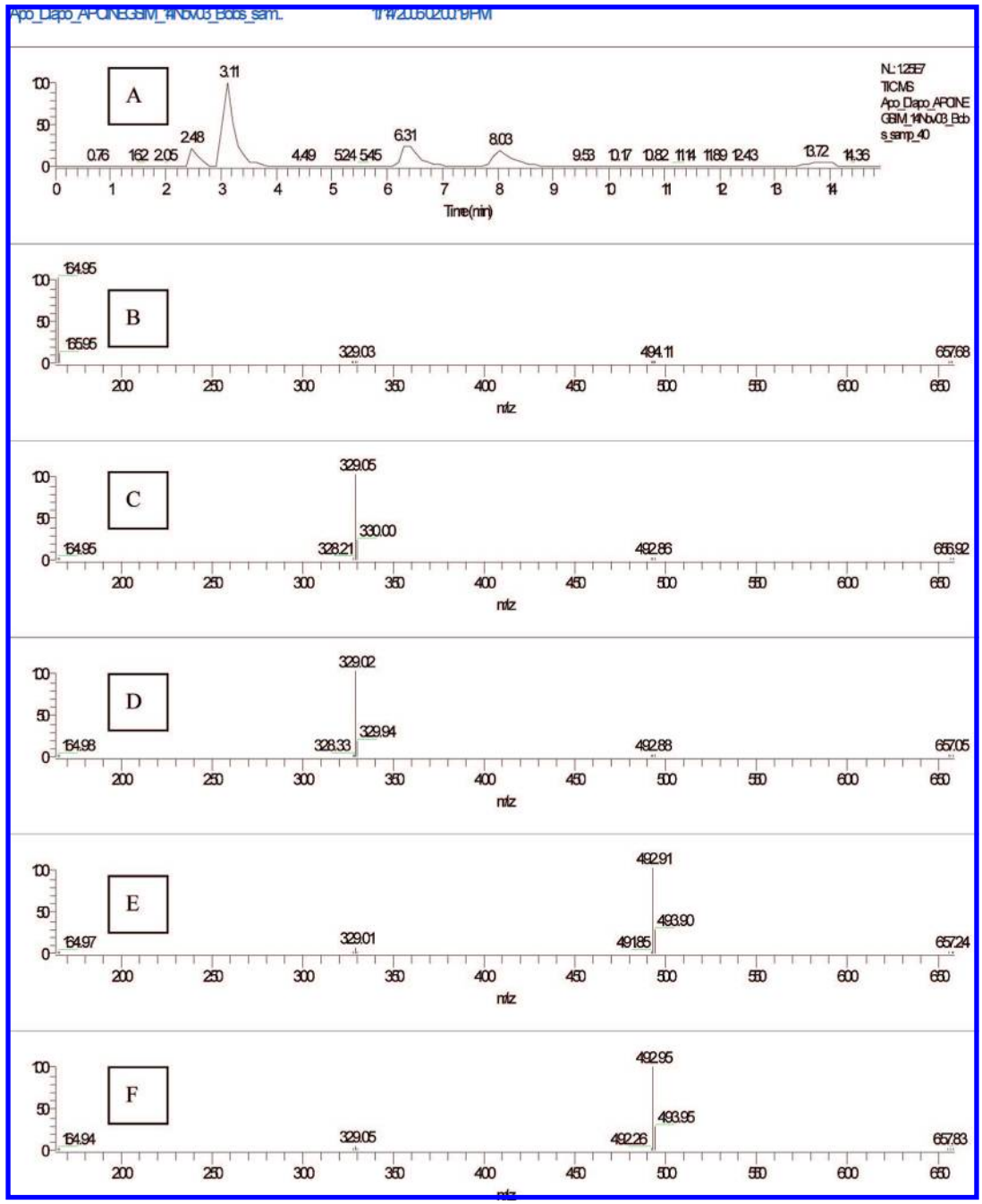

Figure 3. LC−MS analysis of methanol washing of diapocynin. (**A**) chromatogram, using full-scan mass spectral detection. (**B**) *m*/*z* 164.95 negative ion produced by apocynin, eluting at 2.48 min. (**C**) *m*/*z* 329.05 negative ion produced by diapocynin, eluting at 3.11 min. (**D**) *m*/*z* 329.02 negative ion produced by an isomer of diapocynin, eluting at 6.31 min. (**E**) *m*/*z* 492.91 negative ion produced by an isomer of triapocynin, eluting at 8.03 min. (**F**) *m*/*z* 492.95 negative ion produced by another isomer of triapocynin, eluting at 13.7 min.

different reaction times. It was found that only 5 min at 100 °C was needed for the reaction. More triapocynin impurity was produced when the reaction proceeded for 30 min, which was the reaction time used by previous workers (*8, 9*). Most of the small amount of unreacted apocynin was removed by thoroughly washing the diapocynin with boiling water 3 times, as shown in **Figure 2**. However, there were two small peaks that eluted after the apocynin in the chromatogram of the water-washed diapocynin that had reacted for 30 min.

To determine the molecular weight of these two small peaks, LC−MS was used with isocratic elution. The first methanol washing of a sample that reacted for 30 min was diluted 1:1 with water and analyzed by LC−MS, and the results are in **Figure 3**. With the Ace column and 35% methanol, apocynin eluted before diapocynin and three other peaks eluted at 6.31, 8.03, and 13.72 min. The *m*/*z* values of these peaks were 329.0, 492.9, and 492.9, respectively. These were due to $[M-1]^-$ ions; therefore, the molecular weights of these impurities were 330 and 494, corresponding to the expected molecular weights of an isomer of diapocynin and two isomers of triapocynin. The coupling of two apocynin molecules with a molecular weight of 166 occurred through the loss of two hydrogens; therefore, the molecular weight of diapocynin was 330. Similarly, the coupling of an apocynin with a diapocynin to produce triapocynin also happened with the loss of two hydrogens; therefore, the expected molecular weight was 494.

Almost all of the rest of the apocynin and most of the triapocynin was removed by washing with boiling methanol.

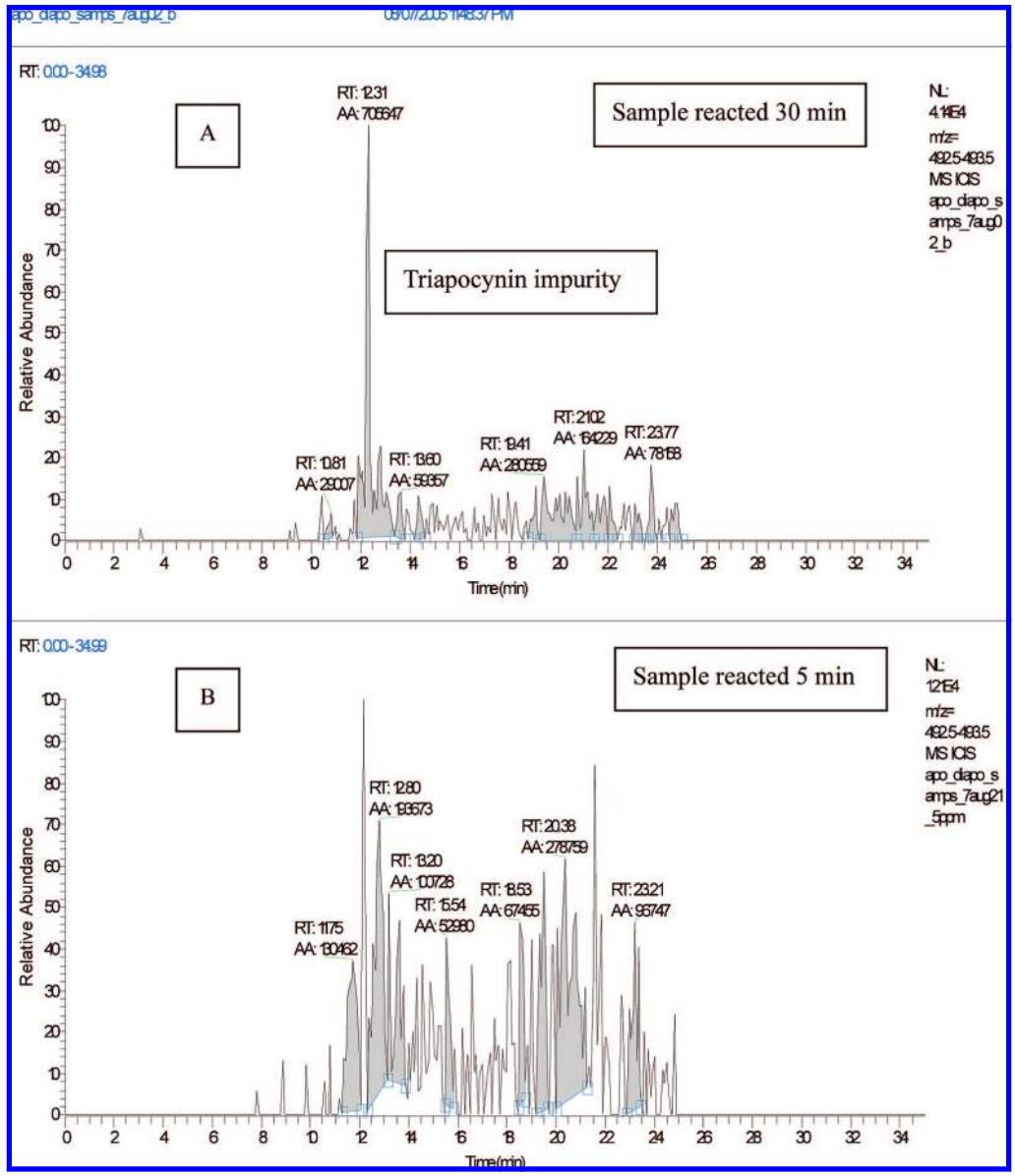

Figure 4. Triapocynin was present in samples reacted for 30 min (**A**) but not in samples reacted for 5 min and washed with boiling methanol (**B**). Only ions with *m*/*z* 492.5–493.5, because of the triapocynin $[M-1]^-$ ion, are shown.

LC−MS chromatograms showing the removal of triapocynin by washing with methanol are in **Figure 4**. A gradient elution and SIM were used in this analysis. For quantitative analysis of apocynin impurity in diapocynin, the calibration curve was linear between 0.05 and 8 μg/mL for apocynin.

Diapocynin was not very soluble in water, but suspensions that passed through 1 μm filters could be made but with relatively poor reproducibility. The concentrations of supersaturated aqueous suspensions of the purified diapocynin in 10 mM phosphate depended upon pH. The amount of diapocynin suspended at pH 7.0 was 14 ± 5 μg/mL. At pH 7.5, it was 42 ± 15 μg/mL; at pH 8.0, it was 206 ± 29 μg/mL; and at pH 8.5, it was 240 ± 17 μg/mL ($n = 5$).

Diapocynin was quite soluble and stable in DMSO. A solution of 5.52 mg/mL (16.7 mM) diapocynin in DMSO was analyzed after 1, 10, and 30 days. The concentrations of diapocynin each day were 5.45 ± 0.09, 5.54 ± 0.13, and 5.44 ± 0.08 mg/mL. Thus, 5.52 mg/mL diapocynin in DMSO was stable for 30 days at room temperature.

The method for synthesizing diapocynin included some changes compared to the previously published methods (*8, 9*). The reaction was conducted in boiling water for 5 min, instead of the original 30 min in hot water (*8, 9*). The sodium salt of persulfate was used, instead of the potassium salt, because the potassium salt produced a lower yield (*8*). Also, the diapocynin was redissolved in 3 M NH_4OH, instead of NaOH, because NaOH is often contaminated with chloride and carbonate (*18*). Finally, this newer method of synthesis included washing the diapocynin 3 times each with boiling water and boiling methanol and analysis of the purified diapocynin by HPLC and LC−MS.

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