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Instability of St. John's Wort (*Hypericum perforatum* L.) and Degradation of Hyperforin in Aqueous Solutions and Functional Beverage

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Several bioactive botanicals including St. John's wort (SJW; *Hypericum perforatum* L.) have been used to formulate functional foods and beverages. This study aimed to investigate the stability of SJW components in aqueous solutions and fruit-flavored drinks. Changes of active marker components (hypericin, pseudohypericin, hyperforin, and adhyperforin) as affected by pH and light exposure were determined by HPLC, and the degradation of hyperforin was analyzed by LC-MS/MS and NMR. SJW components were found to be unstable in acidic aqueous solutions. More changes occurred under light exposure, with hyperforin and adhyperforin decreasing the most. Less severe changes were observed in the drink sample as compared to the pH 2.65 solution. Major degradation products of hyperforin in acidic aqueous solutions were identified as furohyperforin, furohyperforin hydroperoxide, and furohyperforin isomer a. The latter was also found in the drink product containing SJW as an ingredient. Biological activities and potential quality and safety implications of these chemical changes are yet to be evaluated.

KEYWORDS: ***Hypericum perforatum* L.; St. John's wort; fruit drink; stability; hyperforin degradation**

INTRODUCTION

St. John's wort (SJW; *Hypericum perforatum* L.) is one of the herbs most commonly used as a folk medicine or herbal remedy. Historically, SJW leaf/flower preparations were used to treat mental disorders and nerve pain and as a sedative and antimalarial agent (*1*). Products of SJW extracts have been used therapeutically for depression in Germany for a long time. Over the past two decades, SJW extract became popular in treating mild to moderate depression (*2*) and is considered as a drug in Germany, whereas it is a dietary supplement in the United States.

SJW contains many compounds with documented biological activities. The main classes are naphthodianthrones (hypericin and pseudohypericin and their precursors, protohypericin and protopseudohypericin, respectively), phloroglucinols (hyperforin and adhyperforin), flavonol glycosides, and biflavones (*3*, *4*). The structures of naphthodianthrones and phloroglucinols are shown in **Figure 1**.

Initially, hypericin was considered to be the major constituent responsible for the antidepressant activity of SJW (*5*), and many dietary supplement products have been standardized for the hypericin content. Recently, hyperforin has been thought to be the main constituent responsible for the antidepressant activity (*6*, *7*). However, several cases have been reported that SJW induced adverse effects and caused interactions with co-administered drugs (*2*, *8*, *9*), and hyperforin was potentially responsible for the SJW-drug interactions (*10*, *11*).

Several studies have shown the instability of hypericins and hyperforins in SJW preparations with regard to heat, air, and light (*12*−*14*). Under the influence of light, protoderivatives could change to their respective hypericins, and pseudohypericin

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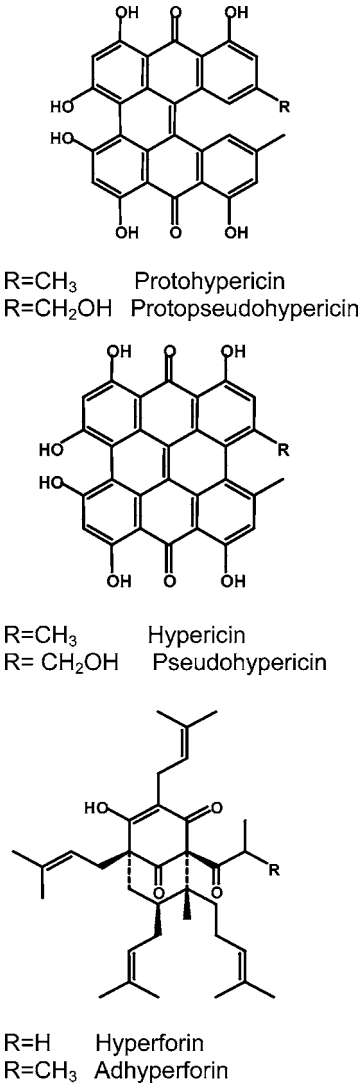

Figure 1. Chemical structures of major active components from St. John's wort.

could be oxidized to cyclopseudohypericin (*3*). The effects of solvent polarity, pH, and light exposure on the stability of hyperforins were also reported (*15*). A number of hyperforin oxidized products were identified, including 2-methyl-3-buten-2-ol (*3*), furohyperforins (1-methyl-1-hydroxyethyl group in the 6β-position) (*16*, *17*), oxepahyperforin, 33-deoxy-33-hydroperoxyfurohyperforin, 8-hydroxyhyperforin-8,1-hemiacetal (*18*), and isomers of furohyperforin (*19*).

In recent years, there has been a growing interest in food ingredients that may provide health benefits. A number of popular bioactive botanicals including SJW have been used as ingredients in conventional foods and sold as "functional foods". However, to our knowledge no information is available concerning the stability and safety of these ingredients in various functional foods. Among these products are nonalcoholic, noncarbonated beverages. The concentrations and the potential chemical changes of these "functional ingredients" are not known. Previously we found that some fruit drink products labeled to contain SJW actually retained extremely low levels of SJW components, if at all (*20*). Our hypothesis for the instability of SJW compounds in these drinks was due to, in part, their low pH and the light exposure and/or temperature during storage.

The objectives of this study were to determine the effects of pH and light exposure on the stability of selected, unique active components of SJW, that is, pseudohypericin, hypericin, hyperforin, and adhyperforin, in aqueous buffer solutions and nonalcoholic, noncarbonated, fruit-flavored beverages. Additional investigations were aimed to investigate the nature of chemical changes of hyperforin and its degradation products in these systems.

MATERIALS AND METHODS

Materials. Packages of SJW plant material composed of dry leaves and flowers were obtained from Frontier Natural Products Co-Op, Norway, IA. The mixture was ground using a coffee grinder (Braun type KSM2, Braun Inc., Woburn, MA), passed through a 20-mesh sieve, sealed in plastic bottles, and stored at −60 °C. These bottles were wrapped in aluminum foil to avoid any possible changes by photoactivity. Noncarbonated, nonalcoholic, fruit-flavored beverages were obtained from a local market. The labels on these bottles indicated that they contained SJW or SJW extract.

Chemicals. All chemicals and solvents were of high-performance liquid chromatographic (HPLC) or analytical grades. Reference standard, hypericin (>85%, LC purity), formic acid (97.8%), and triethylamine (99.0%) were from Sigma Chemical Co. (St. Louis, MO). Pseudohypericin (>79.45% LC purity) was from Chromadex (Santa Ana, CA). Hyperforin and adhyperforin were isolated from SJW leaf/flower mixtures in our laboratory and identified by mass spectrometry (MS) and nuclear magnetic resonance (NMR) methods (*21*, *22*). The purity was determined by LC-MS and LC−photodiode array (PDA). All other reagents were of HPLC grade (J. T. Baker, Phillipsburg, NJ). Water was distilled, deionized using a Milli-Q system from Millipore (Milford, MA).

Part 1: Stability Study. *Preparation of SJW Methanolic Extract.* The procedure of Ang et al. (*20*) was followed. Briefly, 1 g of finely ground SJW plant material was extracted with 40 mL of methanol (MeOH) in an ultrasonic bath (60 Hz, 125 W, Cole-Parmer Instrument Co., Chicago, IL) for 60 min. After centrifugation at 2000*g* for 10 min, the supernatant was transferred to a capped glass vial and stored at −20 °C until used. All operations involved with SJW or it components were carried out under dimmed yellow light unless otherwise indicated.

Preparation of SJW Solutions for pH and Light Exposure Tests. Three buffer solutions were prepared by adjusting the pH of water to 2.65, 4.5, and 6.1, respectively, with ammonium formate and formic acid. For the pH 4.5 solution, 250 mg of ammonium formate was first dissolved in 500 mL of water, and ~25 μL of formic acid was added under stirring while the pH value was monitored with a pH-meter (model 340, Corning Inc., Corning, NY). For the pH 6.1 solution, 460 mg of ammonium formate and ~1 μL of formic acid were used, and for the pH 2.65 solution, the amounts of ammonium formate and formic acid were approximately 1 mg and 500 μL, respectively. The ammonium formate system was selected for the preparation of buffer solutions because of its compatibility with MS.

For fortification of these solutions with SJW extract, 5 mL of the prepared SJW methanolic extract solution was added to each buffer solution to a total of 100 mL (i.e., 5% SJW extract in each buffer solution). The contents were mixed and distributed into 1-mL autosampler clear glass vials (0.5 × 3.9 cm; i.d. × height), which were then closed with polyethylene snap caps and stored at room temperature (22 °C) under normal laboratory light (fluorescent light and indirect sunlight) for 0, 4, 24, 48, and 144 h. Brown vials were used for storage in the dark, and they were placed inside an opaque laboratory cabinet. At designated times, duplicate or triplicate vials from each of the dark and light treatment groups were analyzed directly by HPLC.

Evaluation of SJW Stability in Fruit-Flavored Drink. Drink samples used were those labeled to contain SJW, but actually the amounts were not detectable by our analysis (<10 ng/mL of hyperforin, adhyperforin, or hypericin; <5 ng/mL of pseudohypericin). An aliquot of 200 mL was centrifuged at 3500*g* for 20 min, and the supernatant was fortified with 5% of SJW methanolic extract (5 mL of SJW extract solution in a 100-mL final volume). This fortified drink solution was distributed into 1-mL capped, autosampler vials and subjected to storage tests in the dark and under light as described previously for the buffer solutions. At specific time periods, duplicate vials from each treatment group

were analyzed directly by HPLC. The entire experiment was repeated using another bottle of the drink sample.

HPLC Analysis for Retention of SJW Components. The HPLC system consisted of a Waters 610 E pump with a 717 autosampler and a 996 PDA (Waters, Milford, MA). The instrument control and data processing were accomplished with Millennium M32 Chromatogram Manager software. A Luna C18 column (Phenomenex, Torrance, CA) with 3-μm particle size (150 × 4.6 mm) was used at ambient temperature (22 °C). Mobile phase A was acetonitrile (ACN), and mobile phase B was triethylammonium acetate buffer prepared by adding 1.5 mL of acetic acid and 3.5 mL of triethylamine to ~450 mL of water, adjusting the pH to 6.5 ± 0.1, and making up the volume to 500 mL (*20*). The flow rate was 1.0 mL/min using an isocratic program with 80% A and 20% B. Run time was 15 min. The injection volume was 20 μL, and photodiode array (PDA) data were collected from 190 to 650 nm. The major wavelengths monitored were 290, 544, and 590 nm.

For determination of the initial content of target compounds in the SJW extract, an aliquot of 0.90 mL of prepared extract was mixed with 0.10 mL of internal standard (luteolin, 1.60 mg/mL in MeOH) and analyzed by HPLC (*20*). For determination of the changes of target compounds in aqueous solutions and drinks, aliquots (20 μL) of the test solutions at designated storage times were injected directly onto the LC column. The peak areas of a specific peak (P_t) of the test solutions were compared with the peak area at the initial time (P_0), and the percentages of each remaining (% retention) phytochemical were calculated according to the following formula:

$$\% \text{ retention} = (P_t/P_0) \times 100$$

LC-MS Analysis for SJW Stability Study. For the LC-MS analysis, the SJW solutions were prepared at 10% concentration in pH 2.65 and 4.5 formic acid buffer solutions. Aliquots were stored for 4 h in the dark and under light exposure. Components were separated using an HP 1090 HPLC (Agilent, Palo Alto, CA) with a Luna C8 250 × 2 mm, 5 μm, column (Phenomenex). The mobile phase, delivered at a rate of 0.2 mL/min, was a 30-min linear gradient from 50 to 80% ACN with constant 3 mM ammonium formate, which was held for another 30 min. LC-MS analyses were performed on the HP 5989B mass spectrometer operated in negative ion electrospray mode with the capillary exit voltage at −100 V. Full scans were acquired from *m*/*z* 100 to 950 at 0.92 scans/s.

Part 2: Investigation of Hyperforin Degradation Products. *Assessing Hyperforin Degradation in Buffer Solutions and Fruit-Flavored Drink.* For evaluation of the degradation of hyperforin in a model aqueous solution, 250 mL of hyperforin solution at 0.5 mg/mL was prepared in formic acid buffer solution (pH 2.8) in a conical flask. It was then stored at room temperature (22 °C) in the dark for 2 days. For evaluation of hyperforin degradation in the drink product, one drink sample (same type as used in part 1) was fortified with a high level of hyperforin (0.2 mg/mL), stored for 2 days in the dark, and analyzed by HPLC.

To clean up the drink test solutions before the HPLC analysis, 10 mL of the liquid was loaded onto an SPE column (Waters Sep-Pak cartridge, 3 cm^3, C18), washed with 30% MeOH, and eluted with 2 mL of 80% MeOH, followed by 2 mL of 100% MeOH. Both of the 80 and 100% MeOH fractions were tested for the presence of hyperforin degradation products by HPLC. The 100% MeOH fraction tested positive and was subsequently analyzed by LC-MS/MS.

Isolation of Major Hyperforin Degradation Products from Buffer Solution. Approximately 60 mg of hyperforin was added into 250 mL of pH 2.8 formic acid buffer solution in a 500-mL conical flask to result in 240 μg/mL hyperforin in solution and then stored in the dark. After 4 days in the dark, the solution was transferred onto an ODS column (2 cm × 30 cm, i.d. × height) containing 50 g of octadecyl-functionalized silica gel, 40−63 μm, 60 Å (Supelco, Sigma-Aldrich, St. Louis, MO), washed with 250 mL of water, and eluted successively with 150 mL of each of 80, 90, 95, and 100% MeOH. The 95% MeOH fraction was subjected to normal silica gel (63−200 μm, 40 Å, Supelco, Sigma-Aldrich) column separation (1.5 cm × 30 cm, i.d. × height) and eluted with 100 mL of each of hexane/ethyl acetate of various compositions (12:1, 8:1, 4:1, 2:1, v/v), successively. These fractions were tested for the presence of hyperforin and its analogues by HPLC-PDA method. The 8:1 eluted fraction was found to be positive, and it was used in the preparation of pure degradation products. For this purification purpose, a semipreparative Luna C18 column (30 × 9.0 mm, 5-μm particle size; Phenomenex) was used. The mobile phase consisted of 90% ACN and 10% water (containing 0.1% acetic acid and 0.2% triethylamine). The flow rate was 3.0 mL/min, and UV absorbance was monitored at 274 nm. Three major degradation products were obtained, peak 1 (5.0 mg), peak 2 (6.2 mg), and peak 3 (1.3 mg), for NMR and LC-MS/MS analysis.

Isolation of Hyperforin Degradation Products from Control Drink. One bottle (946 mL) of a drink product labeled to contain 75 mg of SJW extract (standardized to 0.3% hypericin) per 240-mL serving was filtered through Whatman no. 1 filter paper, loaded onto an open ODS column (described earlier), washed with water, and eluted with 200 mL of each of 60, 80, and 100% MeOH, successively. The fractions were concentrated by rotary evaporation, and the presence of hyperforin derivatives in each fraction was tested by HPLC-PDA. The 100% MeOH fraction showing positive for the presence of hyperforin and/or its derivatives was subjected to further analysis by MS and LC-MS/MS.

MS and LC-MS/MS Analysis of Hyperforin Degradation Products. The samples were analyzed by direct exposure probe/electron ionization−mass spectrometry (DEP/EI-MS) on a TSQ 700 mass spectrometer (ThermoFinnigan Corp., San Jose, CA) in the electron ionization (EI), single-quadrupole mode. The ion source temperature was 150 °C and the electron energy 70 eV (uncorrected). The first quadrupole analyzer was scanned from *m*/*z* 50 to 650 in 0.7 s. The rhenium wire of the DEP was heated from 0 to 800 mA with a linear ramp of 5 mA/s.

Components were separated using an HP 1100 HPLC (Agilent) with a Luna C5 2 × 150 mm 3-μm column (Phenomenex). The mobile phase, delivered at 0.2 mL/min, was a 30-min gradient from 50 to 95% ACN with constant 0.1% formic acid, which was held for another 20 min. The PDA was scanned 190 to 650 nm. LC-MS analyses were performed on a TSQ 7000 triple-quadrupole mass spectrometer (ThermoFinnigan, San Jose, CA) with an electrospray ion source operated in positive ion mode. The in-source CID offset (SID) was set at 12.3 V to reduce clustering with acetonitrile. For peaks 1 and 2 and the hyperforin standard, full scans were initially acquired with Q1 from *m*/*z* 150 to 750 s^{-1}. Other conditions were as follows: capillary temperature, 275 °C; sheath gas, 70 psi; auxiliary gas flow, 5 units; electrospray voltage, 4.5 kV. An LC-MS/MS method was set up for fragmentation of *m*/*z* 569, 553, and 537 at 25 eV 1.0 mTorr argon while Q3 was scanned from *m*/*z* 50 to 600 at 2 scans/s.

NMR Analysis of Hyperforin Degradation Products. The NMR spectral analyses of hyperforin degradation compounds were performed at 500 MHz on a Bruker AM-500 NMR spectrometer (Bruker Biospin, Rheinstetten, Germany). Hyperforin and its analogues were dissolved in CD_3OD, and all experiments were run at 300 K. The chemical shifts were defined by assigning the CD_3OD ^1H resonance peak to 3.31 ppm and the ^{13}C resonance peak to 49.15 ppm.

RESULTS AND DISCUSSION

Part 1: Stability Study. The analysis of SJW plant material showed the following composition: pseudohypericin, 0.86 ± 0.05 mg/g; hyperforin, 11.71 ± 0.32 mg/g; adhyperforin, 2.19 ± 0.05 mg/g; and hypericin, 0.39 ± 0.02 mg/g. One gram of this plant material was extracted with 40 mL of MeOH. When 5% of this methanolic solution was added to the test aqueous solutions, the resulting concentrations were 1.07, 14.6, 2.73, and 0.48 μg/mL for pseudohypericin, hyperforin, adhyperforin, and hypericin, respectively. A typical LC chromatogram of this solution at pH 2.65 at the initial time (0 h) is shown in **Figure 2A**,**B**. After storage in the dark, the peak areas of each compound decreased gradually. However, solutions stored under light changed drastically; hyperforin, adhyperforin, and protopseudohypericin all disappeared (**Figure 2C**,**D**).

The initial PDA absorbance spectra showed that both hyperforin and adhyperforin had maximum absorbance (λ_m) at

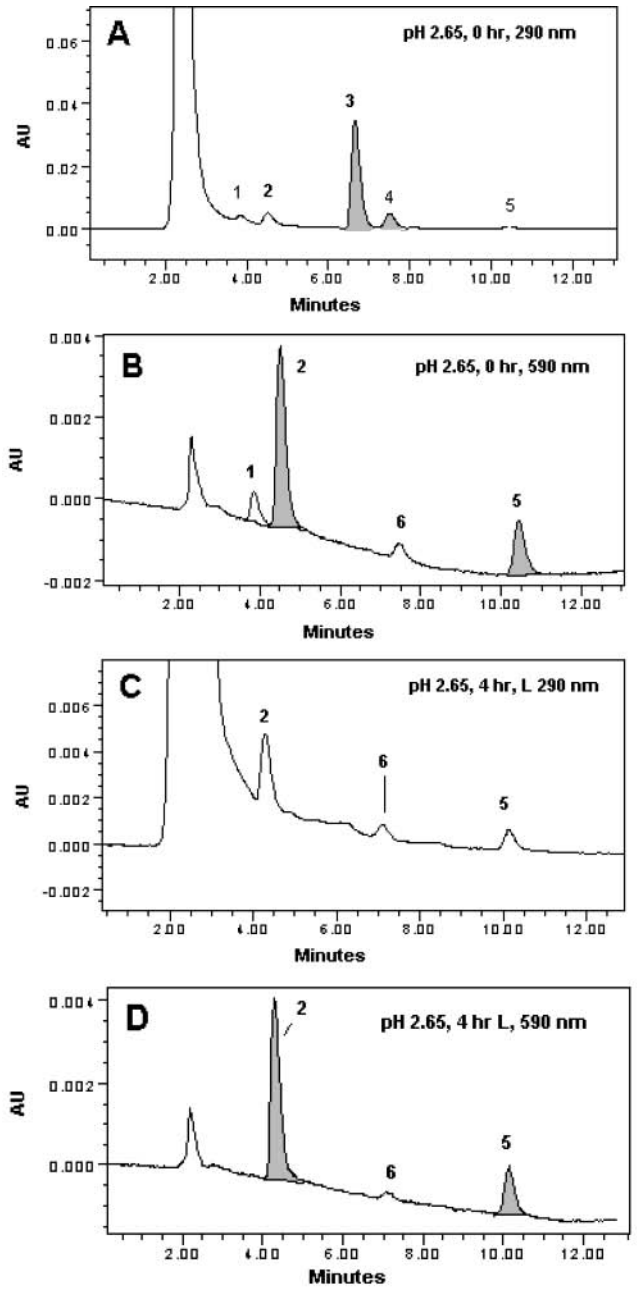

Figure 2. Typical HPLC chromatograms of St. John's wort (SJW) extract in buffer solution at pH 2.65: (**A**) 290 nm, 0 h; (**B**) 590 nm, 0 h; (**C**) 290 nm, 4 h under light; (**D**) 590 nm, 4 h under light. Peak identification: 1, protopseudohypercin; 2, pseudohypericin; 3, hyperforin; 4, adhyperforin; 5, hypericin; 6, unidentified.

290.5 nm and an unknown peak (peak 6) with λ_m at 412 nm. The absorbance of this unknown peak at 290 nm was very small (~5%) in comparison with that of the adhyperforin peak. When the hyperforin and adhyperforin decreased due to the light exposure, some small peaks appeared to have λ_m at 412 nm, with peak 6 the highest (**Figure 2C**). These peaks also disappeared after 24 h of light exposure.

The PDA spectra indicated that pseudohypericin and hypericin had λ_m at 590−592 nm, and the protopseudohypericin λ_m was 544 nm. The quantitative changes of these naphthodianthrones are shown in **Figure 3**. An increase of pseudohypericin was noted at 4 h under light but not in the dark (**Figure 3**). After this initial increase, the levels of pseudohypericin gradually decreased under light exposure. The decrease was not as severe as in the dark. Several studies (*3*, *23*, *24*) have suggested that protopseudohypericin changed to pseudohypericin and protohypericin to hypericin as affected by light. A slight increase of hypericin was noted after light exposure for 4 h. However, the level of protohypericin was below the detection limit of the HPLC procedure used in this study. LC-MS was able to detect both protopseudohypericin and protohypericin and confirmed the degradation of the various SJW natural products (details under LC-MS Confirmation for Stability Study).

The changes of hyperforin and adhyperforin during the storage in the dark and under light are shown in **Figure 4**. Whereas both compounds decreased during storage in the dark, the effect of light exposure was more destructive. Almost no residual hyperforin or adhyperforin could be detected after 24 h under light.

To determine the effect of pH on the stability of SJW components, aqueous solutions of three pH values were tested. The selection of pH 2.65 was based on the pH range of drink products (pH 2.5−2.8), and other pH values (4.5 and 6.1) were selected for comparison purposes at higher pH values. Results showed that within the pH range of this study, the instability was more severe in the lower pH than in the higher pH range. The percentages remaining (% retention) of each component after each time period at different pH values are also demonstrated in **Figures 3** and **4** for naphthodianthrones and phloroglucinols, respectively. The retention curves showed that SJW components in pH 2.65 solutions were the least stable either in the dark or under light exposure as compared to solutions of higher pH values, and all of the target compounds appeared to be most stable in pH 6.1 solution, especially in the dark.

A question regarding whether the higher concentrations of SJW components detected in solutions at pH 6.1 were merely due to the better solubility of these components at pH 6.1 as compared to pH 2.65 was investigated in a preliminary experiment. After exposure to light for 72 h, the pH 2.65 solution containing SJW extract was adjusted to pH 6.1, mixed, and analyzed by HPLC. However, concentrations of SJW components were not changed by adjustment of the pH to 6.1. Thus, the decrease of SJW phytochemicals in pH 2.65 buffer solutions during storage was apparently not due to the solubility effect.

The changes of SJW components in the drink samples were similar to those in the pH 2.65 buffer solutions. Each analyte decreased somewhat during storage in the dark (**Figure 5A**) and decreased more severely under light exposure (**Figure 5B**), especially for hyperforin and adhyperforin. Similar to the changes in buffer solutions, an increase was noted for pseudohypericin after 4 h of light treatment, and hyperforin and adhyperforin decreased the most as affected by light. However, the rate of change of each SJW component was slower in drink solutions than in the buffer solution at pH 2.65.

Fruit-flavored drink products may contain ascorbic acid and several types of fruit juice concentrate. For example, one brand of the drink products contained orange, pineapple, and mango juice concentrates, ascorbic acid, β-carotene, and pyridoxine hydrochloride. Apparently, ingredients such as ascorbic acid could have contributed to the protective effect of the SJW components during storage, even though the pH values of drink products were also low. Additionally, fruit-flavored drinks usually contain some color pigments; they appeared to be yellow, orange, or pinkish. The color pigments might also have a protective effect on the stability of phytochemicals under light exposure. Nevertheless, during long storage under light exposure, the decrease of SJW components in drinks was still severe as observed in the present study. Results from the present study demonstrating the drastic decrease of hyperforin in acidic

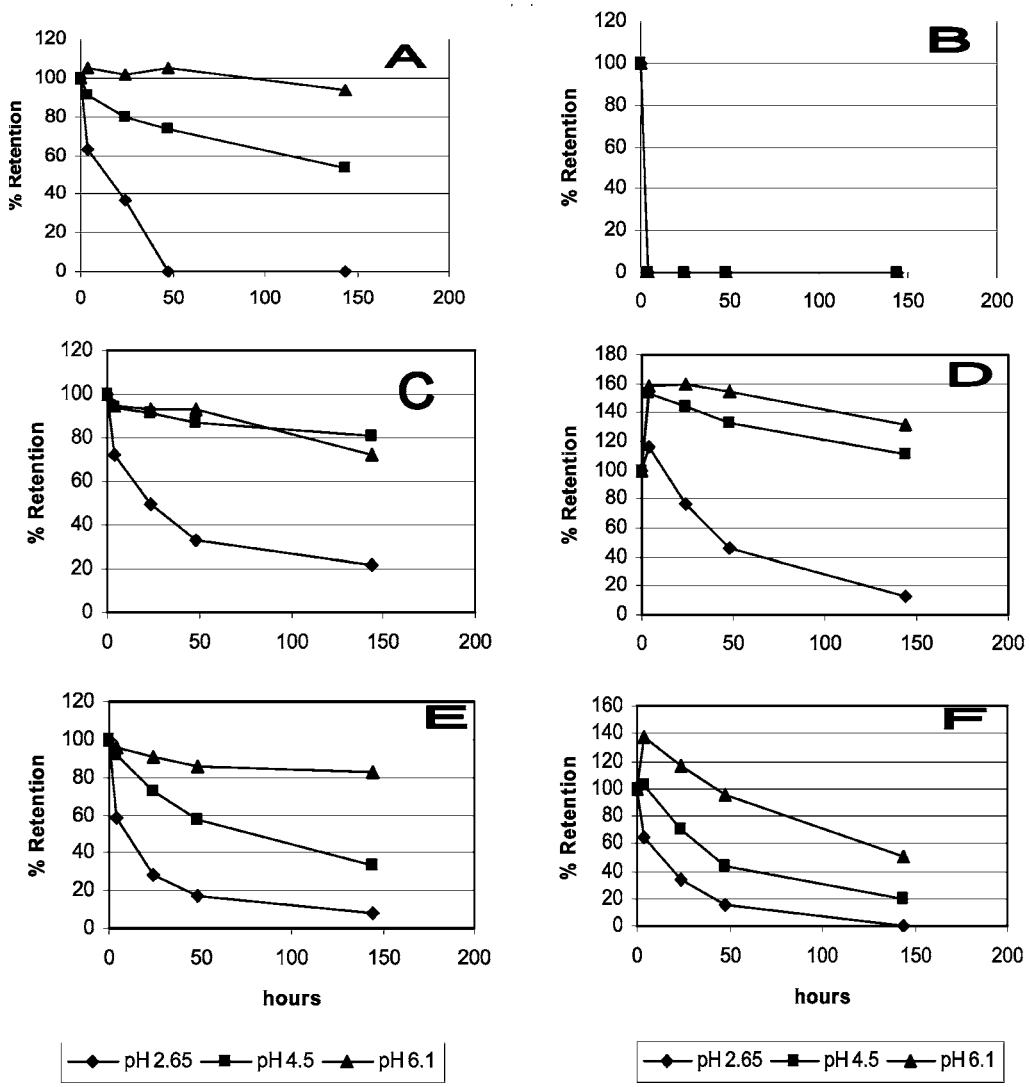

Figure 3. Effect of pH and light on retention of naphthodianthrones in aqueous buffer solutions: (**A**) protopseudohypericin in the dark; (**B**) protopseudohypericin under light; (**C**) pseudohypericin in the dark; (**D**) pseudohypericin under light; (**E**) hypericin in the dark; (**F**) hypericin under light.

solutions, especially under light exposure, provide an explanation of the previous observations that no hyperforin was detected and that only low levels of pseudohypericin were present in the fruit-flavored drink, if any (*20*).

An earlier study (*15*) showed that hyperforin and adhyperforin were more stable in MeOH and MeOH/H_2O (80:20, v/v) than acetone or hexane. Acidified MeOH (pH 2) with ascorbic acid and citric acid appeared to slow the light effect as compared to MeOH alone or the alkaline MeOH (pH 12). The present study focused on the stability of SJW components in aqueous solutions (pH 2.65−6.1) prepared in an ammonium formate and formic acid system. No direct comparison could be made between the published observation (*15*) and the present results.

Limited studies have shown the instability of hyperforins and/or hypericins in SJW products, such as commercial extracts and gelatin capsules (*14*), whereas the present investigation offers the first report on the instability of SJW components in aqueous solutions and fruit-flavored drink as affected by pH and light exposure.

LC-MS Confirmation for Stability Study. The SJW buffer solution at pH 2.65 that was stored in the dark was analyzed by LC-MS using negative ion electrospray ionization. Protopseudohypericin, protohypericin, and other natural products in crude SJW extracts were detected as $[M - H]^-$ ions. A total ion chromatogram is shown in **Figure 6A**. A sample at pH 4.5 that was stored in the dark gave identical results. After 4 h in the light at pH 2.65, protopseudohypericin and protohypericin were totally degraded (**Figure 6B**). The other natural products were greatly reduced.

Part 2. Degradation of Hyperforin. Among the four SJW marker compounds (hypericin, pseudohypericin, hyperforin, and adhyperforin), the hyperforin content is usually the highest. It is also most unstable during storage as shown in part 1. Thus, this part of the investigation focused on the determination of hyperforin degradation products. To obtain adequate quantities of any hyperforin degradation compounds for NMR and LC-MS/MS analysis, it was necessary to use a large amount of hyperforin as the starting material. The purified hyperforin (∼1 g) obtained in our preliminary study was essential for the further investigation of its degradation in buffer solutions and drink samples.

Hyperforin Degradation Products in Buffer Solutions and Fortified Drinks. Preliminary experiments of HPLC analysis showed that the hyperforin degradation compounds were eluted much later than the original hyperforin. The PDA spectra showed these compounds have λ_m at 274 nm. Thus, the HPLC conditions for the analysis of these degradation products were modified as following: The mobile phase was changed to 90%

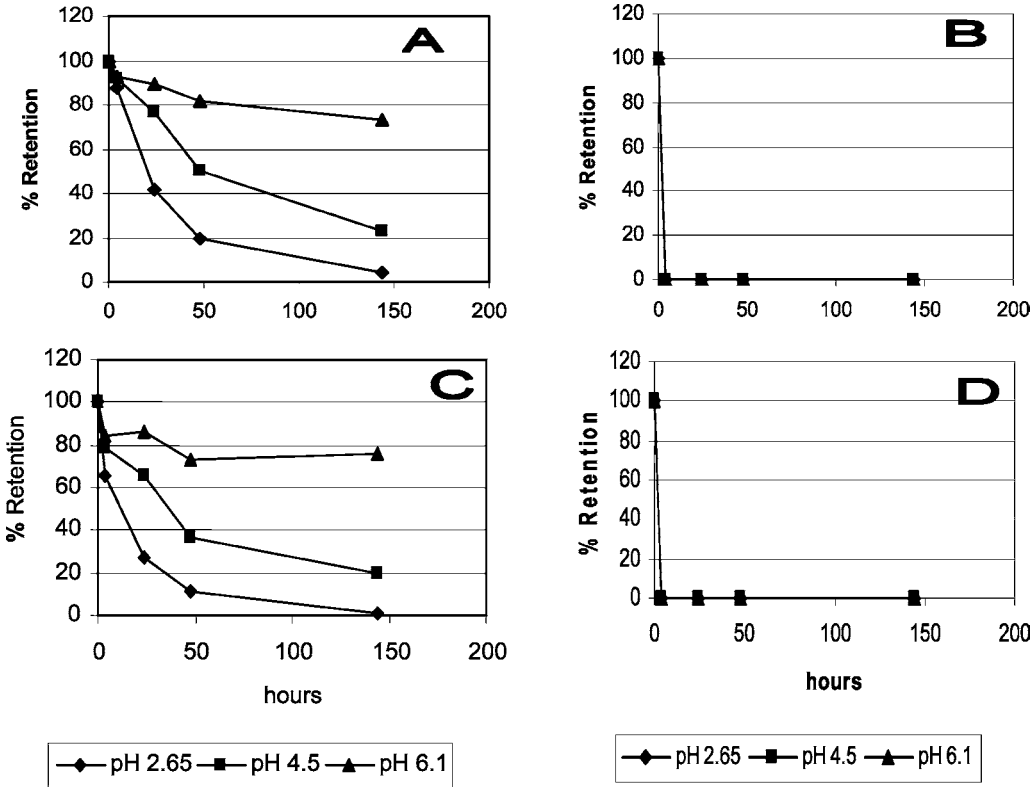

Figure 4. Effect of pH and light on retention of phloroglucinols in aqueous buffer solutions: (**A**) hyperforin in the dark; (**B**) hyperforin under light; (**C**) adhyperforin in the dark; (**D**) adhyperforin under light.

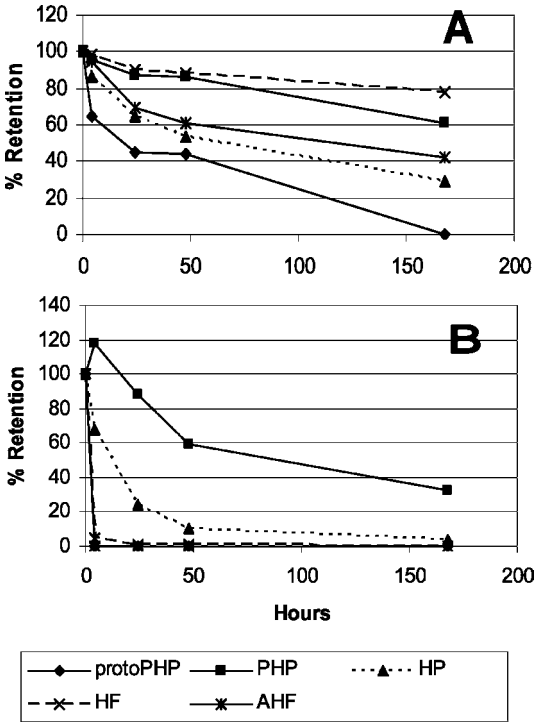

Figure 5. Effect of dark or light treatment on retention of SJW components in fruit-flavored drink sample: (**A**) in the dark; (**B**) under light. protoPHP, protopseudohypericin; PHP, pseudohypericin; HP, hypericin; HF, hyperforin; AHF, adhyperforin.

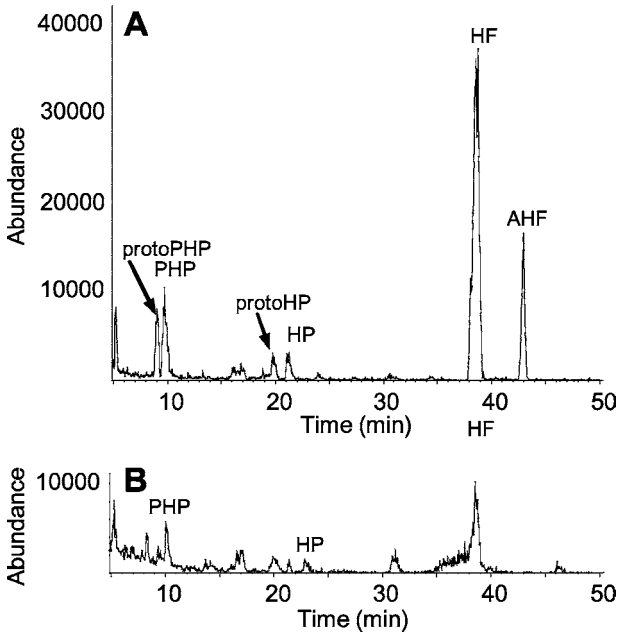

Figure 6. Total ion chromatograms of LC-MS negative ion electrospray analyses of SJW extract at pH 2.65: (**A**) after treatment in the dark; (**B**) after exposure to light for 4 h. protoPHP, protopseudohypericin; PHP, pseudohypericin; protoHP, protohypericin; HP, hypericin; HF, hyperforin; AHF, adhyperforin.

ACN and 10% water (containing 0.1% acetic acid and 0.2% triethylamine), and the detection was monitored at 274 nm. The HPLC analysis of a high concentration of hyperforin (0.5 mg/mL) in buffer solution, which has been stored in the dark for 2 days, showed three major hyperforin degradation peaks (**Figure 7A**). Similarly, a drink sample fortified with pure hyperforin at 0.2 mg/mL and stored for 2 days also produced three similar peaks of hyperforin degradation products (**Figure 7B**).

Subsequent investigations were to verify whether these degradation components could be detected in drinks fortified with lower concentrations of hyperforin. The analysis of these

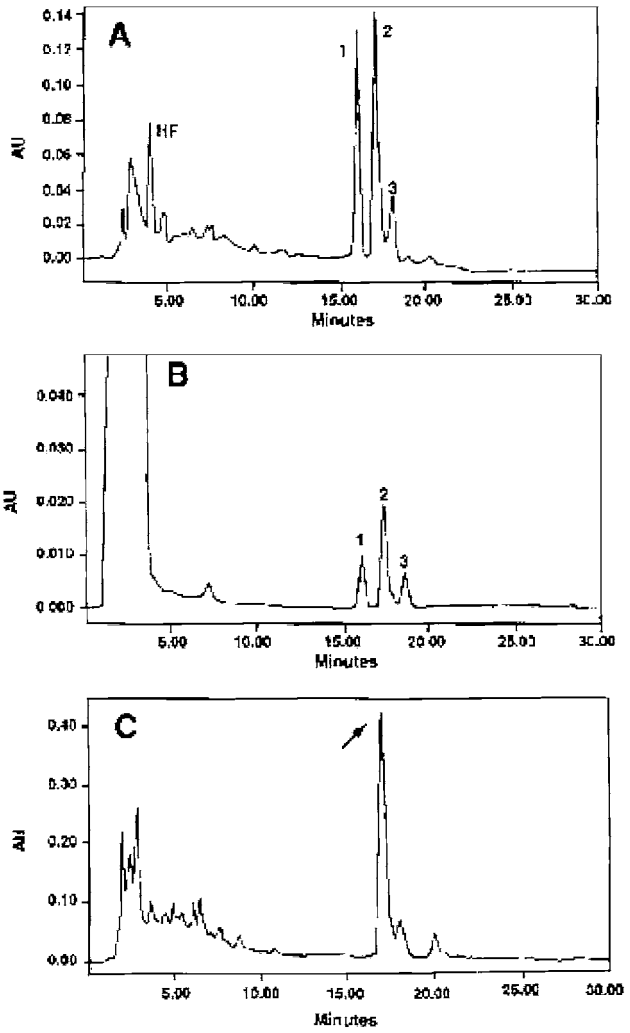

Figure 7. HPLC profiles of hyperforin degradation products: (**A**) oversaturated hyperforin in pH 2.8 buffer solutions (0.5 mg/mL), stored for 2 days in the dark (peaks 1–3 are the major degradation products); (**B**) oversaturated hyperforin in drink sample, stored for 2 days in the dark; (**C**) 100% MeOH fraction of control drink labeled to contain 75 mg of SJW/240-mL serving. Peak at 15 min (marked with an arrow) was identified as degradation product. Peak 2 is furohyperforin isomer a.

samples (fortified with pure hyperforin at 31 μg/mL, or SJW capsules at 9.5 μg/mL of hyperforin in drink, and stored for 5 days) also showed the presence of a small amount of degradation product, mainly peak 2. This peak underwent further degradation when the storage time was extended.

Hyperforin Degradation Products in the Control Drink. One of the drink products tested previously (*20*) contained 10 ng/mL pseudohypericin. This product was labeled to contain 75 mg of SJW extract per 240-mL serving. However, no other SJW components (other than pseudohypericin) and no hyperforin degradation derivatives were detectable by our routine HPLC method for drinks. Different LC procedures including various gradient and isocratic mobile phase solvent systems were tested, but no measurable hyperforin analogues were found. To verify if any degradation products were present in this product, a large volume of the drink sample was used in an open column fractionation. The 100% MeOH fraction was found to contain some hyperforin degradation products, with the major peak similar to peak 2 (**Figure 7C**).

MS and LC-MS/MS Analysis of Hyperforin Degradation Products. Using a high concentration of hyperforin in acidic aqueous solution, large quantities of hyperforin degradation products were obtained. Methods for the isolation and purification of degradation components by preparative HPLC were developed. Three major peaks were isolated and characterized by NMR and LC-MS/MS analysis. Several hyperforin oxidized products have been reported previously (*18*, *19*); examples are shown in **Figure 8**.

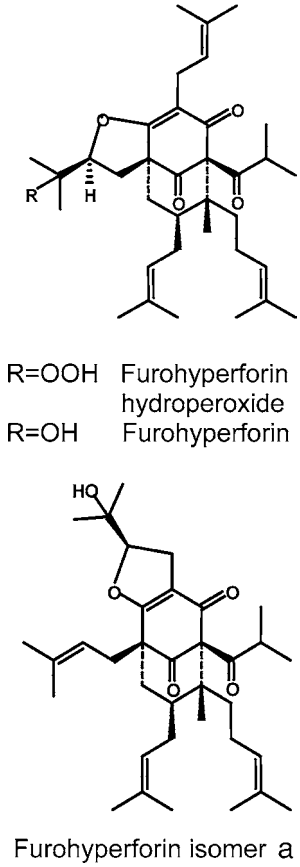

Figure 8. Hyperforin degradation products.

Negative ion electrospray LC-MS analyses of the two isolated peaks (peaks 1 and 2) gave ions at *m*/*z* 597 and 613 that did not fragment well by CID or MS/MS. The two peaks were then analyzed by direct exposure probe with electron ionization at 70 eV. The EI mass spectrum with ions at *m*/*z* 568, 552, 347, 305, 293, 204, 161, 147, 135, 95, 93, and 69 indicated that peak 1 was probably a mixture of at least two compounds with molecular weights of 568 and 552. The ions were consistent with fragment ions mentioned in the literature for furohyperforin (*16*) and furohyperforin hydroperoxide (*18*). An EI spectrum for peak 2 indicated that it was also related to hyperforin with a molecular weight of 552. Because the negative ion electrospray ions did not give typical $[M - H]^-$ ions when extra energy was applied, there may have been a rearrangement resulting in an ion that was more stable than the typical isobaric formate adduct ion.

An LC-MS/MS positive ion electrospray method was set up, and the product ion mass spectra correlated well with a study by Fuzatti et al. (*19*). The PDA chromatogram of the positive ion electrospray analysis of peak 1 is shown in **Figure 9A**. The main peak at 33.64 min was actually two coeluting components with protonated molecules at *m*/*z* 569 and 553. The product ion spectrum of *m*/*z* 569 with ions at *m*/*z* 365 and 309 (**Figure 9B**) was identified as furohyperforin hydroperoxide. The product ion spectrum of *m*/*z* 553 with ions at *m*/*z* 349 and 293 (**Figure 9C**) was identified as furohyperforin. The main PDA peak in

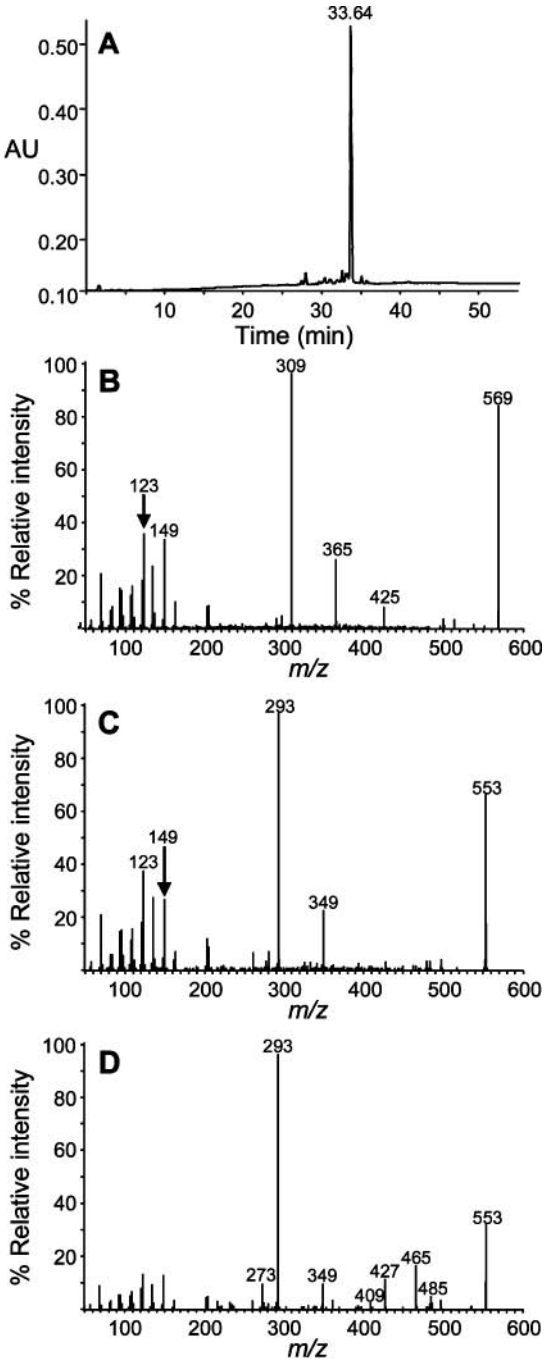

Figure 9. LC-MS/MS positive ion electrospray analyses of hyperforin degradation product peaks 1 and 2: (**A**) PDA chromatogram of analysis of peak 1; (**B**) product ion mass spectrum of *m*/*z* 569 identified as furohyperforin hydroperoxide in degradation peak 1; (**C**) product ion mass spectrum of *m*/*z* 553 identified as furohyperforin in both degradation peaks 1 and 2; (**D**) product ion mass spectrum of *m*/*z* 553 identified as furohyperforin isomer a in degradation peak 2 only.

the LC-MS analysis of peak 2 was at 33.25 min. It was a doublet of two isobaric components that both had protonated molecules at *m*/*z* 553. The product ion mass spectrum of the tailing part of the peak matched **Figure 9C**, which was identified as furohyperforin impurity in peak 1. The product ion mass spectrum of *m*/*z* 553 at the leading edge of the peak with ions at *m*/*z* 553, 485, 465, 427, 409, 349, 293, and 273 (**Figure 9D**) was identified as furohyperforin isomer a. Only weak protonated molecules were seen for hypericins, so positive ion electrospray would not have been suitable for the first part of this study.

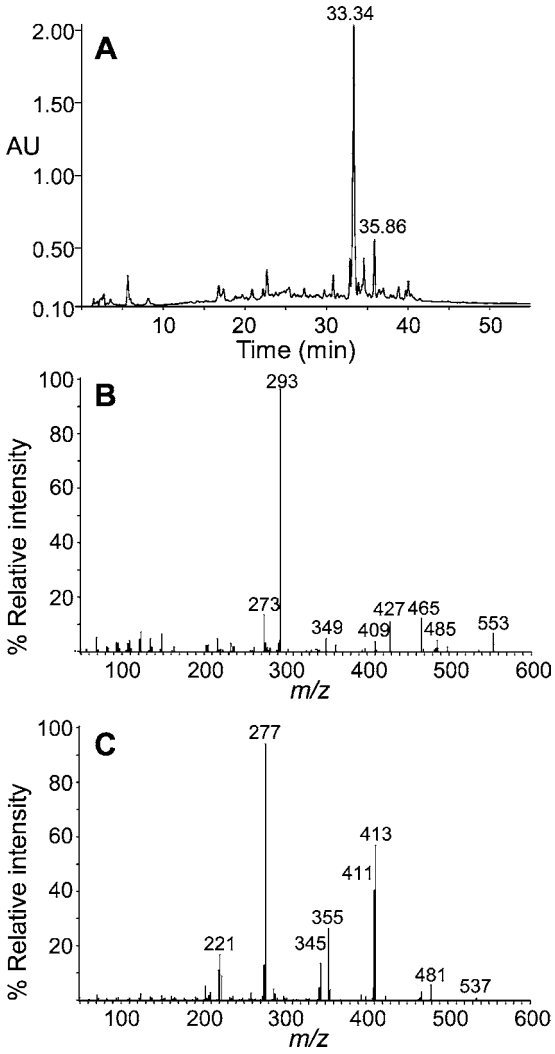

Figure 10. LC-MS/MS positive ion electrospray analysis of SJW drink 946 mL without hyperforin added: (**A**) PDA chromatogram of the analysis; (**B**) product ion mass spectrum of *m*/*z* 553 at 33.34 min identified as furohyperforin isomer a; (**C**) product ion mass spectrum of *m*/*z* 537 at 35.86 min identified as hyperforin.

LC-MS/MS analysis of hyperforin degradation in the pH 2.8 buffer showed a major peak at 33.29 min that was identified as furohyperforin isomer a, a minor peak at 33.69 min was identified as furohyperforin hydroperoxide, and another minor peak at 35.97 min was identified as residual hyperforin.

LC-MS/MS analysis of a control drink (946-mL bottle) showed two peaks in the PDA chromatogram (**Figure 10A**). The product ion mass spectrum of *m*/*z* 553 at 33.34 min (**Figure 10B**) was identified as furohyperforin isomer a. The product ion mass spectrum of *m*/*z* 537 at 35.86 min (**Figure 10C**) was hyperforin itself. The major hyperforin degradation product detected in the drink was furohyperforin isomer a, which was present at 6 times the level of residual hyperforin as estimated by area at 274 nm. Furohyperforin and its hydroperoxide were not seen in the control drink.

NMR Analysis of Hyperforin Degradation Products. The isolated hyperforin degradation products (peaks 1 and 2) were analyzed by NMR. Careful examination of their ^{1}H,^{13}C NMR data established peak 1 as furohyperforin hydroperoxide and peak 2 as furohyperforin. NMR spectra of peak 3 indicated that it was still a mixture. No further identification was conducted for this peak. Proposed structures by NMR analysis for furohyperforin and/or furohyperforin hydroperoxide from dried

SJW extract or aerial parts have been reported (*17*, *18*). The present study represents an attempt to detect hyperforin derivatives formed in aqueous solutions.

Because hyperforin existed as two tautomers in chloroform solution, the proton NMR spectrum of hyperforin in $CDCl_3$ was very complicated to assign. However, hyperforin existed as one tautomer in MeOH solution. Thus, we could completely assign all proton and carbon signals of hyperforin detected in MeOH-d_4 on the basis of $^1H-^1H$ COSY, HMQC, HMBC, and NOESY. The NMR solvent had a great effect on proton data, but little effect on carbon data. Peaks 1 and 2 were analyzed in MeOH solution. Then NMR carbon data of degradation products could be compared with those of hyperforin. Detailed NMR chemical shift assignments including data collected for peaks 1 and 2 will be presented in a separate paper.

Conclusions. SJW marker components, pseudohypericin, hyperforin, adhyperforin, and hypericin, were found to be unstable in aqueous solutions, especially under light exposure. The extent of change varied with specific compound. Degradation of these components was less severe in drink than in buffer solutions of pH 2.65. Initial conversion of proto-derivatives to hypericin and pseudohypericin under light exposure was observed. The major degradation products of hyperforin in acidic aqueous were furohyperforin, furohyperforin, hydroperoxide, and furohyperforin isomer a. The latter was also identified in the control drink sample. The present study provides the first report on the instability of SJW components and the formation of hyperforin degradation products in acidic aqueous solutions and fruit-flavored drink. The implications of these findings for the quality and safety aspects of functional beverages containing SJW are yet to be evaluated.

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