FOOD COMPOSITION AND ADDITIVES

Simultaneous Determination of the Predominant Hyperforins and Hypericins in St. John's Wort (*Hypericum perforatum* L.) by Liquid Chromatography

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Hypericin and hyperforin are believed to be among the active constituents in common St. John's wort (*Hypericum perforatum* L.). Presently, dietary supplements are generally standardized to contain specified levels of hypericin and hyperforin, and the related compounds, pseudohypericin and adhyperforin. A rapid method was developed for simultaneous determination of these 4 active constituents by liquid chromatography (LC). A 1 g portion of dried, finely ground leaf/flower sample is extracted with 20 mL methanol for 2 h. A 0.6 mL aliquot of the crude extract is combined with 5.4 mL acetonitrile–methanol (9 + 1) and passed through a mixed solid-phase cleanup column. The eluate is examined by LC for hyperforin, adhyperforin, hypericin, and pseudohypericin on a Hypersil reversed-phase column by using simultaneous ultraviolet (284 nm) and fluorescence detection (excitation, 470 nm; emission, 590 nm). The compounds are easily separated isocratically within 8 min with a mobile phase of acetonitrile–aqueous 0.1M triethylammonium acetate (8 + 2). Average recoveries of hyperforin and adhyperforin were 101.9 and 98.4%, respectively, for 3 sample mixtures containing concentrations ranging from approximately 0.2 to 1.5% combined hyperforins per gram dry weight. Average relative standard deviation (RSD) values for hyperforin and adhyperforin for all 3 mixtures were 18.9 and 18.0%, respectively. Average recoveries of hypericin and pseudohypericin were 88.6 and 93.3% respectively, from 3 sample mixtures containing concentrations ranging from approximately 0.2 to 0.4% combined hypericins per gram dry weight. Average RSD values for hypericin and pseudohypericin for all 3 mixtures were 3.8 and 4.2%, respectively.

Common St. John's wort (*Hypericum perforatum* L.) is a perennial species of the Hypericaceae family, native to Europe. Dietary supplements and other herbal preparations produced from the leaves and flowers of St. John's wort have gained popularity in the United States in recent years (1, 2). A recent overview of 23 controlled clinical trials concluded that St. John's wort was more effective than a placebo for the treatment of mild depression (3). Commercial extracts from the leaves and flowers are also being investigated for anticancer and antiviral activities (4). The predominant napthodianthrone derivatives, hypericin and pseudohypericin, and the phloroglucine derivatives, hyperforin and adhyperforin, are among the compounds presently being investigated for their biological activities. Standardized dietary supplements of St. John's wort currently contain from 0.3 to 0.5% hypericin(s), and/or approximately 3.0% hyperforin(s). In 1998, St. John's wort herbal products showed exceptional sales growth, increasing nearly 3000% from 1997 to 1998 (2).

Several recent papers on the chemical analysis of St. John's wort have provided the means to measure many of the predominant chemical constituents from diluted, crude extracts (5–12). In general, samples were extracted and filtered, or liquid–liquid extraction was used to remove chlorophylls and other pigments. The use of mixed solid-phase (MSP) cleanup columns has been reported recently in the literature. Wilson and Romer (13) developed a proprietary cleanup column consisting of a mixture of reversed-phase, ion-exclusion, and ion-exchange packing materials used for cleanup of extracts of corn, cottonseed, rice, mixed feeds, and a variety of nuts in the determination of aflatoxins. Similarly, Tacke and Casper (14) developed a C_{18}–alumina (1 + 3) MSP cleanup column for wheat extracts in the determination of deoxynivalenol. The following method was developed to provide a rapid, inexpensive, MSP cleanup with simultaneous determination of the 4 compounds of greatest current interest, hypericin, hyperforin, pseudohypericin, and adhyperforin from flower and leaf mixtures of St. John's wort.

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METHOD

Apparatus

(**a**) *Grinding mill*.—Stein laboratory mill, Model M-2 (Seedboro Equipment Co., Chicago, IL).

(**b**) *Horizontal shaker*.—S/P Rotator V (Baxter Scientific Products, McGaw, IL).

(**c**) *LC system*.—Hitachi (Hitachi Instruments, Inc., San Jose, CA) L-7100 LC pump, isocratic flow at 1.0 mL/min; Hitachi L-7200 autosampler, Hitachi L-7400 UV detector (284 nm), and Hitachi L-7480 fluorescence detector (excitation, 470 nm; emission 590 nm). Data were recorded and processed by a Hitachi D-7000 data acquisition package with ConcertChrom software on a microcomputer.

(**d**) *Analytical column*.—Hypersil, 3 µm, C_{18} (BDS), 100 × 4.60 mm reversed-phase column (Phenomenex, Torrance, CA).

(**e**) *Guard column*.—Phenomenex SecurityGuard C_{18} (ODS) 4.0 × 3.0 mm (Phenomenex).

(**f**) *Syringe filters*.—9.5 mm diffusers (Analtech, Newark, DE).

(**g**) *Borosilicate glass scintillation vials*.—20 mL (Fisher Scientific, St. Louis, MO).

Reagents

(**a**) *Solvents*.—LC grade acetonitrile and methanol (Fisher Scientific).

(**b**) *Water*.—Deionized.

(**c**) *LC mobile phase*.—Acetonitrile–aqueous 0.1M triethylammonium acetate (8 + 2). The mixture was filtered and degassed under vacuum and pumped at 1 mL/min. The final pH of the mobile phase was 7.0.

(**d**) *Standards*.—Hyperforin standard (Addipharma, Hamburg, Germany) and hypericin standard (Sigma, St. Louis, MO). Adhyperforin and pseudohypericin were determined by using previously published results, which included chromatograms and retention times (9, 11, 12). In addition, the published literature indicated that no chromatographic peaks of other known compounds occurred in the immediate vicinity of the hyperforin peak (other than the adhyperforin peak), and that no other known compounds exhibited the unique fluorescence emission of hypericin at 590 nm (other than pseudohypericin).

(**e**) *MSP cleanup column*.—A syringe filter is placed in the bottom of a 3 mL syringe barrel (Sherwood Medical, St. Louis, MO), followed by 1.5 mL MSP material consisting of completely mixed Bakerbond C_{18}, 40 µm particle size, (Mallinckrodt Baker, Inc., Phillipsburg, NJ) and Florisil (60–100 mesh, Sigma) in a 2:1 ratio. A syringe filter is then placed on top.

Sample Extraction

Grind approximately 10 g dried aerial portions of St. John's wort, using Stein mill. Accurately weigh 1.0 g sample into 20 mL vial, and add 20 mL methanol; cap vial and place on horizontal shaker for 2 h. Let samples settle for several minutes. Pipet 0.6 mL methanol extract into 10 mL test tube, and dilute with 5.4 mL acetonitrile–methanol (9 + 1) to a final volume of 6 mL.

Column Cleanup and Liquid Chromatography

Apply 5 mL diluted sample extract to MSP cleanup column. No preconditioning of the column is necessary. Collect ca 2 mL eluate, and place 0.5 mL in sealed autosampler vial. Using instrument parameters listed above, inject 20 µL into liquid chromatograph. Hyperforin, adhyperforin, hypericin, and pseudohypericin are determined simultaneously by UV and fluorescence detection.

Standard Preparation

Prepare primary standards of hypericin and hyperforin by dissolving 1 mg hypericin in 10 mL methanol (making 100 ppm hypericin), and 5 mg hyperforin in 5 mL methanol (making 1000 ppm hyperforin). From initial 1000 ppm hyperforin and 100 ppm hypericin standard mixtures, prepare combined hyperforin:hypericin working standards of 250:25, 100:10, 50:5, and 20:2 ppm. Combine 0.5 mL 1000 ppm hyperforin with 0.5 mL 100 ppm hypericin to produce 1 mL 500:50 ppm hyperforin:hypericin. Combine equal parts standards mixture with methanol for 250:25 hyperforin:hypericin standards mixture. Continue dilution of 500:50 standards mixture with methanol to produce 100:10 (dilute 1 part standards

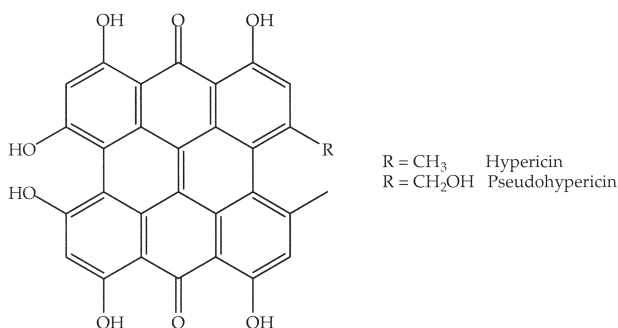

Figure 1. Structures of hypericin and pseudohypericin.

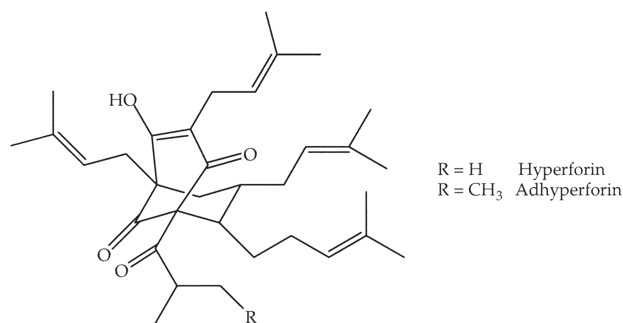

Figure 2. Structures of hyperforin and adhyperforin.

Table 1. Mean concentrations ± SD[a] (% dry weight) of hyperforin, adhyperforin, hypericin, and pseudohypericin found in St. John's wort extracts after MSP cleanup, and mean recoveries ± SD (%)

Parameter	Hyperforin	Adhyperforin	Hypericin	Pseudohypericin
Sample mixture 1[b]				
Mean ± SD	1.2 ± 0.2	0.2 ± 0.04	0.2 ± 0.008	0.2 ± 0.004
Recovery ± SD	104.5 ± 2.5	96.3 ± 5.1	91.2 ± 1.2	95.3 ± 2.3
Sample mixture 2[b]				
Mean ± SD	0.6 ± 0.08	0.1 ± 0.02	0.07 ± 0.003	0.08 ± 0.005
Recovery ± SD	91.4 ± 1.3	86.8 ± 4.6	82.4 ± 5.1	89.7 ± 7.0
Sample mixture 3[b]				
Mean ± SD	0.2 ± 0.05	0.04 ± 0.009	0.1 ± 0.005	0.2 ± 0.006
Recovery ± SD	109.9 ± 4.5	111.9 ± 10.1	92.1 ± 2.1	95.1 ± 1.1
Mean recovery from all mixtures[c]	101.9 ± 8.5	98.4 ± 12.5	88.6 ± 5.5	93.3 ± 4.9

[a] SD = standard deviation.
[b] $n = 7$ samples per mixture.
[c] $n = 21$ samples.

mixture with 4 parts methanol); 50:5 (dilute 1 part standards mixture with 9 parts methanol); and 20:2 (dilute 1 part standards mixture with 24 parts methanol).

Because of the nearly identical structures and optical properties of hypericin and pseudohypericin, and hyperforin and adhyperforin (Figures 1 and 2, respectively), the concentrations of pseudohypericin and adhyperforin are calculated from the hypericin and hyperforin standard curves, respectively.

Calculations

Use working standards described above to create standard curve regressions based on peak areas of hyperforin and hypericin (x_1 and x_2) and concentrations of hyperforin and hypericin (y_1 and y_2), and use a zero-intercept model, slope forced through origin (15). From this model, the equation $y = \beta_1 x$ can be interpreted as ppm = slope (sample peak area), where the result is multiplied by 200, the dilution factor, to obtain the chemical concentration in µg/g dry weight.

Results and Discussion

This method was developed to provide an inexpensive, single-step cleanup for the rapid quantitation of the hypericins and hyperforins in St. John's wort. Commercial St. John's wort samples contain various ratios of flower and leaf material, and prestandardized mixtures therefore contain a range of hyperforin and hypericin concentrations. In this study, 3 leaf and flower mixtures, with 7 replicate extractions per mixture, were prepared to represent general concentration ranges. The 3 sample mixtures were based initially on high (mixture 1, approximately 1.5%), average (mixture 2, approximately 0.8%), and low (mixture 3, approximately 0.2%) combined concentrations of hyperforin and adhyperforin per gram dry weight (Table 1). The concentrations of hypericin and pseudohypericin also varied within these 3 mixtures, ranging from high (mixture 1, approximately 0.4%), average (mixture 3, approximately 0.3%), and low (mixture 2, approximately 0.2%) combined concentrations per gram dry weight (Table 1).

Samples were extracted with methanol at room temperature for 2 h on the basis of a 24% increase in hypericin concentration and a 31% increase in pseudoypericin concentration

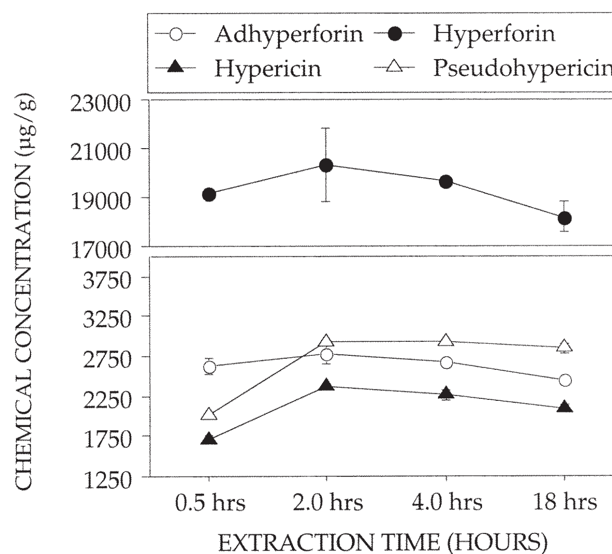

Figure 3. Mean ± standard deviation of differences in chemical concentrations (µg/g) of hyperforin, adhyperforin, hypericin, and pseudohypericin extracted from flower and leaf mixtures of St. John's wort from 0.5 to 18 h ($n = 2$).

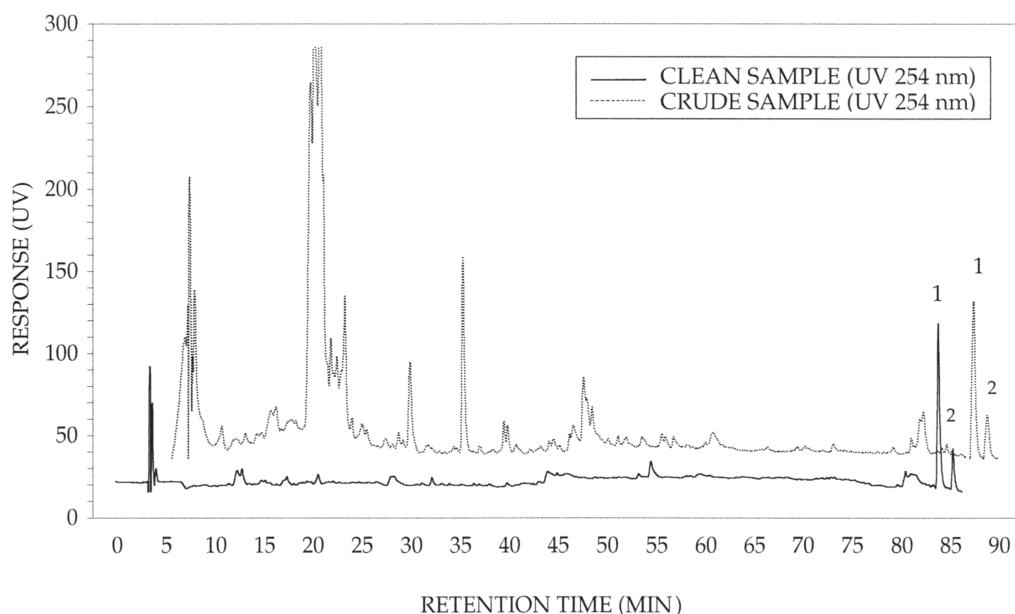

Figure 4. Comparison of liquid chromatograms obtained for crude and MSP-cleaned extracts of the same flower/leaf sample of St. John's wort, with UV detection at 254 nm [1, hyperforin; 2, adhyperforin; mobile phase: (A) acetonitrile–water with 1% acetic acid (1 + 10); and (B) acetonitrile. Linear gradient from 90 to 5% (A) over 90 min].

observed after extracting for >0.5 h (Figure 3). After 2 h extraction time, no substantial concentration changes were observed for the hypericins with up to 18 h extraction time. The hyperforin concentrations, however, were essentially unchanged from 0.5 to 18.0 h extraction time. It is generally believed that the hypericins are sequestered within specialized glands located predominantly along the leaf margins, and the minimum 2 h extraction time observed for a dried and powdered sample under these extraction conditions may be necessary for this reason.

Passing the acetonitrile–methanolic extract through the MSP cleanup column retains pigments and other coextracted compounds on the column (Figure 4), while allowing the hypericins and hyperforins to elute from the column in a single step. The advantages of the MSP cleanup columns over commercial reversed solid-phase extraction (SPE) columns and cartridges are (*1*) multiple activation and elution steps are eliminated (no preconditioning is necessary with MSP columns); (*2*) pigments and extraneous compounds are retained on the column from 100% methanol or acetonitrile extracts; and (*3*) the column eluate can be easily concentrated or directly analyzed.

When methanol flower/leaf extract was applied to the MSP column, pigments leached from the column and the hypericins were retained. An acetonitrile flower/leaf extract applied to the MSP column retained both the pigments and the hyperforins. A mixture of acetonitrile–methanol (8 + 2) flower/leaf extract [which includes approximately 10% methanol each from the crude extract, and from the acetonitrile–methanol (9 + 1) dilution mixture] applied to the MSP column retained the pigments and allowed quantitative recovery of the hypericins and the hyperforins in the eluate. Increasing total concentration of methanol to >20% did not increase either hyperforin or hypericin recoveries, but increased pigment leaching.

Exposure to air and light (in an open test tube after column cleanup) caused both hyperforin and adhyperforin to deterio-

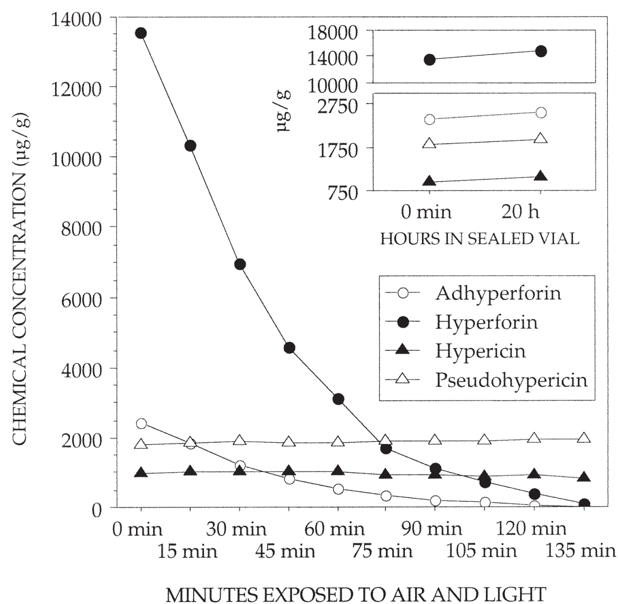

Figure 5. Stability of hyperforin, adhyperforin, hypericin, and pseudohypericin after exposure to light and air in an open test tube (main graph, *n* = 1); stability of the same compounds in the dark in air-tight autosampler vials (inset graph, *n* = 1).

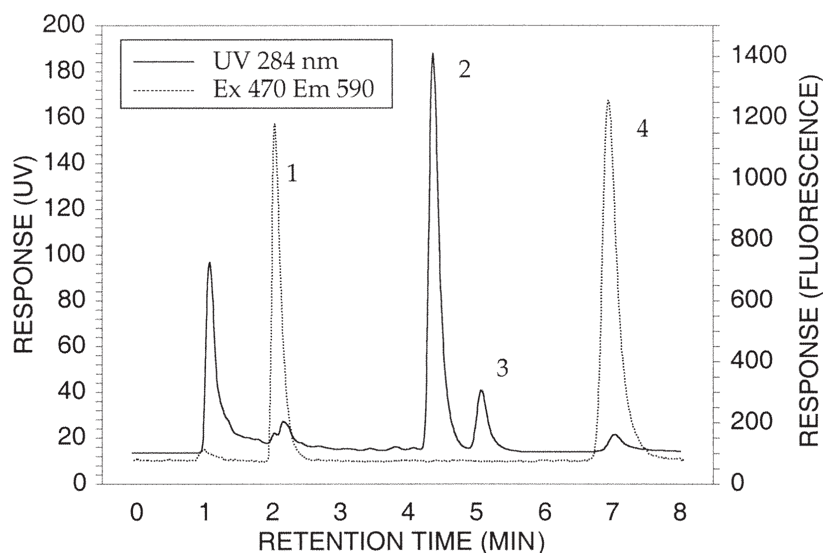

Figure 6. Simultaneous LC fluorescence detection at excitation wavelength of 470 nm and emission wavelength of 590 nm (1, pseudohypericin; 4, hypericin) and UV detection at 284 nm (2, hyperforin; 3, adhyperforin) of MSP-cleaned sample extract of St. John's wort [mobile phase: acetonitrile–aqueous 0.1M triethylammonium acetate (8 + 2), isocratic over 8 min].

rate rapidly, while hypericin and pseudohypericin remained stable (Figure 5). Similar decomposition results for hyperforin in *n*-heptane have been previously reported (11), in which nearly 100% of the compound was lost after 25 min. To avoid deterioration of the hyperforins, the eluate should be placed in a sealed vial, in the dark immediately. When samples were directly placed in sealed autosampler vials in the dark (i.e., in a contained autosampler), no deterioration of the hyperforin and adhyperforin occurred (Figure 5, inset).

This MSP cleanup procedure produces clean liquid chromatograms from flower and leaf mixtures of St. John's wort (Figure 6). The determination of the 4 compounds was achieved by using a modification of an LC mobile phase previously reported by Piperopoulos et al. (9). However, the combined use of Phenomenex columns and a mobile phase of acetonitrile–aqueous 0.1M triethylammonium acetate (8 + 2) allowed for the isocratic determination of all 4 compounds easily within 8 min and produced excellent peak shape and resolution. Additionally, the simultaneous determination of the hypericins and hyperforins by using both UV and fluorescence detection provided a much more sensitive method than did the use of UV alone. The unique emission wavelength for both pseudohypericin and hypericin effectively screens out competing napthodianthrone derivatives that appear with UV detection (Figure 6).

Recoveries were determined by directly comparing calculated concentrations (μg/g) of all 4 compounds, from each clean and crude sample in the 3 mixtures. Average recoveries for all compounds were high across mixtures, ranging from 82.4 to 111.9% (Table 1). In general, recoveries and RSD values of the hyperforins tended to be higher for the samples that contained both the highest and lowest concentrations (sample mixtures 1 and 3, respectively; Table 1). Recoveries averaged >100% for both hyperforin and adhyperforin from these 2 mixtures, with RSD values of 18.8 and 23.3% (hyperforin, sample mixtures 1 and 3, respectively) and 16.6 and 23.6% (adhyperforin, sample mixtures 1 and 3, respectively). The lowest recoveries of both hyperforins were found for sample mixture 2, which contained intermediate levels of these compounds. The RSD values for sample mixture 2 at 14.6% (hyperforin) and 13.8% (adhyperformin) were lower than, but comparable to, the RSD values found for sample mixtures 1 and 3.

Recoveries of the hypericins tended to be greatest from the samples that contained the highest and the average concentrations (sample mixtures 1 and 3; Table 1). Recoveries averaged >93% for both hypericin and pseudohypericin from these mixtures, with RSD values of 3.3 and 3.7% (hypericin, sample mixtures 1 and 3, respectively) and 3.1 and 3.2% (pseudohypericin, sample mixtures 1 and 3, respectively). The lowest recoveries of both hypericins were found for sample mixture 2 (which contained the lowest levels of these compounds), possibly because of retention of the hypericins on the MSP column when present in relatively low amounts. The RSD values for recoveries from sample mixture 2 at 4.4% (hypericin) and 6.3% (pseudohypericin) were comparable to the other RSD values found for sample mixtures 1 and 3. Overall mean recoveries were >85% for all compounds when averaged across mixtures, with overall recovery RSD values ranging from 3.1 to 23.6%.

This isocratic LC method with MSP cleanup provides a rapid, inexpensive, and reliable procedure for the determination of the 4 primary compounds of current interest from St. John's wort: hyperforin, adhyperforin, hypericin, and pseudohypericin. The application of this method in industry, especially when heterogeneous sample mixtures are involved,

could prove to be a valuable analytical tool for quick cleanup and compound elucidation.

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