Extraction and Quantitation of Digoxin and Acetyldigoxin from the *Digitalis lanata* Leaf via Near-Supercritical Methanol-Modified Carbon Dioxide

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An extraction process is reported that employs a near-supercritical mixture of CO_2 and MeOH to extract the cardiac glycoside, digoxin, from the *Digitalis lanata* leaf. The method development of the sample preparation procedure is presented in detail, and reasons for trends that occur in the natural products extraction are given.

Recent concerns about the toxic properties of many conventional liquid solvents have fueled a sizable growth in the field of supercritical fluid (SF) technology. SF rates of diffusion increase by approximately 1 order of magnitude when compared to that of conventional liquids, and viscosities are decreased by 1 order of magnitude. Correspondingly, the surface tension of the fluid is near zero, which allows the supercritical medium to enter areas that may be impenetrable to liquid solvents. Perhaps the biggest advantage of SF is the selectivity realized, as these fluids possess variable solvating power that can be increased by raising the density through varying the temperature and pressure of the system. In addition, small percentages of polar liquid modifiers and additives can be employed, which can dramatically increase the solubility of compounds in SF−CO_2.

One area of chemistry that has benefited from the advantageous properties of SF is natural product chemistry.[1] SF extraction (SFE) has been used in the isolation of alkaloids, triglycerides, lipids, and steroids.[2−16]

The primary objective of this work was to develop an SFE to isolate the glycoside digoxin (**3**) and its acetylated form, acetyldigoxin (**2**), from *Digitalis lanata Ehrh.* (Scrophulariaceae) leaf. The impetus for developing such an extraction method was to decrease the time and costs and, perhaps as important, to eliminate the hazards involved with the current extraction method, which involves the use of H_2O, MeOH, and a chlorinated solvent. A literature search reveals only one paper dealing with the extraction of glycosides with supercritical fluids. Shibuta reported the cumulative glycoside amounts that could be recovered from various leaf types, achieving the highest recoveries from the *D. lanata* leaf approximately 0.4% total glycosides with 10 mol % EtOH in SF−CO_2).[17] Digoxin is a moderately polar compound containing numerous hydroxyl functionalities known to present problems of solubility for SF−CO_2. Therefore, it was proposed that relatively high amounts of modifier would be needed for the SFE of digoxin. Consequently, this project was carried out following an extensive SFE trapping study, designed to learn more about the various mechanisms responsible for analyte loss in the trapping stage of SFE, when high levels of modifier are used, so as to minimize loss of digoxin and acetyldigoxin.[18] In addition to digoxin, we were also interested in acetyldigoxin, which differs from digoxin only by the presence of an acetyl group instead of a hydroxyl group located on the 3‴ position of the terminal sugar unit and is readily converted to digoxin through base hydrolysis.

Results and Discussion

Preliminary experiments showed that soaking of the *D. lanata* leaf was essential for enzymatic hydrolysis of lanatoside C (**1**) into acetyldigoxin (**2**) and, subsequently, to digoxin (**3**) prior to successful SFE (Chart 1).[19] Consequently, several extractions of the wet leaf, macerated in H_2O−alcohol, were performed. Residual H_2O left within the leaf resulted in low extraction recoveries, as the moderately polar digoxin partitions into the liquid medium to a greater extent as opposed to the extracting CO_2 medium. Therefore, after maceration (H_2O−EtOH (80:20, v/v) over 24 h), the leaf material was lyophilized at −60 °C and 128 mTorr to remove H_2O and alcohol, prior to extraction. With each of the trapping parameters optimized, as well as the extraction flow rate determined, the remaining two areas of the SFE procedure that needed attention are the amount of modifier needed for optimized solubility and suppression of matrix effects and the extraction temperature needed for optimized diffusion.

The effect of modifier on solvating power is well known; however, modifiers also play other very important roles, especially for matrices, where the analyte is strongly bound through chemisorption and physisorption. Another advantage realized by employing a polar liquid modifier such as H_2O or MeOH is the swelling of the matrix, thereby increasing the internal volume, which in turn increases the amount of surface area accessible to the near SF mixture. It has been shown that solid matrix materials exhibit a higher degree of swelling when liquids are used as opposed to when a gas or SF is used.[20] As a general rule, it was observed that the swelling power of a liquid decreased as its dielectric constant decreased. For our work, however, H_2O had a detrimental effect on SFE efficiencies due to the diminished partitioning into the near SF phase.

A modifier can be introduced either into the CO_2 (in-line) or the matrix (off-line). To study the effect of in-line addition, MeOH in volumes of 10, 15, 20, and 25% were added with all other conditions remaining the same. A plot of percentage recovery versus in-line

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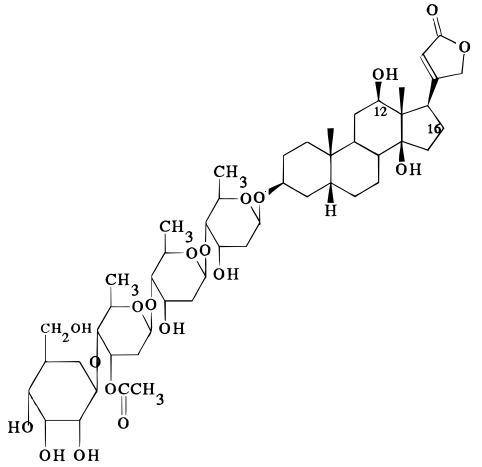
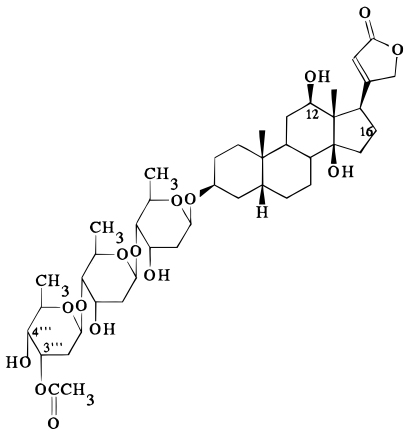
Chart 1



1



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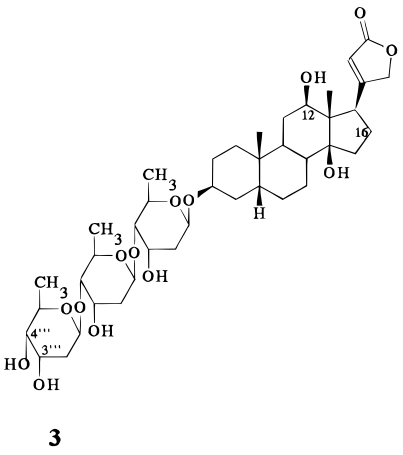

3

modifier amount is presented in Figure 1a. As can be seen, the percentage of recovery increases dramatically from 10 to 20%, but reaches a plateau after 20%, which means that 20% MeOH-modified CO_2 is the optimum volume to solubilize the amount of free digoxin and acetyldigoxin without experiencing trapping problems.

Because the majority of the initial digoxin recovered appeared to be of limited solubility, it was theorized that by using the off-line approach of spiking a specific quantity of liquid MeOH into the vessel prior to extraction digoxin could be extracted more quickly than was realized with the optimal in-line extraction (0.2 mL of MeOH/minute). The effect of three different spike volumes (0.5, 1.0, and 1.5 mL) on percentage of recovery of digoxin from the leaf was investigated with the same conditions as in the initial experiment. As can be seen in Figure 1b, increased amounts of spiked MeOH in the extraction vessel prior to SFE resulted in a decreased recovery of digoxin. This trend is not too surprising as it further supports the conclusions of the trapping study that was alluded to earlier. At larger spike volumes, trapping efficiency is decreased due to aerosol formation and modifier elution mechanisms arising from modifier condensation and CO_2 decompression. This trend could also be partially explained by the fact that, at higher ratios of liquid modifier to CO_2, a loss in diffusion could occur as the overall fluid becomes more liquid-like and less SF-like, but as a result of our previous trapping study, it was believed that the majority of the problem stemmed from trapping inefficiencies.

At this point, density, flow rate, trapping parameters, modifier amount and method of modifier introduction, and trap rinse parameters have been optimized. The only parameter yet to be optimized is the temperature of the extraction. It is known that, although a temperature increase at fixed pressure results in a less dense SF, the diffusivity of the SF increases and its viscosity decreases. Even though a loss in solvating power is realized as the temperature is raised, the presence of the large amount of polar modifier should raise the solvating power back to an acceptable level. Three different temperatures were studied and their effect on percentage recovery noted. As is seen in Figure 1c, as the temperature increases, the amount of digoxin recovered increases from 77% to approximately 100% (relative to the 0.25% recovered with the current liquid extraction method). Even though the extraction fluid at these conditions is not supercritical and our ability to vary solvating power of the fluid has diminished, the advantages of high diffusion and low viscosity remain. The extraction conditions remained the same as previously outlined with the exception of oven temperature and elimination of the static step. Therefore, 100% recovery of digoxin was achieved in 45 min with 20% MeOH-modified CO_2 at an extraction temperature of 100 °C and a pressure of 380 bar. This extraction was exhaustive, as the method was repeated once more on the raffeinate and no additional recovery of digoxin was achieved.

With all parameters optimized, five replicate extractions were performed with two different types of traps: stainless steel beads and octadecylsilica. Extraction conditions employed in this reproducibility study were: sample size, 200 mg; CO_2 pressure, 380 bar; temperature, 100 °C; liquid flow, 1.0 mL/min; fluid, 20% MeOH-modified CO_2, trap temperature, 80 °C; extraction time, 45 min; thimble volumes, 8.3; trap, C-18 or stainless steel beads; rinse solvent, 4.5 mL of MeOH; trap temperature during rinse, 25 °C. Although the stainless steel trap resulted in recoveries of 105% with 9% RSDs relative to the liquid extraction method, the C-18 trap resulted in 95% recoveries with 3% RSDs. Statistically, the recoveries of the two methods exhibited little difference. Although the C-18 trap method exhibits a tighter recovery range, as well as a cleaner extract, the stainless steel trap tends to resist plugging to a better extent over longer periods of time as highly nonpolar materials are rinsed off more effectively.

The objective of the project was to replace the current liquid-liquid extraction method, which utilizes $CHCl_3$, with automated SFE in order to minimize or eliminate, if possible, the large amounts of hazardous chlorinated liquid wastes and, if possible, decrease the extraction time and costs involved in the isolation of digoxin and acetyldigoxin. This goal was achieved with equivalent recoveries of digoxin from the leaf relative to the current extraction method. The technique of lyophilization of macerated plant material worked well at setting up a favorable partition of digoxin into the SF. Temperature was shown to have a dramatic effect on extraction

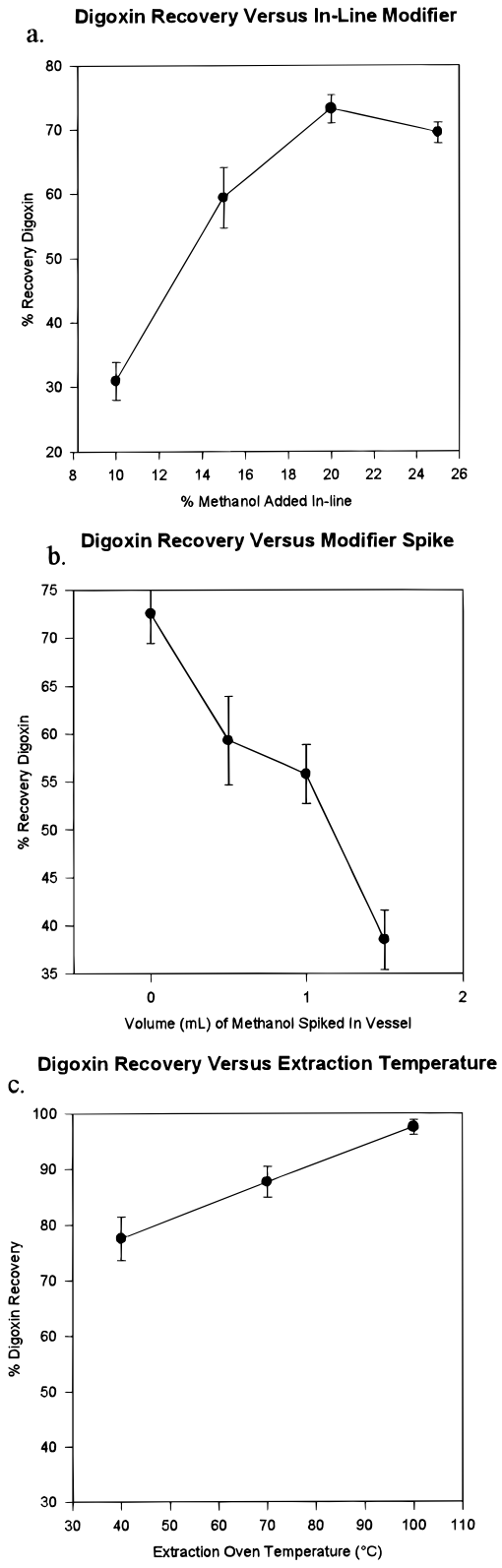

Figure 1. (a) Digoxin recovery as a function of in-line modifier amount. (b) Digoxin recovery as a function of spiked modifier amount. (c) Digoxin recovery as function of extraction temperature.

efficiency, as the extraction of the leaf was proven to be diffusion limited. Two traps were employed, and both performed well under optimized trapping conditions. SFE of the *D. lanata* leaf as a way of isolating digoxin and acetyldigoxin has proven to be a quantitative and reproducible sample preparation technique.

Experimental Section

General Experimental Procedures. Chromatographic separation of the extracts was performed using a Hewlett-Packard 1050 quaternary gradient pump along with an HP multiwavelength UV detector at 215 nm. A 4.6- × 250-mm Hypersil ODS (5 μm) chromatographic column (Keystone Scientific, Inc., Bellefonte, PA) was employed. A Valco Model EQ-60 LC injector (Austin, TX) fitted with a 10-μL sample loop was used. The mobile-phase solvents for HPLC were purchased from EM Science (Gibbstown, NJ) and consisted of solvent A (MeCN−H_2O 75:25, v/v) and solvent B (100% MeCN). The method employed a mobile phase of 100% A for 4 min; linear gradient to 90% A/10% B in 1 min, hold for 5 min; linear gradient to 80% A/20% B in 1 min, hold 6 min; linear gradient to 100% B in 1 min, hold for 15 min. The flow rate was 1.5 mL/min.

Plant Material. The *D. lanata* leaf material used in this study was collected at Verenigde Nederlandse Kruidencoöperatie in the Netherlands. A voucher specimen is deposited at Virginia Polytechnic Institute and State University in Blacksburg, Virginia. The leaves were ground in a Fitzpatrick mill at Burroughs-Wellcome prior to their arrival. To increase surface area, the received leaf was ground further using a Miracle Mill prior to extractions.

Extraction and Isolation. Extractions were performed on the Hewlett-Packard supercritical fluid extractor model 7680T (Wilmington, DE). Modifier is introduced into the system using Hewlett-Packard 1050 HPLC isocratic pump. Celite (Supelco, Bellefonte, PA) was used both to fill any dead volume remaining in the vessel and also to act as a filter at the exit end of the vessel in order to prevent leaf particulates from plugging the exit frits and the system tubing. SFE/SFC grade CO_2 (Air Products and Chemicals, Inc., Allentown, PA) was used for all extractions. All spike solvents and modifiers were HPLC grade and were obtained from EM Science (Gibbstown, NJ). All extractions used 1.0 mL/min liquid flow and a solid-phase trap of stainless steel beads, with the exception of a final study, which compared the trapping efficiency of stainless steal with octadecylsilica trapping material.

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