CHAPTER I

Introduction and Historical Background

1. Supercritical Fluid Extraction Technology

Generally, the analytical process can be defined as four primary operations: sampling, sample preparation, measurement, and data processing. Data show that sample preparation takes up ~ 60% of the overall time devoted to the analytical process.[1] This operation is the most labor-intensive task and it is a major source of error which may critically influence the quality of the results. Usually the specific method of sample preparation is determined by the sample matrix. The most well-known of these techniques is Soxhlet extraction which primarily uses an organic solvent. Another extraction method that was initially used in large-scale industrial applications and has been investigated in last two decades as an analytical technique is supercritical fluid extraction (SFE), with primarily carbon dioxide (CO_2) as the extraction medium.[2]

Supercritical fluid extraction is a technique that employs a fluid phase having properties intermediate between a gas and liquid, to effect the solubilization of solutes in a matrix.[3] The advantages that are gained by employing SFE can be traced to the unique physical properties that the fluids possess. A pure supercritical fluid is a substance above its critical temperature (T_C) and pressure (P_C). Above its critical temperature, it does not condense to form a liquid, but is a fluid (dense gas), with properties changing continuously from gas-like to liquid-like as the pressure increases at fixed temperature.[4] The solvating power of a supercritical fluid (SF) depends on the density, which is determined by its pressure and temperature. Since the solvating power (density) of a SF can be altered over a wide range by changing the pressure, the temperature or both,

extraction can be made to be selective to some extent. Compared to conventional organic liquids, supercritical fluids have higher diffusivity and lower viscosity, thus allowing more efficient mass transfer of solutes from sample matrices.[5] CO_2 has so far been the most widely used fluid in SFE, because of its low critical parameters, cheapness, non-explosive character, and non-toxicity. Its relatively low critical temperature (T_C = 31.3 °C) permits extraction of thermally labile compounds.[3] Most conventional extraction techniques require the use of large amounts of toxic organic solvents. SFE is an environmentally friendly technique which uses CO_2 as the major solvent and it can offer the same or even better extraction efficiencies in a much shorter time.[6] Even though supercritical CO_2 preferentially extracts non-polar compounds, the polarity of supercritical CO_2 can be enhanced by the addition of a miscible polar compound (such as methanol) as modifier.

2. SFE-Chromatographic Hyphenation Techniques

SFE extracts are cleaner due to lower concentrations of interfering co-extracts. This feature permits the direct introduction of extracts into an analytical system without a further clean up step. On line coupling of SFE and separation techniques is highly beneficial for trace analysis.[7] It offers higher sensitivity since all of the extract maybe transferred to the separation column. In addition, this method is far less labor intensive than off-line analysis and the opportunity for the sample to become contaminated, or volatilize or degrade is minimized. On line coupling of SFE to gas chromatography (GC) and supercritical fluid chromatography (SFC) [8-12] is relatively straightforward due to the nature of the chromatographic mobile phases employed. For on-line SFE/GC analysis,

the presence of depressurized supercritical CO_2 (from the extraction process) in the interface is not a problem since the GC mobile phase is usually helium. The extracts can be trapped either at the head of the column or at the split/splitless injection port. Decompressed CO_2 gas is purged, then thermal desorption of the extracts is initiated to introduce analytes into the column in the vapor phase.

For on-line SFE/SFC, the same fluid is used for both extraction and chromatography. For most analytes and matrices an intermediate trap is required to concentrate the analyte prior to chromatography because of less than optimal extraction kinetics. An inert cryocooled stainless steel surface has been employed. Thermal desorption is employed to initiate the SFC step.

A logical extension of the above described hyphenated techniques would be the interfacing of SFE to high performance liquid chromatography (HPLC). This technique should be popular since it is aimed at addressing the analysis of extracts that are inaccessible to GC and SFC, due to their extensive polarity, high molecular weight, or thermal lability.[13] On-line SFE-HPLC would be particularly helpful for the analysis of samples which are: (a) light or air sensitive; (b) limited in quantity; and (c) at the trace level.[14] Unfortunately, up to now only a few articles [14-25] have reported such a technique. Even fewer cases have accomplished quantitative transfer of analytes to the LC column for analysis. Experimentally the problem lies in the poor miscibility of most liquids and gaseous CO_2. For a conventional HPLC system, maintaining the mobile phase as gas-free as possible is vital for optimum pump and detector performance.[4] The pressure of the mobile phase becomes erratic if gas is present in the LC's delivery system. If gas is introduced into the column, irregular baseline noise and variable detector response will

be observed, since gas bubbles will perturb the ultraviolet absorbance (UV) detector. The above problems thus suggest that on-line SFE/HPLC is the most challenging of SFE-Chromatography hyphenated techniques.

3. SFE-HPLC Research Background

During the process of SFE, supercritical CO_2 is decompressed to CO_2 gas through a restrictor (backpressure regulator) thus allowing for the deposition of the extracts into a liquid or solid phase trap. At this time CO_2 gas may be introduced into the LC system. Based on this observation, previously reported interfaces can be divided into two basic types: (1) interfaces with introduction of CO_2 into the LC system (Section 3.1) and (2) interfaces without introduction of CO_2 into the LC system (Section 3.2). In Section 3.1, we have also divided the interfaces into two subgroups: 1) fractional analyte transfer; and 2) total analyte transfer. In Section 3.2, we have divided these interfaces into two subgroups: 1) back pressure method; and 2) solvent displacement method.

3.1 Interface With Introduction of CO_2 Into the LC

3.1.1 Fractional Transfer Interface

The first SFE-LC system was reported in 1983, by Unger and Roumeliotis.[14] The schematic diagram of this interface is shown in Figure 1: where 1 is the back pressure regulator; 2 is the extraction vessel; 3 is the two-way valve; 4 and 8 are two six-port valves; 5 and 7 are the microbore columns; 6 is the rotameter; 9 is the heated zone; and 10 is the LC column. [14]

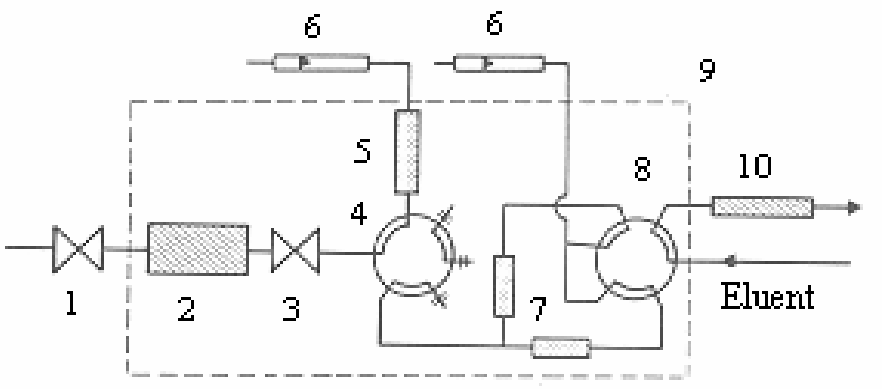


Figure 1. Schematic diagram of on-line SFE-LC interface of Unger's design.[14] 1, back pressure regulator; 2, extraction vessel; 3, two-way valve; 4 and 8, two six-port valves; 5 and 7, microbore columns; 6, rotameter; 9, heated zone; 10, LC column. (Reproduced with permission from Elsevier Ltd.)

The system has three operational modes: release, load, and inject.[14] In the release position (Figure 2a), valve 4 was switched so that expansion of the CO_2 gas took place into the waste column (5). The second valve (8) was set to allow mobile phase to enter the LC column (10). In the load position (Figure 2b), the valves were switched so that the analytes could be trapped on two microbore ODS columns. This valve position was kept open for 10 ~ 60s, then valve 8 was switched to the inject mode (Figure 2c). The extracts which had been loaded into the loop were rinsed with mobile phase and eluted into the chromatographic column. The sample studied was an extraction of valepotriates from Radix Valerianae. During the extraction-separation, only a small fraction of the extracts were introduced into the LC. The amount was determined by the loop capacity and loading time. No quantitative results were reported since there was not a quantitative transfer. In addition, during the transfer, CO_2 must have been introduced into the LC column accompanying the extracts, but no chromatogram was reported to show how CO_2 bubbles interfered with the baseline.

The next paper concerning on-line SFE-LC was presented in 1988, by Nair and Engelhart. [15, 16] A Milton Roy supercritical sample-preparation accessory (SPA) was used, which was the first and only commercial SFE-HPLC coupling system on the market, although it was marketed only for solubility measurements.[13] The SPA used a single switching valve to connect the extractor and chromatograph. A variable-wavelength UV detector was placed before the valve to monitor the extracts. In the load position, the sample loop became part of the flowing stream. When the UV response became constant (which meant that exhaustive extraction was achieved), then the valve

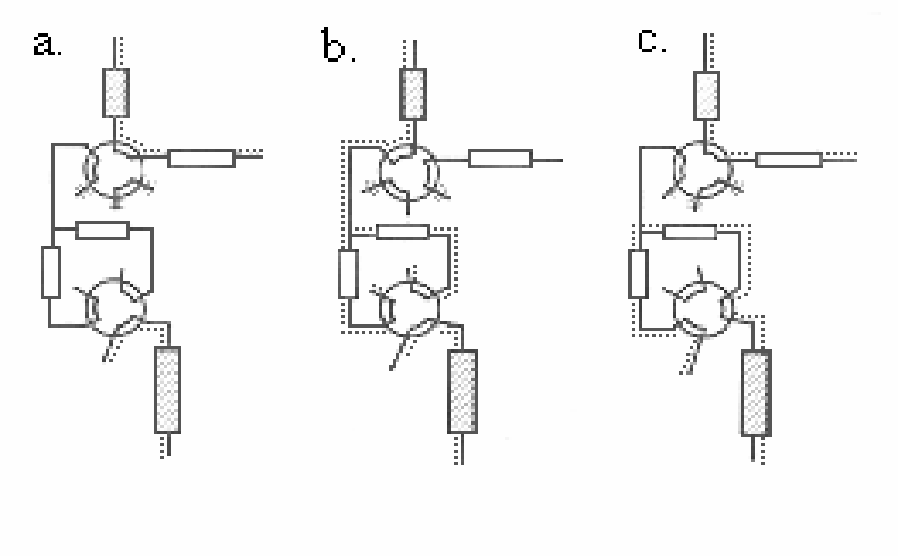


Figure 2. The operation mode of Unger's on-line SFE-LC. a: Release, b: Load, c: Inject.[14] (Reproduced with permission from Elsevier Ltd.)

was switched to the inject position, the mobile phase passed through the loop and brought the SF and extracts into the LC. The amount transfer was decided by the volume of the loop. Unlike Unger's interface, it allowed for an on-line injection of an analyte-containing CO_2 plug into the HPLC. As a result, only static mode extraction rather than dynamic extraction was possible. This interface was interfaced with reversed phase LC to analyse various drugs. All chromatograms had a huge peak in the first 3 minutes due to CO_2, which indicated that CO_2 eluted as a plug, rather than diffused throughout the mobile phase.[13]

Ashraf-Khorassani et al. [17] reported a similar type of interface for the analysis of four polynuclear aromatic hydrocarbons (PAHs) and five linear alkylbenzene sulfonates. After the extraction equilibration was achieved, the supercritical fluid containing analytes were injected directly into the HPLC system from the injection loop (9 μL), which is a part of the SF flowing stream. The supercritical CO_2 in the loop injected into HPLC system dissolved in the mobile phase (methanol/water) if the percentage of methanol was higher than 80%. Under these conditions, the baseline of the UV detector signal was unaffected. However, at higher percentages of water, CO_2 solubility in the mobile phase decreased and caused baseline interferences, because while supercritical CO_2 is miscible in methanol, it has solubility of only 6.0% in water.[18]

Jinno et al. reported a similar interface.[19] They used solid phase microextraction (SPME) to extract organophosphorus pesticides spiked in water, and then used supercritical CO_2 to desorb the extracts from the SPME fiber before on-line introduction into HPLC. All 5 μL of SF CO_2 containing extracts that were injected into the HPLC loop were dissolved in methanol/water (80/20) mobile phase without affecting the baseline.

To realize quantitative analysis in SFE-HPLC system, we must quantitatively transfer analytes from the extractor to the chromatograph. The above mentioned interfaces are thus not suitable for trace quantitative analysis, since only a fraction of the extract was transferred to the LC system.

3.1.2 Total Transfer Interface

Johansen [20] et al. reported an on-line SFE-LC/GC method to determine polychlorinated biphenyls (PCBs). The schematic diagram of this interface is shown in Figure 3: where 1 is the CO_2 gas reservoir; 2 is the SFE pump; 3 is the extraction oven; 4 is the extractor cell; 5 is the alumina column; 6, 10 and 15 are switching valves, V1, V2 and V3 respectively; 7 is the restrictor; 8 is the restrictor heater; 9 is the Valco T; 11 is the HPLC pump; 12 is the restrictor tip; 13 is the steel tubing; 14 is the union; 16 is the fused silica capillary; 17 is the LC column and 18 is the UV detector. The on-line system as accomplished by connecting a 1/16 "T" (9) coupling to the linear restrictor (7). No trap was used in this system. The cooling effect due to the decompression of the pressurized fluid helped the analytes concentrate in a narrow band in the steel tubing (13). A six-port valve (V2) was placed between the T piece and the HPLC mobile phase reservoir to avoid back-diffusion of CO_2 to the mobile phase during extraction. Valve V3 was placed at the column inlet to prevent CO_2 from entering the column during extraction. Before the extraction began, the valves V2 and V3 were placed in the locked position so that the CO_2 could not enter the mobile phase reservoir or the column. After the extraction was completed, valve V2 was switched to the flow mode to introduce LC mobile phase (hexane) and the extracts that were deposited on the tubing (13) were then transferred to

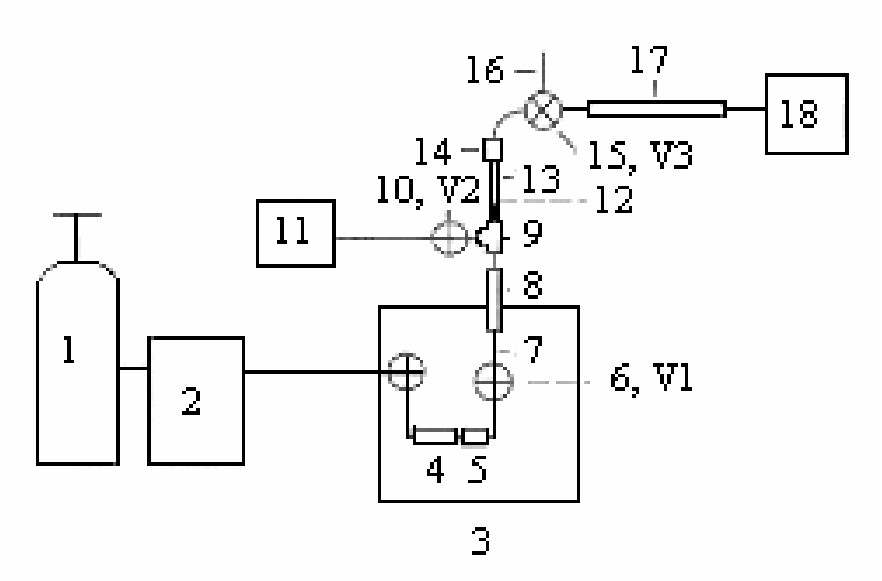


Figure 3. Schematic diagram of on-line SFE-LC of Johansen's design.[20] 1, CO_2 gas reservoir; 2, SFE pump; 3, extraction oven; 4, extractor cell; 5, alumina column; 6, 10 and 15, switching valves, V1, V2 and V3 respectively; 7, restrictor; 8, restrictor heater; 9, Valco T; 11, HPLC pump; 12, restrictor tip; 13, steel tubing; 14, union; 16, fused silica capillary; 17, LC column; 18, UV detector. (Reproduced with permission from American Chemical Society.)

the LC. Dissolved CO_2 in the hexane phase resulted in large peaks near the front of the chromatogram. The authors also found (when compared with direct injection of the components) that 7 ~ 12 % lower peak area with the on-line method and a 12 ~ 36 % loss in the number of theoretical plates.

Stone and Taylor [7] reported another type interface in 2001. Wide-bore open-tubular columns with various wall-coated stationary phases and lengths were tested as an interface for SFE/HPLC. During the extraction, analytes were trapped on the open tubular column. The pressure at the outlet of the interface was controlled by a back-pressure regulator. Only moderate back pressures were generated as supercritical fluid CO_2 was decompressed and channeled through the open-tubular column. A 15 m open-tubular column with a 95% methyl-5% phenyl stationary phase was found to be the preferred trapping phase. This phase allowed for good analyte focusing onto the LC column which exhibited low reactivity. This approach allowed for quantitative transfer of the analytes to the LC even when 10% modifier was used in the extraction. During the chromatographic stage, liquid mobile phase forced CO_2 from the interface onto the LC column, giving rise to a huge CO_2 peak in chromatogram.

3.2 Interface Without Introduction of CO_2 into the LC

To date, only two types of methods have been developed which prevents depressurized CO_2 gas from entering the LC column: 1) back pressure method; 2) solvent displacement method.

3.2.1 Backpressure Method

Liu et al. reported another type interface in 1993.[21] They used SFE/LC to extract and analyse chlorinated phenols in soil, wood, and biological tissue. Two three-way valves and three six-port valves were used as the interface to connect the SFE and LC. A 50 μL sample loop without absorbent became part of the stream and functioned as the SFE trap. When extraction equilibration was reached, the SF and extracts in the loop were transported into the LC by mobile phase. These workers found that introduction of such a high amount (50 μL) of supercritical CO_2 led to considerable deterioration in chromatographic performance (Figure 4). The primary reason was bubble formation (entrapment of CO_2) in the chromatographic column and the detector cell. High diffusivities of solute molecules also led to peak broadening. To prevent formation of CO_2 bubbles, two linear restrictors consisting of 8 cm x 25 μm I.D. fused-silica tubing were attached at the end of the detector and the vent tube. In this study an over pressure of 850 p.s.i. was found to be adequate for preventing bubble formation. Recovery and detection limit were also reported (all the recoveries were higher than 84% and the detection limit was in the sub ppm level). Due to a fractional transfer, this technique is not suitable for analysis of sample at the trace level.

Cortes et al. reported SFE on-line coupling with a microcolumn LC for analysis of chlorpyrifos insecticide.[22] An impactor interface was constructed for the coupling of SFE to LC (Figure 5). The interface consisted of a union (5) and a tee (3). The tee was connected to a linear restrictor (1), which was pushed through the tee so that it would pass into the impactor tube (4). The tube contained a 0.5 mm long deactivated porous ceramic frit (6) which served to trap and focus the solutes during the extraction. When extraction was done, the LC mobile phase entered the tee (from inlet 2) and carried the

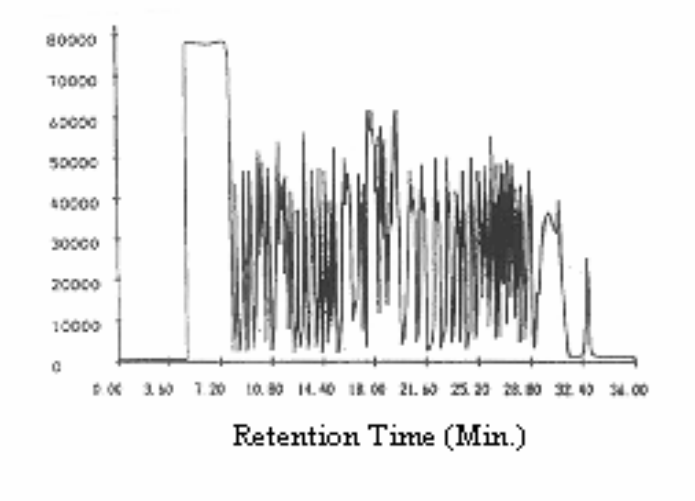


Figure 4. Chromatogram of SFE/LC when CO_2 entered the LC. [21] (Reproduced with permission from Elsevier Ltd.)

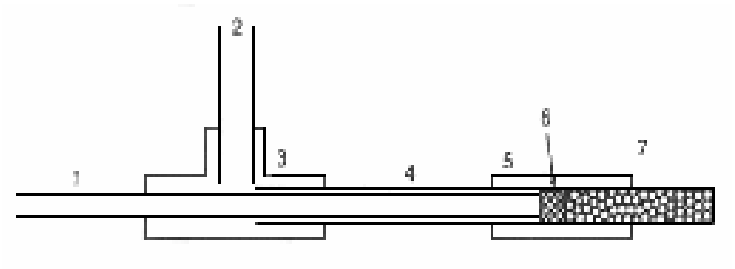


Figure 5. SFE-LC interface reported by Cortes. [22] 1, linear restrictor; 2, inlet tubing; 3, tee; 4, impactor tube; 5, union; 6, deactivated porous ceramic frit; 7, LC column. (Reproduced with permission from American Chemical Society.)

extracts from the impactor to the LC column (7). Initially, the authors observed that during the extraction step, CO_2 gas would enter the LC column and thereby flush out LC eluent from the column packing. As a result, the detector signal remained unstable for a long time due to entrained gas in the system after the valves were switched from the extraction mode to the separation mode.

In further research, the valve at the LC outlet was plugged to achieve a high backpressure. As a result no CO_2 was found to enter the LC column during the extraction, and a stable signal was obtained.[22] The supercritical fluid was decompressed through a linear restrictor, allowing for the deposition of the extracted components in the interface. Expansion of supercritical fluids is generally accompanied by a positive Joule-Thompson coefficient, resulting in a cooling effect. The impactor interface used in this work was designed to provide a highly porous surface which would dissipate the kinetic energy of the components in the gas jet and thus aid in their deposition as a narrow band.[22] The impactor not only helped to focus the extracts, but it also helped to prevent loss of microcolumn packing during valve switching between extraction mode and separation mode. The authors also found that the LC bandwidth for chlorpyrifos with unmodified CO_2 was smaller than the bandwidth obtained when methanol-modified CO_2 was used. When pure CO_2 was used, the supercritical fluid was decompressed into a gas which had minimal solvating power for the analytes. But when modified CO_2 was used, a portion of the methanol fraction in the fluid condensed to the liquid phase during decompression and was vented as liquid. This resulted in some small droplets of methanol with analytes traveled from the restrictor to the impactor and along the tube toward the vent (which disturbed in a relatively broad band), therefore a broadened bandwidth was found.[22]

3.2.2 Solvent Displacement Method

Mougin et al. [23] reported an SFE-HPLC interface for the determination of triazines in soil. One ten-way valve was used in the interface. Two pumps were used to deliver: 1) trap rinse solution (methanol/water); and 2) LC mobile phase (acetonitrile/water), respectively. During the extraction, the depressurized CO_2 from the restrictor and the rinse solution from the rinse pump were confluent at a "T" connector which passed through the first collection column (function as a trap). After the extraction was completed, the MeOH/H_2O mixture continuously rinsed the system for 5 minutes, in order to remove any residual CO_2 from the system. The ten-way valve was then switched, thus allowing the mobile phase to pass through the first collection column which in turn carried analytes to the analytical column for separation. At the same time, the second collection column could be subsequently placed for trapping during the next sample extraction. A short unstable UV signal during the first 5 minutes of separation was observed. No peak due to CO_2 was found. Unfortunately, no quantitative results were given in this paper.

Recently, Batlle et al. reported on-line coupling of SFE-HPLC for the determination of explosives.[24] The SFE and HPLC were hyphenated by using two six-port valves. When extraction was done, the supercritical fluid CO_2 was depressurized onto a 1-cm porous graphitic carbon trap, allowing for the deposition of analytes. Then, the first valve was switched to fill the trap and connecting tubes with distilled water, which was delivered by an HPLC pump. Any gas remaining in the system was displaced before ignition of the separation. By switching the second valve to the inject position, the mobile phase from another pump passed through the trap. The analytes were desorbed

and carried to the LC column for separation. Two six-port injection valves and associated tubing were used in the interface. Broadened LC peaks were observed due to the introduction of the additional extra column dead volume to the separation system.

4. Summary

Table 1 compared the reported interface and their applications. For those interfaces reviewed in Section 3.1, CO_2 was introduced into HPLC system, when analytes were transferred from SFE to HPLC. From the point of maintaining the LC system and obtaining accurate integration, an ideal interface should avoid CO_2 entering the LC, when analytes are transferred. Only two types of methods were reported to prevent CO_2 from entering the LC system (Section 3.2). One was the backpressure method (Section 3.2.1), where usually a valve or a restrictor was put at the LC outlet to increase the backpressure, so no CO_2 can enter the LC system. Another method was the solvent displacement method (Section 3.2.2). Solvent was introduced into the trap to displace the remaining CO_2, after supercritical fluid was depressurized. To achieve quantitative trace analysis, we need to transfer all the extracts to the LC system when extraction is performed. The reported interfaces with such functions had at least two valves or a tee. The more valves that are used, the more dead volume will be introduced. This will result in a dramatic loss in separation efficiency and make this type of hyphenation system impractical. To make SFE-HPLC a routine method in the analytical lab, we need to simplify the interface as much as possible.

Table 1. Comparison of reported SFE-HPLC interfaces and their application

References	Total transfer	CO_2 enters LC	SFE Modifier	Interface	Trap	Loop	LC	Sample
[14]	No	Yes	No	Two six-port valves	Two 2.5×1 mm RP-18 columns	Trap worked as loop	Normal (silica)	Radix valeriane
[15]	No	Yes	No	One six-port valve	loop worked as trap	Size no mention	Reversed (C-18)	Caffeine, ibuprofen loaded on celite
[17]	No	Yes	Methanol up to 5%	One three-way valve and one six- port valve	loop worked as trap	9 μL	Reversed (C-18, C-18)	PAHs, alkylbenzene sulfonates spiked on sand
[19]	No	Yes	No	Two six-port valves	loop worked as trap	5 μL	Reversed (C-18)	Organophosphorus pesticide
[20]	Yes	Yes	No	One tee and three six-port valves	200 × 0.5 mm i.d. empty steel tubing	Trap worked as loop	Normal (PYE)	PCBs spiked on a glass fiber filter **(quantitative)**
[7]	Yes	Yes	Methanol up to 10%	Three six-port valves	Open-tubular column with various stationary phase	No	Reversed (C-18)	Nitrotoluenes, PAHs, lorazepam etc. spiked on sand **(quantitative)**
[21]	No	No[1)]	No	Two three-way valves and three six-port valves	loop worked as trap	50 μL	Reversed (C-18)	Chlorinated phenols spiked on glass beads **(quantitative)**
[22]	Yes	No[1)]	No	One tee and one six-port valve	0.5 × 0.25 mm i.d. deactivated porous ceramic frit	No	Reversed (C-18, microcolumn)	Chlorpyifos insecticide in a grass sample **(quantitative)**
[23]	Yes	No[2)]	No	One tee, one ten-port valve and one three-port valve	30 × 4 mm id. MCH column	Trap worked as loop	Reversed (C-18)	Triazine compound in soil
[24]	Yes	No[2)]	Methanol up to 10%	Two six-port valves	1-cm porous graphitic carbon	Trap worked as loop	Reversed	explosive in vapor phases **(quantitative)**

1)"back pressure method"
2)"solvent displacement method"

HPLC, especially reversed phase LC is the major separation technique in separation science. The purpose of this research was to develop a simple, novel interface for on-line coupling SFE with RPLC, and explore its ability for quantitative analysis of trace level compounds at different matrices.

In Chapter II, a simple interface was developed via only one six-port injection valve to connect the SFE and LC system. Water displacement method was utilized to eliminate decompressed CO_2 gas in the solid phase trap and connection tubes. To evalute this novel hyphenated system, spiked polynuclear aromatic hydrocarbons (PAHs) from a sand matrix was used as target analytes and extraction condition were optimized in oder to achieve quantitative result. PAHs in naturally contaminated soil were then extracted and determined by this hyphenated system. The results were compared to the EPA method (Soxhlet extraction following by GC-MS).

Based on this hyphenated technique, Chapter III reported the extraction and analysis of hyperforin in St. John's Wort under air/light free condition. Hyperforin is a major active constituent of antidepression herbal medicine — *Hypericum Perforatum* (St. John's Wort). Hyperforin is very sensitive to oxygen and light. Isolated hyperforin is more likely to undergo degradation than when existing in the plant. Since all proposed analytical methods are off-line extraction-analysis modes and have an extract-processing step, it is unavoidable that the extracts come into contact with air before chromatographic analysis. There appears to be no way to determine if degradation has occurred during an analytical extraction or extract-processing step. In hopes of determining the answer to this question, we developed for the first time an air/light free extraction-separation-detection hyphenated system. It involves on-line coupling of SFE-LC with UV

absorbance/electrospray ionization mass spectrometry (SFE-LC-UV/ESI-MS). The feasibility of quantitative extraction and analysis of hyperforin by on-line SFE-LC was explored by optimizing the extraction pressure, temperature, and modifier content.

Chapter IV extended its application to aqueous sample by using a liquid-fluid extraction vessel. Quantitative extraction and transfer were achieved for the target analytes (progesterone, phenanthrene, and pyrene) spiked in water, as well as in real samples (urine and environmental water). During each extraction, no restrictor plugging was realized. Extraction temperature and pressure were optimized. Different amounts of salt were added to the aqueous matrix to enhance ionic strength and thus extraction efficiency. Methanol and 2-propanol were used as CO_2 modifiers. Two methods were compared, e.g. dynamically mixing modifier and pre-spiking modifier.

Chapter V aimed at explores the possible limitation of this hyphenated technique through the analysis of highly polar phenolic compounds in grape seeds. Five types of SFE trapping materials were evaluated in order to enhance the collection efficiency for the extracted polar components. Pure supercritical CO_2 was used first to remove the oil in the seeds. The traditional off-line SFE/LC method was also studied to compare with the on-line method. Both advantages and disadvantages were observed for the on-line mode.

CHAPTER II

Design for On-line Coupling of Supercritical Fluid Extraction with Liquid Chromatography: Quantitative Analysis of Polynuclear Aromatic Hydrocarbons in Solid Matrix

1 Introduction

Supercritical fluid extraction (SFE) is an environmentally friendly technique because CO_2 is employed as the major extraction medium. Compared to extraction with conventional organic solvents, SFE can offer the same or even better extraction efficiencies in a much shorter time. SF extracts are cleaner due to the presence of lower concentrations of interfering co-extractables. This feature permits the direct introduction of extracts into a chromatographic or spectroscopic system without a further clean up step. On line coupling of sample preparation and separation should be highly beneficial for trace analysis, since all of the extract is transferred to the separation column.[7] In addition, the opportunity for the sample to become contaminated, volatilized, degraded is minimized.

On line coupling of SFE to gas chromatography (GC) and supercritical fluid chromatography (SFC) is relatively straightforward due to the nature of the chromatographic mobile phases employed.[8-12] A logical extension of these two hyphenated techniques which could afford a greater sample base would be the interface of SFE to reversed phase liquid chromatography (RP-LC).[13] On-line SFE-LC would be particularly helpful for the analysis of samples which are (a) light or air sensitive, (b) limited in quantity, and (c) in trace quantity.[4] Unfortunately, only a limited number of articles have reported the feasibility of such a technique.[14-25] Even fewer cases have accomplished quantitative extraction and transfer of analytes to the LC column and

detector. Experimentally the problem concerning quantification lies in the poor miscibility of most liquid mobile phases and gaseous CO_2. Residual CO_2 gas is retained by the solid phase SFE trap during CO_2 decompression and may be introduced into the LC system when mobile phase sweeps through the trap to remove the analytes. For a conventional LC system, maintaining the mobile phase as gas-free as possible is vital for optimum pump and detector performance.[4] In other words, irregular baseline noise and variable detector response will be observed.

To date, only two methods have been reported which prevent depressurized CO_2 gas from entering the LC column. One is referred to as the "back pressure" method and the other is termed the "solvent displacement" method. Liu installed a restrictor (8 cm × 25 μm i.d. fused-silica tubing) at the outlet of the LC detector.[21] A back pressure of 850 p.s.i. was found to be adequate for preventing bubble formation (i.e. entrapment of CO_2). Similarly, Cortes put a valve at the LC outlet.[22] Mougin et al. first reported the "solvent displacement" method wherein a mixture of methanol/water (10/90) was introduced to rinse the solid phase trap of CO_2.[23] Unfortunately, no data were provided concerning efficiency of extraction and analyte transfer. Recently, Batlle et al. reported another type of hyphenation whereby two six-port valves were used to connect the SFE and LC.[24] Distilled water from a second LC pump was delivered to replace the gas in the trap, prior to RP-LC. Quantitative results were achieved for the determination of explosives.

For hyphenated techniques related to sample preparation and separation, one key point should be always kept in mind: the interface should be as simple as possible. The more valves and connection tubes that are used to create the hyphenation, the more extra-column dead volume introduced into the system. A complicated design will sacrifice the

separation efficiency and make the hyphenated system less desirable. Each of the reported interfaces had at least two valves or a tee. A much simpler design is provided in this chapter. A single six port injection valve was used to connect two commercial systems (SFE + LC). A solvent displacement method was employed to eliminate residual decompressed CO_2 gas. In this study, polynuclear aromatic hydrocarbons (PAHs) from spiked sand was successfully extracted and quantitatively analyzed by this hyphenated system. High recoveries were achieved under optimized conditions wherein there was no compromise of normal SFE nor RP-LC parameters. Finally certified reference materials with naturally contaminated PAHs were chosen as the real sample. Compared to the EPA method (Soxhlet extraction following by GC-MS), our hyphenated system achieved precise results in a much shorter time and has the potential to be used as an efficient alternative in PAHs analysis.

2. Experimental Section

2.1 Apparatus

A Suprex prepmaster SFE system (Isco-Suprex, Lincoln, NE) equipped with Accutrap and modifier pump was used for all parts of the study. SFE/SFC grade carbon dioxide with 2000 p.s.i. helium head pressure was obtained from Air Products and Chemical Inc. (Allentown, PA). Extractions were performed using a 2.5mL stainless steel extraction vessel (Keystone Scientific, Bellefonte, PA). A 10cm × 0.2cm i.d. stainless steel column filled with C-18 (Isolute SorbentTM, 40 -70 μm particles) was used as the SFE trap.

An Agilent 1050 LC system (Agilent, Wilmington, DE) with programmable multi-wavelength UV detector was used to analyze the extracts. A Phenomenex C-18 column (Torrance, CA), 250 × 4.6 mm with 5 μm particles was used for the separation.

2.2 Reagents and Environmental Sample

LC grade methanol, acetonitrile, acetone and water (Burdick & Jackson, Muskegon, MI) were used. Acenaphthene, anthracene, benzo(a)anthracene, benzo(b)fluoranthene, benzo[α]pyrene, chrysene, dibenzofuran, fluoranthene, fluorene, naphthalene, phenanthrene, and pyrene were bought from Aldrich Chemical. Co. (Milwaukee, WI). Benzophenone (Fisher Scientific, Fair Lawn, NJ) was used as the internal standard for off-line SFE/LC. Environmental sample was soil contaminated with PAHs, provided as natural matrix certified reference material (CRM115-100) by Resource Technology Corp. (Laramie, WY).

2.3 Design for SFE-LC Interface

The SFE and LC systems were interfaced by a single six-port injection valve (Valco, Houston, TX). At original position (Figure 6a) mobile phase was continuously and directly introduced into the LC column by the LC pump. During this time dynamic extraction was conducted and the CO_2 containing extracted analytes were depressurized via the restrictor in the solid phase trap. When the extraction was completed, water was admitted to the restrictor and trap from the Accutrap rinse pump, which was a component of the SFE instrument. Residual CO_2 gas was thus eliminated from the trap. Analytes which exhibited low water solubility continued to be absorbed on the trap material. The

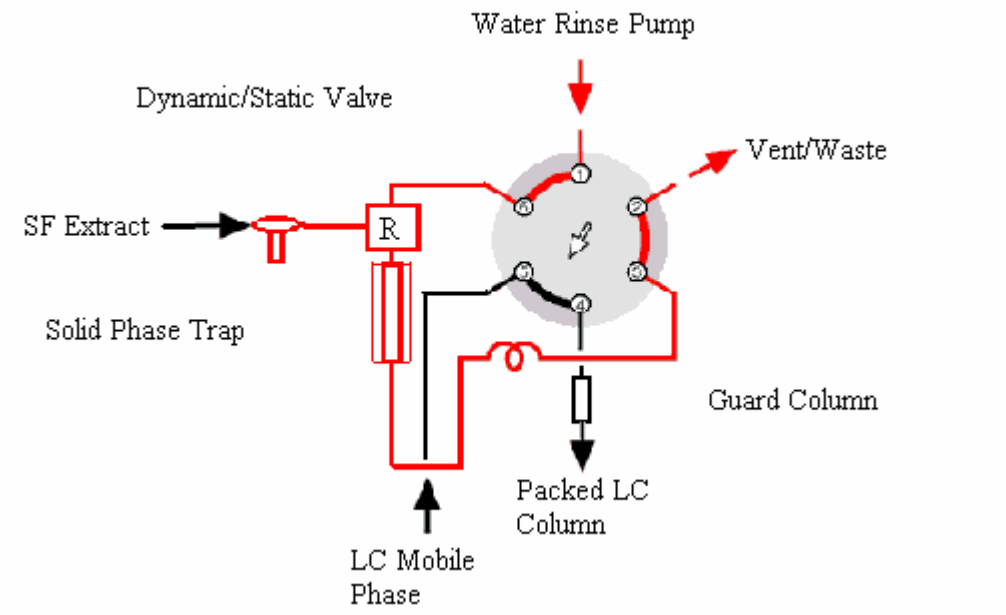


Figure 6a. The interface position for removal of residual gas from solid phase trap by water.

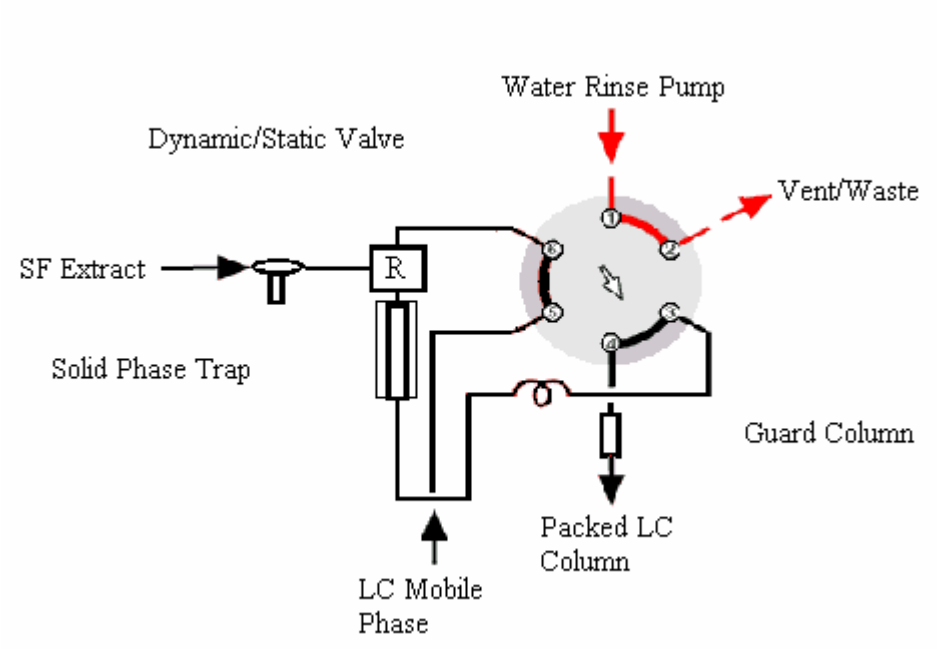


Figure 6b. The interface position for removal of extracted analytes from trap onto LC column by LC mobile phase.

valve was next re-positioned such that mobile phase passed through the trap and carried the analytes onto the LC column (Figure 6b). A guard column was placed before the LC column as a caution to prevent the escape of any particles from the trap that may plug the LC column.

2.4 Extraction Procedure

For spiked sample, the extraction vessel was loosely packed with Ottawa sand (Fisher Scientific, Fair Lawn, NJ) and spiked with 10 μL of a PAH stock solution at the beginning of each run. The SFE variable restrictor was heated to 55°C for all extractions. If not noted, the trap temperature was kept at 0°C for collection and 20°C for desorption of analytes. The flow rate of liquid CO_2 was set at 1 mL/min. Prior to analyte desorption from the trap, the flow rate of rinse water was 1 mL/min for 5 minutes.

For environmental sample, 0.1g soil was put into the vessel and sand was used to fill the remaining space. Two step extractions were employed. Initially, 60 gram pure CO_2 was used as the extraction fluid. The extraction pressure was 450 atm at 80°C and the flow rate of liquid CO_2 was 1 mL/min. the trap temperature was kept at -10°C for collection and 10°C for desorption of analytes. While in the second step, 10% acetone-modified CO_2 was employed. 100g liquid CO_2 was consumed and the flow rate was 2 mL/min. The trap temperature was set at 60°C for desorption of analytes. Other conditions were same with step one.

2.5 Separation Procedure

For spiked sample, the mobile phase was methanol/water (80:20, v/v) for the first 2 minutes, then ramped to 87:13 at 9 minutes, to 100% methanol at 13 minutes and held there for 3 minutes. Flow rate was 1.5 mL/ min. The detection wavelength was set at 250 nm. Direct injection of standards into the LC column was accomplished using a Valco injection valve (Houston, TX) with a 10 μL sample loop.

For environmental sample, the mobile phase was begun at 45% acetonitrile and 55% water, ramped to 95:5 at 45 minutes and kept for 5 minutes, then to 100% acetonitrile at 55 minutes and held there for 10 minutes. Flow rate was 1.0 mL/ min. The detection wavelength was set at 254 nm.

3. Research and discussion

3.1 Spiked Sample

A solution of five PAHs spiked onto a sand matrix was used to evaluate the interface. As a point of reference, direct injection of the stock sample solution to the column provided a chromatogram with excellent peak shapes and reasonably good resolution between peaks (Figure 7a). On-line SFE/LC of the same analytes spiked into the extraction vessel filled with sand, extracted with pure CO_2, collected on the trap, mobilized from the trap by the mobile phase, and analyzed via LC yielded the trace shown in Figure 7b (SFE: 350 atm CO_2 @ 60°C, 1 minute static extraction and 20 minutes dynamic extraction). The artifact peak (U) was corresponding to the transient change of mobile phase composition and resulted in the change of refractive index, when the water in the trap entered the LC system at inject position. When acetonitrile/water was used as the mobile phase, this artifact peak was not observed.

a.

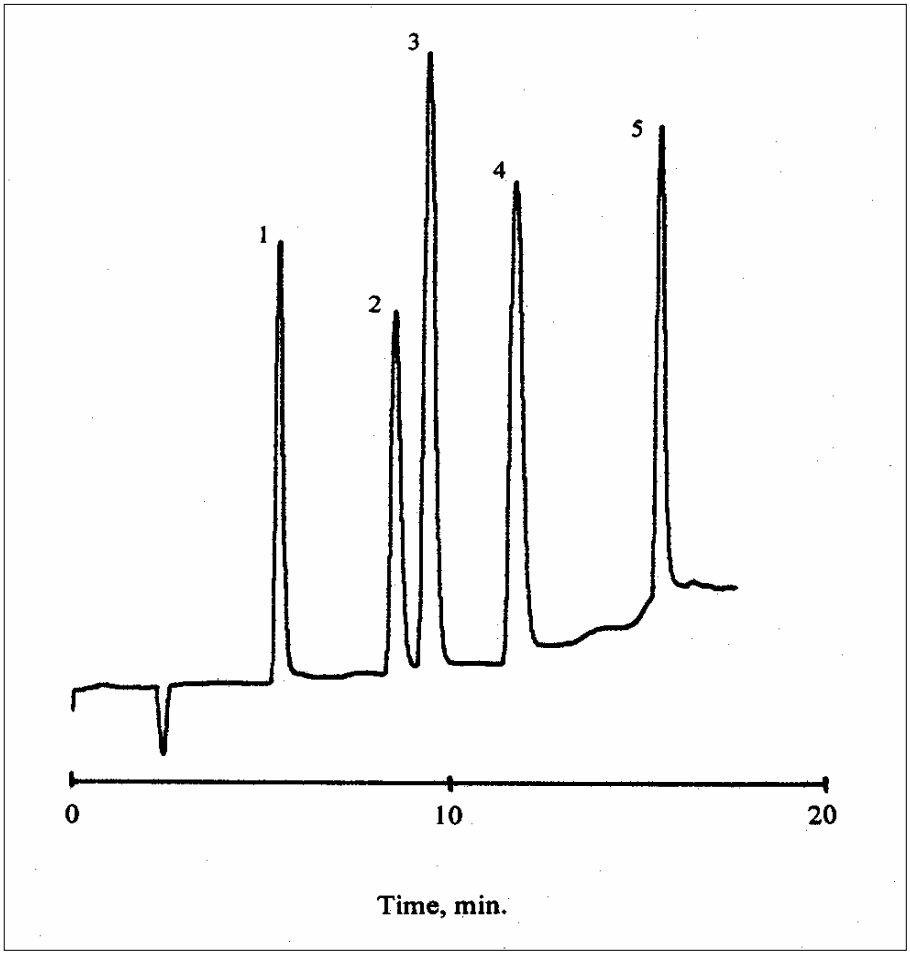


Figure 7a. Chromatograms of spiked PAHs sample by direct injection through 10 µL sample loop. (1) naphthalene, (2) fluorene, (3) anthracene, (4) pyrene, and (5) benzo[α]pyrene. A C-18 LC column (250 × 4.6 mm × 5 µm) was used for separation. The mobile phase was methanol/water (80:20, v/v) for the first 2 minutes, then ramped to 87:13 at 9 minutes, to 100% methanol at 13 minutes and held there for 3 minutes. Flow rate was 1.5 mL/min. The UV detection wavelength was set at 250 nm. The concentration was 40 µg/mL for naphthalene, 10 µg/mL for fluorene, 2 µg/mL for anthracene, 30 µg/mL for pyrene and 6 µg/mL for benzo[α]pyrene.

b.

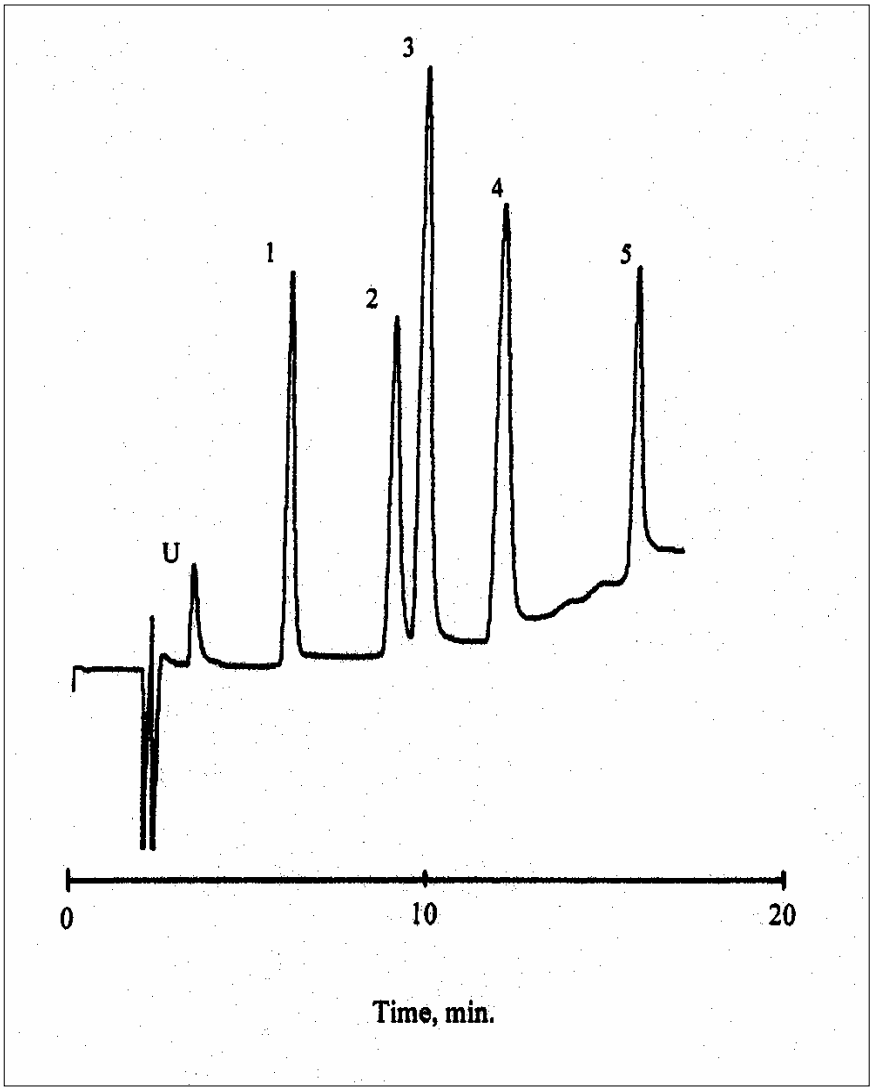


Figure 7b. Chromatograms of spiked PAHs sample by on-line SFE-LC. (U) unknown, (1) naphthalene, (2) fluorene, (3) anthracene, (4) pyrene, and (5) benzo[α]pyrene. SFE: 350 atm CO_2 @60°C, 1 minute static extraction and 20 minutes dynamic extraction. 10 µL stock solution was spiked into sand. C-18 was used as sorbent in the trap. Trap temperature was 0°C for collection and 20°C for desorption. 5 mL water was delivered to replace residual CO_2 in the trap. See Figure 7a for LC conditions.

Table 2 gives the comparison of the two methods relative to five chromatographic parameters: retention time (t_r), theoretical plates (N), retention factor (k), selectivity (α) and resolution (R_s). Compared to the direct LC method, the retention time of analytes via on-line SFE-LC naturally was a little longer. Slight band broadening was also observed for the on-line method compared to direct injection. Such band broadening could be attributed to the additional dead volume introduced into the system by the extra valve and associated connection tubing. Compared to the regular LC method, the on-line coupling of SFE to LC resulted in only 9.9% loss in theoretical plates, 19.2% loss in retention factor, 1.2% loss in selectivity, and 11.9% loss in resolution (average values) under these initial conditions.

A series of experiments were thus undertaken to ensure optimal conditions were being used. The trap efficiency was tested first. The collection temperature of the trap was varied from -10°C to 60°C and recovery for both on-line SFE/LC and off-line SFE/LC methods was compared. For both methods, when collection temperature was increased from -10°C to 60°C, the major difference was in the recovery of the more volatile naphthalene. For the off-line method, recovery was dramatically reduced to 60% at a trap temperature of 60°C, compared to 83% at -10°C. For the on-line method, there was almost no change in recovery. One possible reason for this difference is that the on-line method incorporated a ~30 cm long stainless steel tube which connected the six-port valve to the solid phase trap. This tube was always kept at room temperature although the temperature of the trap was varied. At high trap collection temperatures (above ambient), naphthalene may deposit on the cooler inter-wall of the tubing. This would not present a problem since mobile phase would carry this naphthalene as well as that absorbed in the

Table 2. Comparison of chromatographic parameters via on-line SFE-LC and via direct injection.

Compound	Direct Injection					On-line SFE/HPLC				
	t_r (min)	N	k	α	R_s	t_r (min)	N	k	α	R_s
Naphthalene	5.40	1620	2.11	~	~	5.94	1460	1.77	~	~
Fluorene	8.45	2240	3.87	1.83	4.26	8.87	1890	3.13	1.77	3.59
Anthracene	9.31	2140	4.37	1.13	1.08	9.69	1960	3.51	1.12	0.92
Pyrene	11.61	1870	5.69	1.30	2.12	11.80	1690	4.53	1.29	1.89
Benzo[α]pyrene	15.46	7480	7.91	1.39	5.39	15.64	6960	6.28	1.39	5.05

t_r: retention time;
N: theoretical plates;
k: retention factor;
α: selectivity;
R_S: resolution.

trap into the LC column. In the off-line SFE method, the outlet of the trap was connected with a short plastic tube to transfer the extracted analytes to the collection vial. When the trap collection temperature was increased to 60°C, no doubt naphthalene was inefficiently trapped and may have been lost from the system into the atmosphere.

After depressurization of CO_2 and analyte trapping, water was routinely delivered into the trap to displace any remaining CO_2 gas which could have entered the LC system when analyte was desorbed from the trap. Data showed that when the volume of rinse water was increased, from 5 mL to 15 mL, there was almost no loss of recovery for fluorene, anthracene, pyrene and benzo[α]pyrene. This is due to their very low solubility in water (2, 0.073, 0.14 and 0.0058 mg/L, respectively).[26] For naphthalene, recovery decreased about 3% with increasing amounts of rinse water, which agreed with the higher solubility of naphthalene in water (32 mg/L).[26]

It has been reported that when the supercritical fluid decompresses through the restrictor, the sharp drop in density results in a high gaseous flow rate which can in turn result in considerable loss of trapping efficiency.[7] We, therefore, have studied the influence of CO_2 fluid flow rate on extraction recovery. It was found that when the measured CO_2 liquid flow rate at the pump was increased from 1 mL/min to 3 mL/min[a], no loss in trapping efficiency was observed for both on-line and off-line SFE/LC methods when trapping temperature was kept at 0°C. On the contrary, there was about a 6% increase in recovery for benzo[α]pyrene when the flow rate increased. For each run, extraction time was kept at 20 minutes, so a higher flow rate would mean more CO_2 was used for extraction. From the extraction profiles (Figure 8), we can observe that

naphthalene needed only 5 minutes to reach maximum recovery, while anthracene required 20 minutes. Benzo[α]pyrene, however, need 30 minutes to reach equilibration, thus a higher recovery was achieved when the higher flow rate was used.

Table 3 shows the recovery of the five PAHs at four different extraction conditions. The extraction efficiency for PAHs seems to be more dependent on temperature than on pressure. At both operating pressures, a slightly higher extraction recovery for all PAHs was obtained at 100°C, even though the CO_2 fluid density was lower at 100°C than at 60°C. A similar result was reported in Langenfeld's research.[27]

Different amounts of matrix with a fixed mass of sample also have been tested for their recovery. No decrease in recovery was found when the matrix (sand) mass increased from 1g to 10g. This suggested that when the concentration of analytes is very low, larger amounts of real sample matrix can be extracted.

Even at optimized conditions when pure CO_2 was used as the extraction fluid, the recoveries of PAHs were less than 92%. In certain cases, it is desirable to add a co-solvent (modifier) to the supercritical fluid to enhance the solvating power of the fluid thus enhancing the solubility of an analyte in the extracting medium. The addition of modifier may also improve the separation factor between solutes as they are selectively partitioned into the supercritical fluid phase.[3] We investigated the modifier effect on the extraction efficiency of PAHs by using different percentages of methanol (Table 4). The best results were obtained with 5% methanol. Recovery increased ~3% for naphthalene,

---

[a] Supercritical fluid low rates would be 25-50% greater.

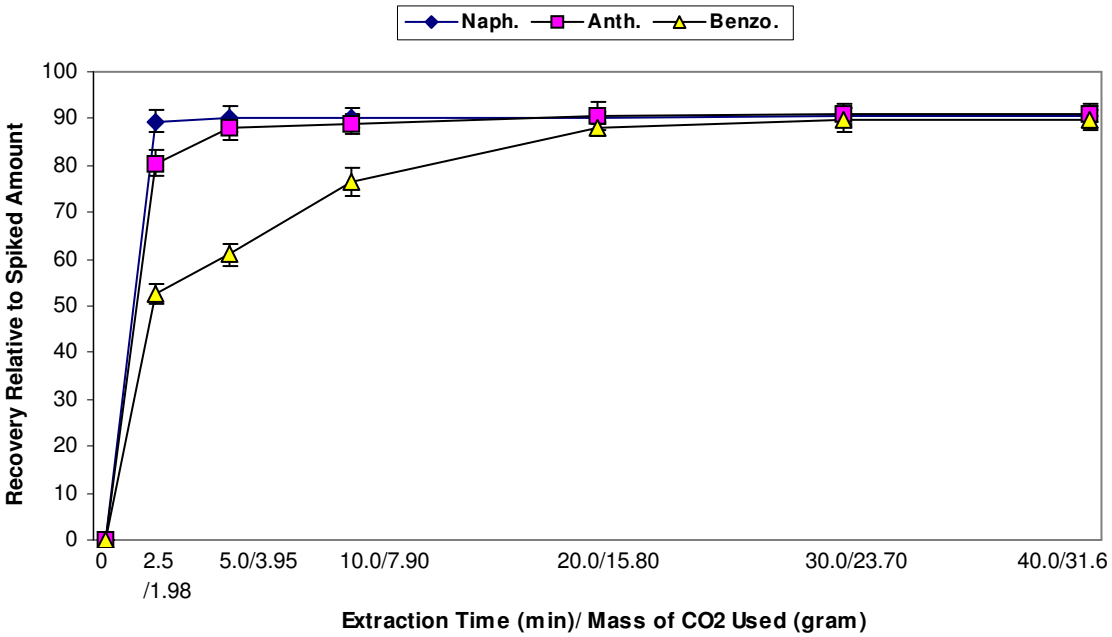


Figure 8. Extraction profiles for naphthalene, anthracene, and benzo[α]pyrene from spiked sample (on-line SFE-LC). SFE: 450 atm CO_2 @100°C, other conditions are same as Figure 7.

Table 3. The influence of extraction temperature, CO_2 pressure, and density on extraction efficiency. (See Figure 7 for HPLC conditions and SFE conditions.)

Compound	350 atm 60 °C (0.87 g/mL)	350 atm 100 °C (0.71 g/mL)	450 atm 60 °C (0.90 g/mL)	450 atm 100 °C (0.79 g/mL)
Naphthalene	80.4 (5.4)	88.4 (5.1)	89.9 (4.9)	90.3 (5.7)
Fluorene	85.8 (5.9)	89.0 (5.9)	91.5 (5.3)	91.7 (5.5)
Anthracene	83.6 (5.2)	89.2 (5.0)	90.0 (5.2)	90.5 (5.8)
Pyrene	80.6 (6.3)	87.6 (6.0)	87.5 (5.8)	87.5 (5.9)
Benzo[α]pyrene	63.0 (5.9)	77.4 (6.1)	84.8 (6.3)	87.9 (6.2)

* Numbers in parenthesis are % relative standard deviation (n = 3)

Table 4. The influence of modifier (methanol) on extraction efficiency. (See Figure 7 for HPLC conditions; SFE: 450 atm CO_2 @ 100°C)

Compound	Recovery				
	0%[a]	1%[a]	5%[a]	10%[b]	20%[b]
Naphthalene	90.3 (4.7)	90.4 (4.9)	93.5 (5.0)	44.3 (5.2)	36.5 (4.8)
Fluorene	91.7 (4.8)	91.9 (4.5)	96.1 (3.9)	96.1 (5.5)	96.3 (4.1)
Anthracene	90.5 (5.1)	93.2 (5.9)	95.4 (4.6)	95.3 (5.2)	97.1 (5.4)
Pyrene	87.5 (5.7)	95.4 (5.3)	96.1 (4.9)	96.1 (5.1)	96.3 (6.0)
Benzo[α]pyrene	87.9 (6.2)	90.6 (4.7)	92.4 (5.8)	93.5 (5.7)	98.1 (5.6)

* Numbers in parenthesis are % relative standard deviation (n = 3)
[a]. Trap temperature: 0°C
[b]. Trap temperature: 60°C

~5% for fluorene, anthrancene and benzo[α]pyrene, and ~9% for pyrene, compared to the results achieved with pure CO_2. When higher percentages modifier were used, the trap temperature had to be increased from 20°C to 60°C to avoid modifier condensation in the trap. The increased modifier resulted in a big loss in naphthalene recovery (about 50%), but for the other four PAHs, recoveries were higher than 96%.

The linear range of C-18 trap capacity (10cm × 0.2cm i.d.) was also investigated (Table 5). Samples with series concentrations were extracted and analyzed. The linear range was ~10^2 for anthracene, benzo[α]pyrene, ~10^3 for naphthalene and fluorene. All the correlation coefficients were larger than 0.99. When higher amounts of analytes were extracted and introduced into the LC, recovery was not quantitative due to limited trap capacity. When lower amounts of PAHs were extracted, no chromatographic peak was detected due to relatively low sensitivity of the UV detector. If a higher capacity trap or a more sensitive detector were used, a broader linear range would be anticipated.

3.2 Environmental Sample

Finally, natural PAHs contaminated soil (certified reference material) was used to evaluate this hyphenated system. It is well-known that recoveries obtained with spiked samples may not be representative of those obtained with real sample. The analytes in real samples can be strongly absorbed inside the porous matrix, whereas spiked analytes are generally lightly coated on the surface of the matrix. Therefore, it is necessary to use longer extraction time for certified reference materials. Here two extraction steps were

Table 5. Linear range, correlation coefficient, and limit of detection of SFE-LC data. (See Figure 7a for HPLC conditions; SFE: 450 atm CO_2 @ 100°C, 5% methanol was used as modifier.)

Compound	Linear range [a]	Correlation coefficient (n = 5)	LOD [b]
Naphthalene	40 - 60,000 ng	0.9992	4 ng
Fluorene	10 - 10,000 ng	0.9997	1 ng
Anthracene	2 - 200 ng	0.9998	0.2 ng
Pyrene	30 - 4500 ng	0.9996	6 ng
Benzo[α]pyrene	6 - 600 ng	0.9994	1 ng

[a.] Amount of analytes spiked in sand.
[b.] Limit of detection (signal to noise = 3).

employed, where pure CO_2 was used in the first step to remove the low molecular weight PAHs, and acetone-modified CO_2 was employed to remove the high molecular weight PAHs. Figure 9a and 9b are the LC chromatogram for these two step extractions, respectively. Final quantitative determination was based on the overall recoveries. Due to the poor separation ability for PAHs, this C-18 column could not separate acenaphthene and fluorene, chrysene and benzo(a)anthracene, they eluted as two single peaks, and their amounts were calculated as the summations.

Table 6 gave the data for reference values and standard deviation by EPA method 3450C (Soxhlet extraction) and 8270C (semivolatile organics by GC-MS), and results by this on-line SFE-LC method. The *t*-test (p=0.05) shows that for most of the results, there is no statistically significant difference between two approaches; however, on-line SFE-LC achieved quantitative result in much shorter time. The whole extraction-analysis time was about 3 hours, much shorter than related EPA methods (24 hours Soxhlet extraction following by GC-MS analysis), and also much more labor-saved than off-line SFE-LC method, which concentration step is essential.

4. Conclusion

In this study, a novel, simple interface for on-line SFE-LC was designed. Only one injection valve was used to interface two commercial systems. We successfully solved the problem of the depressurized CO_2 gas by using water displacement method. No CO_2 gas was entered into LC system. The simple design provided the technical guarantee that less sacrifice in separation efficiency was introduced to the whole system due to the additional extra column dead volume. Quantitative results were achieved for

PAHs in spiked sample and real environmental sample. The method proved to be more efficient and labor-saving than the standard EPA method.

a.

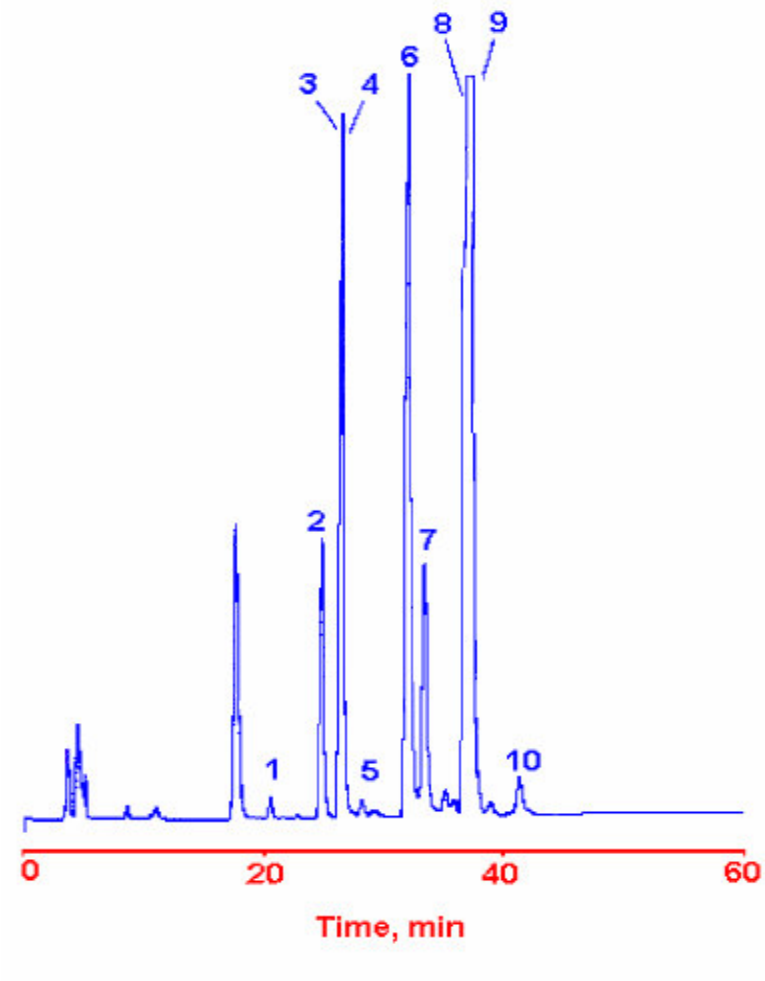


Figure 9a. Chromatograms of environmental sample by on-line SFE-LC: the first step extraction. 0.1g soil was put into the vessel and sand was used to fill the remaining space. During the first step extraction, 60 grams of pure CO_2 was used and the extraction pressure was 450 atm @ 80°C. The flow rate of liquid CO_2 was 1 mL/min. The trap temperature was -10°C for collection and 10°C for desorption. While in the second step, 10% acetone-modified CO_2 was employed. 100g liquid CO_2 was consumed and the flow rate was 2 mL/min. The trap temperature was 60°C for desorption. Other conditions were the same as step one. (1) Naphthalene, (2) Dibenzofuran, (3) Acenaphthene, (4) Fluorene, (5) Phenanthrene, (6) Fluoranthene, (7) Pyrene, (8) Chrysene (9) Benz[a]anthracene, (10) Benzo[b]fluoranthene.

b.

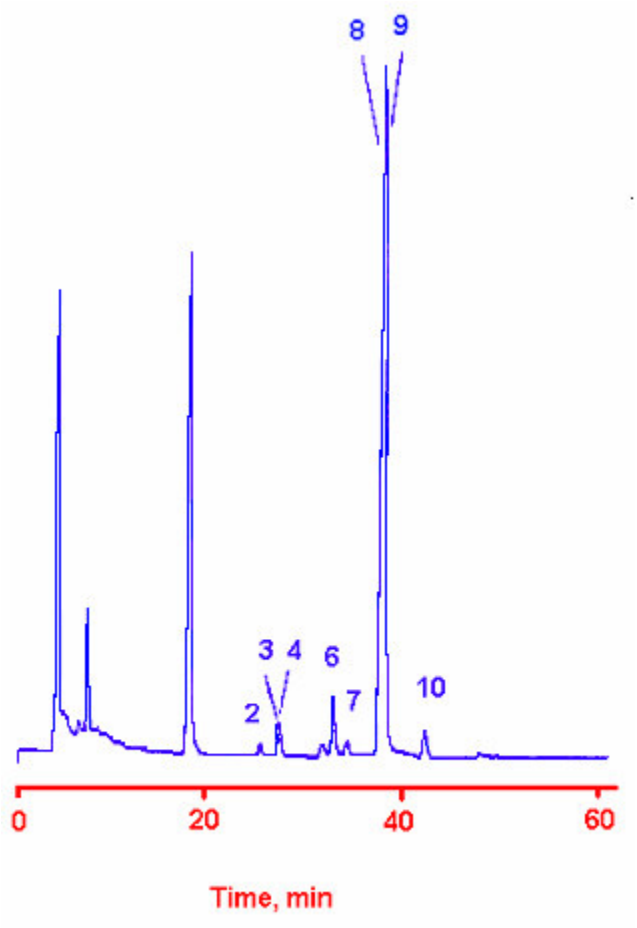


Figure 9b. Chromatograms of environmental sample by on-line SFE-LC: the second step extraction. See Figure 9a for conditions.

Table 6. Comparison of EPA method and on-line SFE-LC for the analysis of PAH contaminated soil.

Compounds	Reference Value[a] (mg/kg)	Standard Deviation[a] (n = 15) (mg/kg)	Value by SFE-LC (mg/kg)	Standard Deviation (n = 3) (SFE-LC) (mg/kg)	Statistical Difference of the two results (*t*-test, $P = 0.05$)
Naphthalene	1.34	0.29	**1.26**	0.06	No
Dibenzofuran	10.6	2.53	**17.02**	0.54	Yes
Acenaphthene/ Fluorene	17.60	3.81	**19.99**	0.45	No
Phenanthrene	0.08	0.01	**0.072**	0.007	No
Fluoranthene	22.1	4.75	**26.60**	1.17	No
Pyrene	7.66	1.98	**9.83**	0.96	No
Chrysene/ Benz[a]anthracene	28.9	5.63	**37.78**	6.59	Yes
Benzo[b]fluoranthene	0.93	0.25	**0.78**	0.16	No

[a]. The reference values were determined by USEPA SW846 (3rd edition) method 3450C (Soxhlet extraction) and
8270C (semivolatile organics by GC-MS).

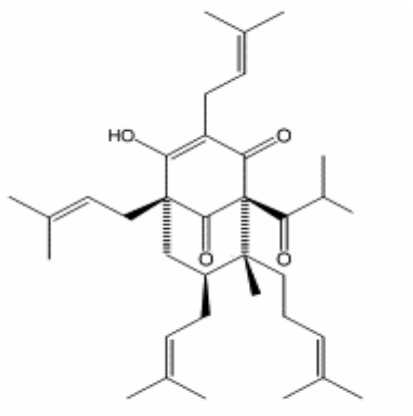
CHAPTER III

Determination of Hyperforin and its degradation products in *Hypericum Perforatum* Under Air/Light-Free Environment

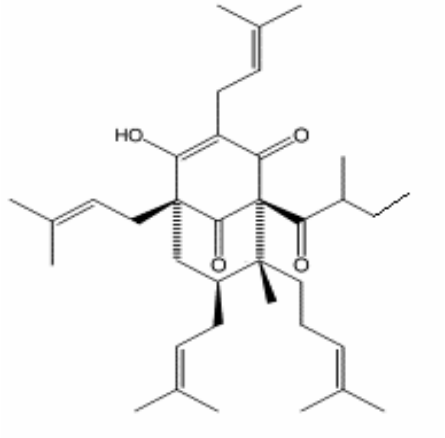
1. Introduction

St. John's Wort (SJW), with the botanical name *Hypericum Perforatum*, is a short, yellow-flowering plant native to Europe, West Asia, and North Africa. It has a 2,400-year history of safe and effective usage in folk herbal medicine. In the last two decades many studies have demonstrated that extracts of SJW are as effective as prescription anti-depressants for the treatment of mild to moderate depression, and with fewer side effects and at considerably lower cost.[28-32] Typically, SJW is administrated as capsules or teas. Originally, the naphthodianthrones (hypericin) were thought to be responsible for the antidepressant activity of hypericum extracts, thus the hyperforin content was used for standardization of SJW products.[33] Hyperforin and adhyperforin (Figure 10) are the major phloroglucinol constituents found in the lipophilic fraction of the SJW extract. Recent research, however, has found that hyperforin might be the critical component to account for the antidepressant activity of SJW, working as a potent inhibitor of uptake of serotonin (5-HT), dopamine (DA), noradrenaline (NA), and L-glutamate.[34, 35] Hyperforin is very sensitive to oxidation and light, which may account for differences in the pharmacological activity of the extracts.[36, 37] The major degradation product of hyperforin is furohyperforin, with a molecular weight of 552 amu (Figure 10).[38]

a.



b.



c.

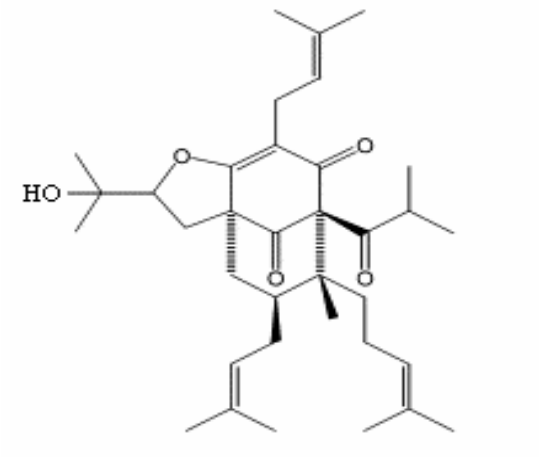


Figure 10. Chemical structures of hyperforin, adhyperforin, and furohyperforin. (a) hyperforin (536 amu), (b) adhyperforin (550 amu), and (c) furohyperforin (552 amu).

For analysis of SJW, typical extraction methods are liquid-liquid extraction (LLE) and ultrasonic extraction (USE). Supercritical fluid extraction provides another approach. Recently, supercritical fluid CO_2 was used to extract phloroglucinols (hyperforin and adhyperforin) in SJW.[39] Compared with data obtained by ultrasonic extraction, supercritical fluid CO_2 extracted only about 60.8% hyperforin under the optimized conditions.

Isolated hyperforin may be more likely to undergo degradation than when existing in the plant. Since all proposed analytical methods are off-line extraction-analysis modes and have an extract-processing step, it is unavoidable that the extracts come into contact with air before chromatographic analysis. There appears to be no way to determine if degradation has occurred during an analytical extraction or extract-processing step. To answer this question, an air/light free extraction-separation-detection hyphenated system has been designed for the first time. It involves on-line coupling of supercritical fluid extraction with liquid chromatography-UV absorbance/electrospray ionization mass spectrometry (SFE-LC-UV/ESI-MS). SFE with a CO_2-based fluid yields extracts that are cleaner due to lower concentrations of interfering co-extractives. This feature permits the direct introduction of extracts into an analytical system without a further clean up step. Supercritical CO_2 is an inert extraction media with a low critical temperature. The anayltes, therefore, can be extracted out at very mild conditions (moderate temperature). On line coupling of SFE and liquid chromatography is consequently highly beneficial for the trace analysis of air/light-sensitive, thermal liable compounds since all the extract is directly transferred to the analytical system in an air/light-free environment. Thus, the opportunity for sample degradation or loss during sample processing is avoided. The

feasibility of quantitative extraction and analysis of hyperforin by on-line SFE-LC was also explored. CO_2-modifier and a solid C_{18} trap were utilized to improve extraction and collection.

2. Experimental Section

2.1 Chemicals and Sample

Dried *Hypericum Perforatum* leaf/flower was ground using a coffee grinder. The grinding was halted for ~15 sec at periodic intervals to prevent heating of the sample. All the materials were stored at -20°C. To avoid any possible light degradation, all the sample vials were wrapped with aluminum foil. Hyperforin standard and formic acid were purchased from Sigma-Aldrich Chemical Co., (Milwaukee, WI). HPLC grade methanol, acetone, acetonitrile, and water (Burdick & Jackson, Muskegon, MI) were used. Before use, the solvent was purged with helium for 30 minutes to eliminate any dissolved oxygen.

2.2 SFE-LC-UV/ESI-MS Apparatus

An Isco-Suprex (Lincoln, NE) Prepmaster supercritical fluid extraction system equipped with Accutrap and modifier pump was used for the study. SFE/SFC grade carbon dioxide with 2000 p.s.i. helium head pressure was provided by Air Products and Chemical Inc. (Allentown, PA). Extractions were performed using a 2.5 mL stainless steel extraction vessel (Keystone Scientific, Bellefonte, PA). In the on-line SFE-LC mode, 5.0 mg of ground sample (50 mg sample in off-line SFE-LC mode) was mixed with enough Ottawa sand (Fisher Scientific, Fair Lawn, NJ) to fill the vessel. The SFE

variable restrictor was heated to 55°C for all extractions. A 10 cm × 0.2 cm i.d. stainless steel column filled with C_{18} (Isolute Sorbent™, 40 -70 μm particles) was used as the SFE trap. If not noted, the trap temperature was kept at 0°C for collection and 20°C for desorption of the analytes. In this study, the flow rate of liquid CO_2 prior to being heated passed the critical temperature was set at 1 mL/min. Prior to analyte desorption from the SFE trap, the flow rate of rinse water was 1 mL/min for four minutes in order to replace any CO_2 gas remaining in the trap. The trap rinse solvent in off-line SFE was methanol.

HPLC analyses were performed with an Agilent (Wilmington, DE) 1050 quaternary HPLC pump and a programmable multi-wavelength UV detector. The HPLC was interfaced to a MicroMass (Milford, MA) Platform quadrupole mass spectrometer equipped with an APCI/ESI ionization chamber. The HPLC column output was spilt 1:10 with 1 part going to the MS and 9 parts going to the UV detector. The mass spectrometer was operated in the positive electrospray mode. ESI conditions: electrospray voltage 3.5 kV, cone voltage 31V, source temperature 120°C, nebulizing gas (N_2) 20L/h, scan range *m/z* 250 to 700.

Figure 11 shows the schematic diagram of the hyphenated SFE-LC-UV/MS system. Experimental details concerning the SFE-LC interface see Chapter 2. Approximately, one tenth of the mobile phase flow exiting the LC column was introduced to the mass spectrometer via a splitter (m). The waste tube (p) was always submerged into the liquid in the waste bottle (q) to prevent air from entering the system. Before each extraction, the system was run in the dynamic extraction mode for 1 minute in order to sweep out any air that might remain in the vessel and tubing.

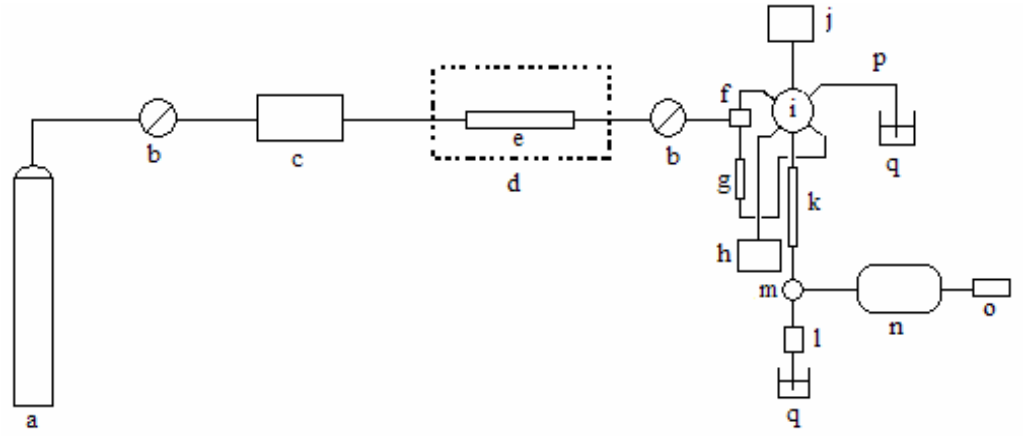


Figure 11. Schematic diagram of the hyphenated SFE-LC-UV/MS system. (a) CO_2 tank; (b) two-way valve; (c) SFE pump; (d) extraction oven; (e) extraction vessel; (f) restrictor; (g) collection trap; (h) LC pump; (i) SFE-LC interface; (j) water rinse pump; (k) LC column; (l) UV detector; (m) splitter; (n) ESI-MS; (o) acquisition system; (p) waste tube; (q) waste bottle. See Chapter 2 for the operation of the interface in detail.

2.3 Determination of Hyperforin Concentration via USE and ESE

The concentration of hyperforin first was determined by two liquid-solid extraction methods, ultrasonic extraction (USE) and enhanced solvent extraction (ESE). An Aquasonic model 75HT ultrasonic extractor (VWR Scientific Products) was used for off-line USE. A 20 mL extraction vial tightly wrapped with aluminum foil was located in an ice-water bath in order to maintain the temperature during extraction. Methanol was used as the extraction solvent. For each extraction, 50 mg of sample was extracted with 5 mL of methanol. When extraction was completed, extracts were centrifuged for 10 minutes and then the supernatant was passed through a 0.45 um PTFE filter into an amber vial for analysis. Figure 12 illustrates the typical liquid chromatogram of a methanol ultrasonic extract. Exhaustive extraction was achieved after four cumulative extraction cycles (cycle time = 30 min) were performed. The concentration of extractable hyperforin in the dried plant was determined to be 8.74±0.26 mg/g (n =3).

ESE was then applied in order to verify the above result. It was performed off-line with an Isco SFx-3560 extractor with capability for both a supercritical extraction and enhanced solvent extraction function (Lincoln, NE). At the beginning of each run, 50 mg of ground sample was mixed with Ottawa sand (Fisher Scientific, Fair Lawn, NJ) to fill a 10 mL extraction cartridge. The vessel was pressurized with methanol to the desired pressure. After attaining equilibrium conditions (2000 p.s.i., 60°C), static extraction was initiated. When it was completed, the analyte valve was open to allow 8 mL of solvent (methanol) to wash the extracted material out of the cartridge with a flow rate of 1 mL/min. Then pressurized CO_2 was used to flush the remaining solvent out. Flush time was 5 minutes. All the solution that eluted from the vessel was collected and the volume

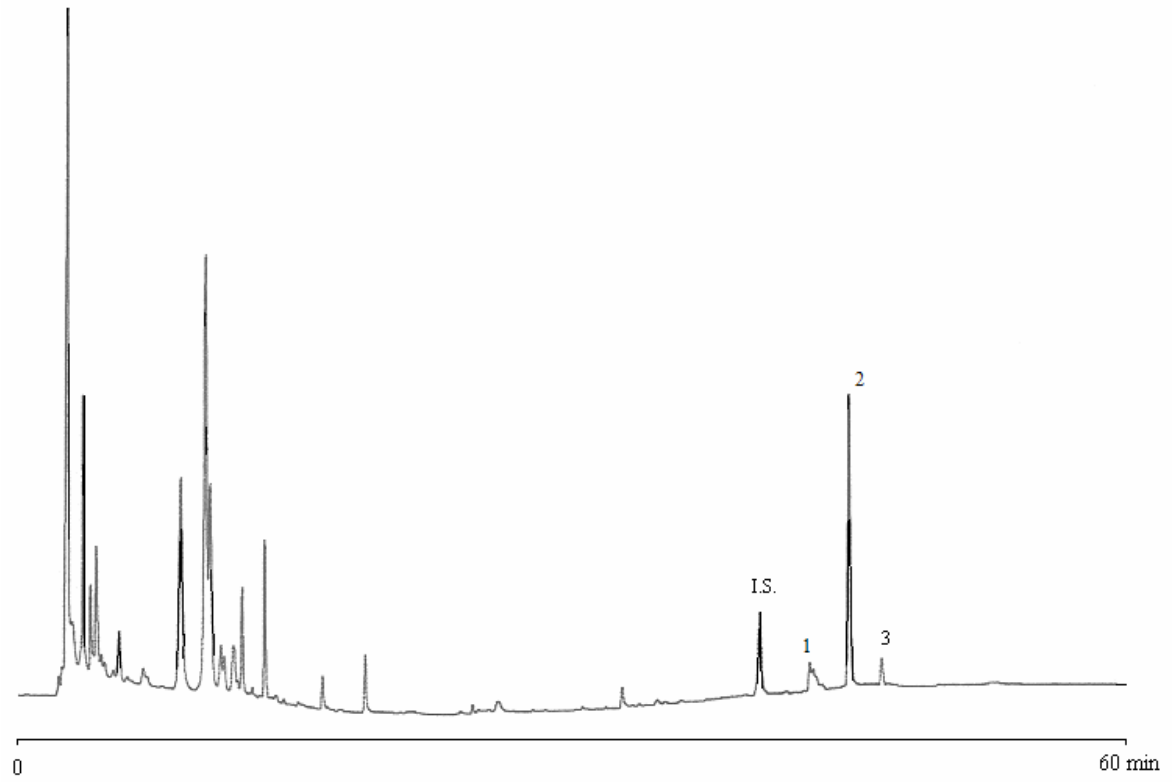


Figure 12. Chromatogram of the ultrasonic methanol extracts of St. John's Wort. (I.S.) benzo[k]fluoranthene (internal standard), (1) furohyperforin, (2) hyperforin, and (3) adhyperforin. YMC-ODS AQ column (250 × 4.6 mm × 5 μm) was used for separation. 20% acetonitrile-80% water (containing 0.5% formic acid, v/v) kept for 5 minutes, then increased to 70% over the next 20 minutes (ramp rate 2.5% per min.), then increased to 90% over 10 minutes (ramp rate 2% per min.), and then to 100% acetonitrile from 35 to 45 minutes (ramp rate 1% per min.) and held there for 15 minutes. Flow rate was 1.0 mL/ min. The UV detection wavelength was set at 272 nm. Ultrasonic extraction: 50 mg of sample was extracted with 5 mL methanol for 30 min. 10 μL benzo[k]fluoranthene (1400 μg/mL) was added as the internal standard after the extraction.

of the solution was made up to 20 mL with methanol for LC analysis. Figure 13 shows a typical chromatogram of an ESE methanol extract. The total ES extractable hyperforin was comparable to the result of ultrasonic extraction (8.74 mg/g sample). All the SFE recovery data in this paper are relative to the USE result.

2.4 Separation Procedure

An YMC ODS-AQ column (Waters, Milford, MA), 250 × 4.6 mm with 5 μm particles was used for the separation. The mobile phase was acetonitrile and water (containing 0.5% formic acid, v/v). A short LC gradient was developed for the separation of SF extracts. The gradient began at 85% acetonitrile for 5 minutes, then increased to 100% acetonitrile over the next 15 minutes (ramp rate 1% per min.) and held there for 5 minutes, with a total analysis time of 25 minutes. Flow rate in each case was 1.0 mL/min. The UV detection wavelength was set at 272 nm. Direct injection of analyte standards into the LC column was accomplished by using a Valco injection valve (Houston, TX) with a 10 μL sample loop.

2.5 Calibration Methods

An internal standard calibration method was used in the off-line SFE-LC mode as well as in USE and ESE experiments. Benzo[k]fluoranthene was added as the internal standard. The external standard calibration method was used in on-line SFE-LC.

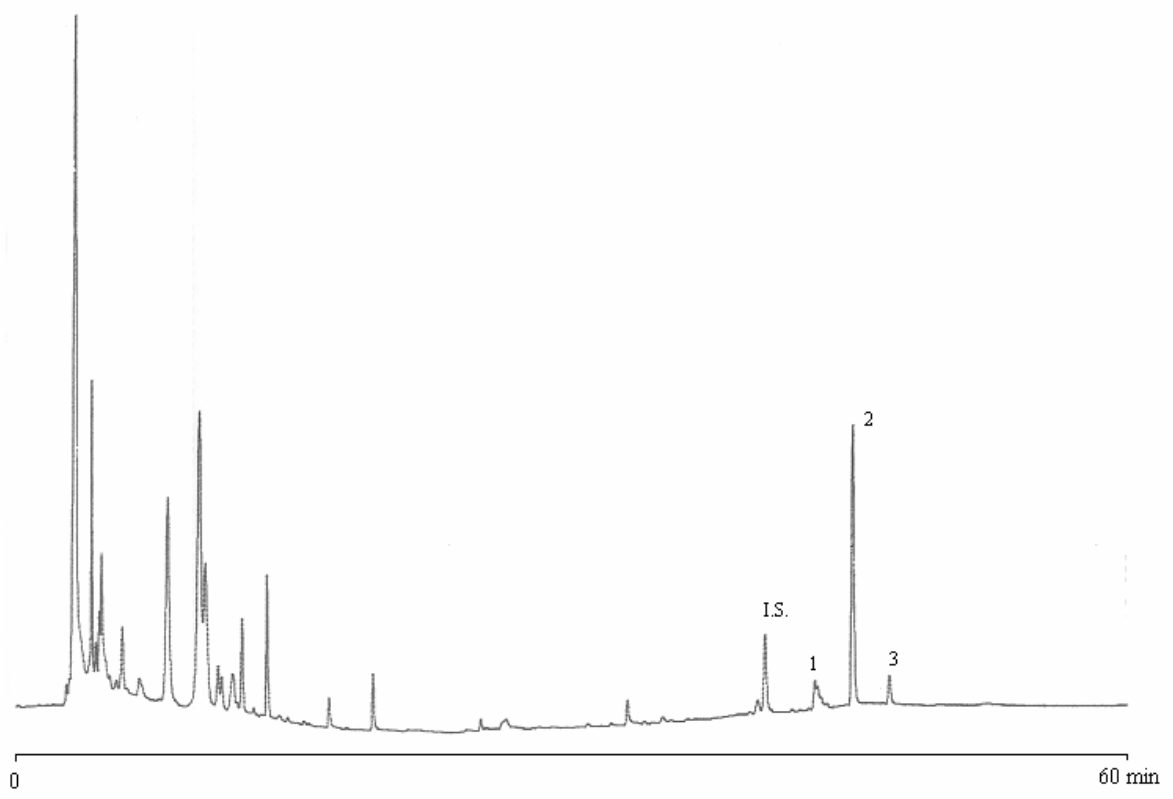


Figure 13. Chromatogram of the enhanced solvent extracts of St. John's Wort. (I.S.) benzo[k]fluoranthene (internal standard), (1) furohyperforin, (2) hyperforin, and (3) adhyperforin. ASE condition: 50 mg of sample was extracted with methanol at 2000 p.s.i., 60°C for 10 min. Wash volume 8 mL (methanol), flush time 5 minutes. 10 µL benzo[k]fluoranthene (1400 µg/mL) was added as the internal standard after the extraction. (See Fig. 12 for LC conditions.)

3. Results and Discussion

3.1 Quantitative Extraction and Analysis of Hyperforin via On-line SFE-LC-UV

Figure 14 describes the UV trace after on-line SFE-LC. SFE is a highly selective extraction technique, especially for low polarity compounds. Compared to ultrasonic extraction and enhanced solvent extraction, SFE extracts are much cleaner as evidenced by fewer chromatographic peaks due to lower concentrations of interfering co-extractives. The major parameters that affect the extraction efficiency in SFE include extraction temperature, pressure, extraction mode (dynamic or static), extraction time, modifier, and collection temperature.

Collection temperature was optimized first. Many low recoveries in the older literatures can be traced to inadequate trapping instead of insufficient extraction.[40] In our design, a C_{18} solid trap was used since the target compound, hyperforin, has relatively low polarity. The collection temperature of the trap was varied from -10°C to 40°C. The recovery was slightly decreased when trap temperature was increased to 40°C, probably due to diminished trap efficiency at the higher collection temperature. If not noted, the SFE trap temperature was kept at 0°C for collection in future experiments.

Extraction pressure and temperature were then optimized. The variation of pressure and temperature will not only result in a change of fluid density (solvating power), but also a change in fluid diffusivity, which will in turn affect mass transfer. Increased pressure results in an increase in density but a decrease in diffusivity; while an increase in temperature has the reverse result. Figure 15 compares the extraction efficiency at three pressures and three temperatures during a series of 30 min dynamic extractions. Within the investigated range, extraction pressure had a positive effect on

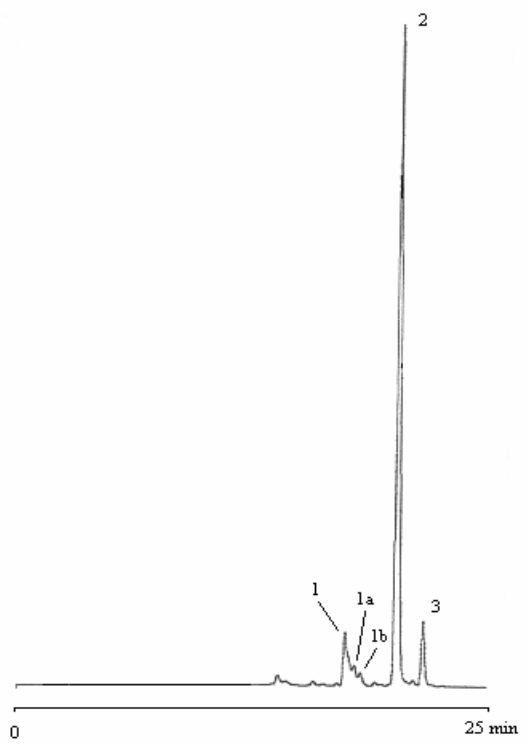


Figure 14. UV trace of on-line SFE-LC of St. John's Wort. (1) furohyperforin, (1a), furohyperforin analogue a, (1b), furohyperforin analogue b, (2) hyperforin, and (3) adhyperforin. SFE: 400 atm CO_2 @ 60°C, 60 min dynamic extraction, 5 mg of sample. Trap temperature for collection was 0°C. 4 mL water was delivered to replace residual CO_2 in the trap. (LC conditions see section 2.4)

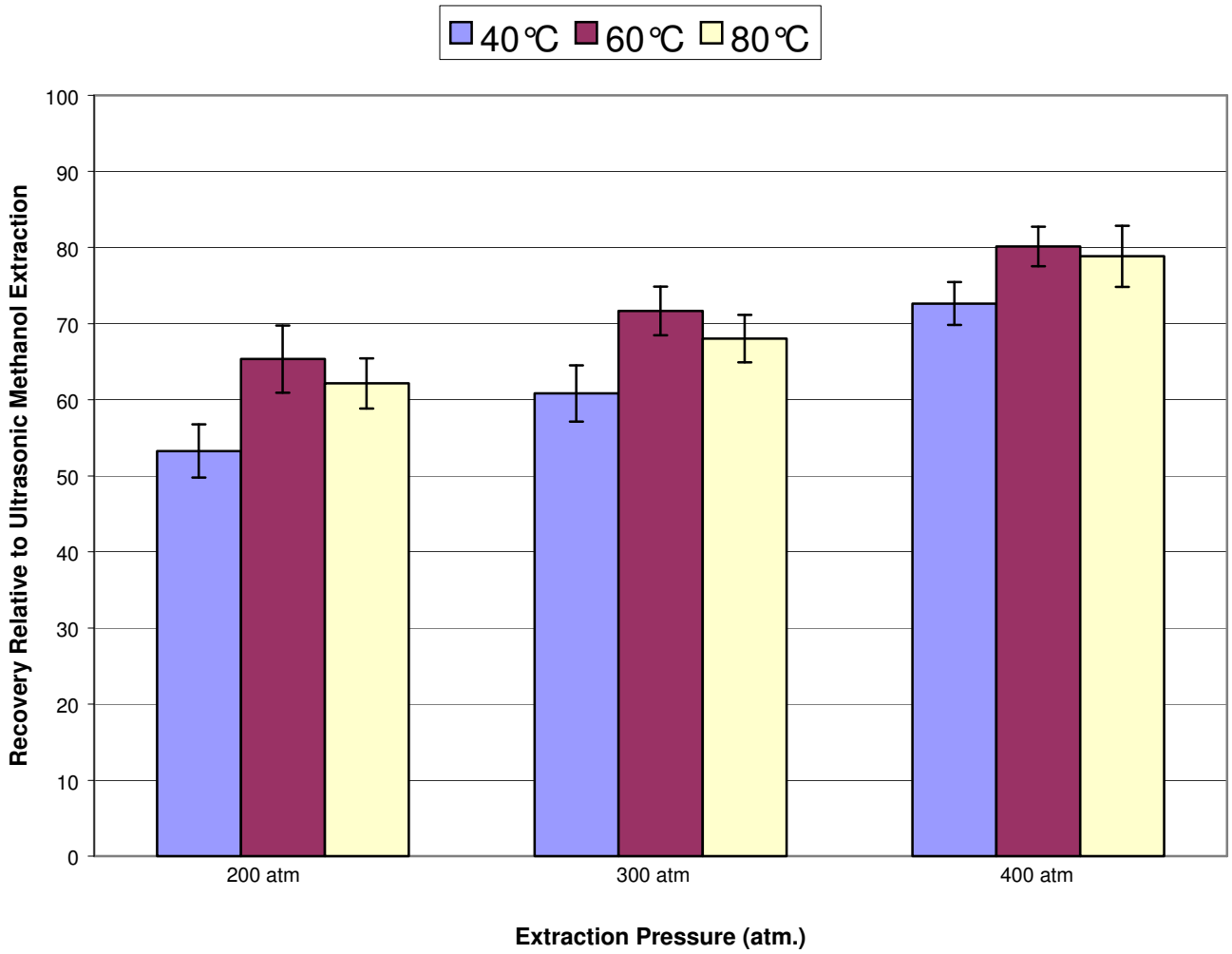


Figure 15. The influence of extraction temperature and CO_2 pressure on SFE extraction efficiency, 30 min dynamic extraction. (See Figure 14 for SFE and LC conditions.)

extraction efficiency. As the pressure increased from 200 atm to 400 atm, a higher recovery was achieved. For the extraction temperature study, both 60°C and 80°C gave a higher recovery than 40°C. The intermediate temperature (60°C) seemed more efficient than the higher temperature (80°C) at low pressure, but the difference between the two temperatures diminished when extraction pressure was increased. For further extractions described in this report, 400 atm/60°C were employed.

Extraction time was first optimized for the dynamic mode. Recovery was 85% when a 60 min dynamic extraction was performed. No further increase was observed when longer extraction times (90 min and 120 min) were used. Introduction of a static step in our case resulted in no positive effect either.

Even under optimized conditions, recovery of hyperforin never exceeded 85% when pure CO_2 was used as the extraction fluid. However, many times addition of a co-solvent (modifier) to the supercritical fluid enhances the solvating power of the fluid thus enhancing the solubility of an analyte in the extracting medium. Consequently, methanol and acetone were dynamically mixed with supercritical CO_2 at 10% and 30%, respectively (e.g. trap temperature was increased to 60°C to avoid a loss of trapping efficiency.) Unfortunately, lower recoveries were found in both the on-line and off-line modes under these conditions. Some polar compounds, nevertheless, were also extracted out of the matrix when higher percentages of modifier were employed. It was concluded from these findings that the limiting factor was not the solubility of hyperforin in the supercritical medium, since hyperforin is a natural product, strong interactions between the matrix and the analyte may exist. In order to disrupt the analyte-matrix interaction and to facilitate the diffusion step, various amounts of modifier were spiked directly into

the vessel before the extraction. For experiments where the matrix is spiked, a certain amount of static extraction time is desirable in order to allow more interaction between modifier and sample. A small amount (100 μL) of methanol was thus tried first with 10 minutes static extraction followed by a 60 min dynamic extraction. Unfortunately, even less recovery was observed, compared to the extraction without modifier. Then 100 μL of acetone was tested. In this case, an enhanced recovery (90%) was achieved. When more acetone (200 μL) or a longer static extraction time (30 min) was applied, no further increase in recovery was observed. Thus, the various experiments described in this section have led to what we believe to be the optimized extraction conditions: 400 atm pressure at 60°C, 10 min static extraction followed by 60 min dynamic extraction, 100 μL acetone pre-spike in the vessel. The resulting recovery of hyperforin was ~90% (7.79±0.32 mg/g) relative to the ultrasonic methanol extraction. The linearity of hyperforin was in the range of 0.5 – 80 μg (r^2 = 0.998). The limit of detection (signal/noise ratio 3:1) of hyperforin was 0.2 μg.

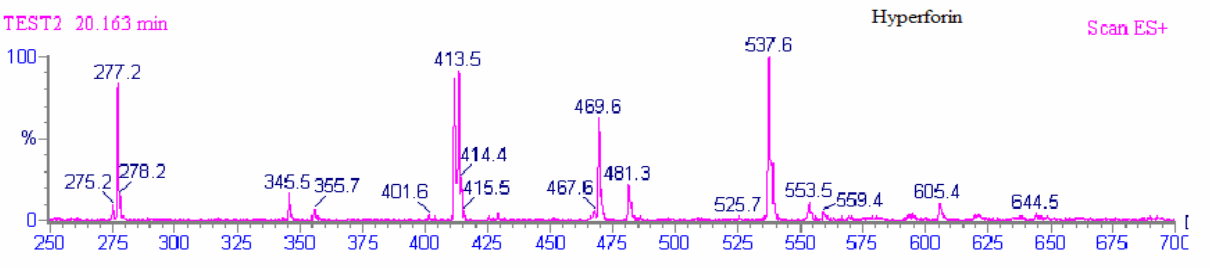
3.2 Determination of Hyperforin and its Degradation Products by on-line SFE-LC/MS

ESI-MS was coupled with SFE-LC to provide specific extract compound information. It is important to remember that the entire extraction-separation-detection system operates under air/light free conditions, thus no analyte oxidation or thermal degradation should have occurred during extraction and analysis. Mass spectral data confirmed that the major peak ($^{\#}$2) in the UV trace (Figure 14) was hyperforin. The mass spectrum (Figure 16a) displayed an intense signal for the protonated molecule $[M+H]^+$ at

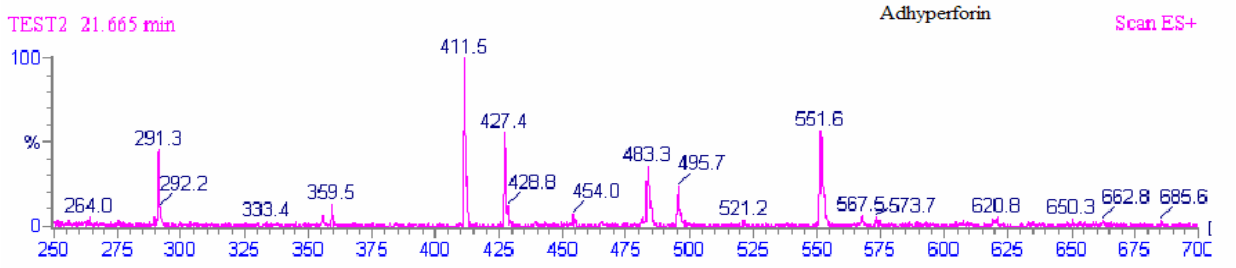
m/z 537. The ions at m/z 481, 469, and 467 may be due to the loss of an alkyl chain, e.g. isobutene (-56 amu), isoprene (-68 amu) and dimethylketene (-70 amu) respectively. The signal at *m/z* 413 may be due to the successive loss of isoprene (-68 amu) and isobutene (-56 amu).[37] The mass spectrum of peak #3 in the UV trace (Figure 14) displayed an intense signal for the protonated molecule $[M+H]^+$ at *m/z* 551 (Figure 16b), accompanied by fragment ions at *m/z* 493, 411, and 291, thus allowing the inference of peak #3 as adhyperforin.[41] The mass spectrum of peak #1 in the UV trace (Figure 14) displayed an intense signal for a protonated molecule $[M+H]^+$ at *m/z* 553 (Figure 16c). It is believed to be furohyperforin (also called orthhyperforin), the major degradation compound of hyperforin.[37] There are 2 other tiny peaks (#1a and #1b in Figure 14) that almost co-elute with furohyperforin. Their mass spectra displayed intense signals for a protonated molecule $[M+H]^+$ at *m/z* 553 (Figure 16d, 16e). These peaks may be analogues of furohyperforin.

In a stability study of supercritical fluid extracted hyperforin, it was found that hyperforin (in methanol) was fully degraded after 4 hours of exposure at natural daylight (Figure 17). It was also slowly degraded when exposed to fluorescent light, which is used for ordinary illumination in the laboratories. 60% of hyperforin was degraded after 6 hours of exposure. No degradation was observed after 6 hours of storage in the dark. In addition, in our off-line SFE-LC procedure, the extracts were protected to avoid light, by wrapping the sample vial with aluminum foil. After adding internal standard, the extracts were immediately analyzed by LC. The amount of hyperforin extracted via off-line SFE-LC is comparable to the amount extracted via on-line SEF-LC, which tells us that there

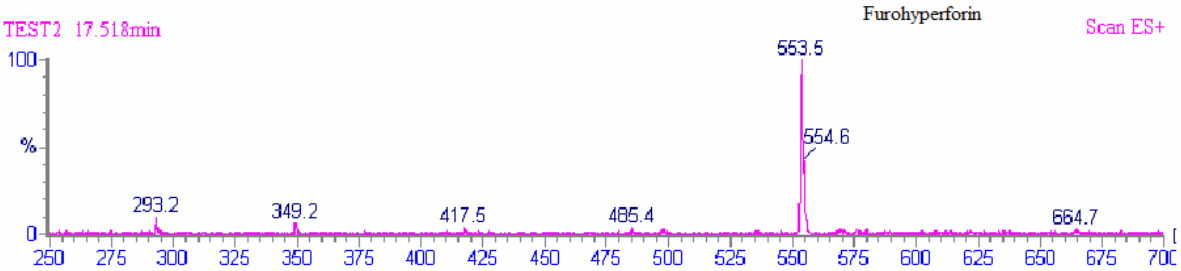
a.


TEST2 20.163 min
Hyperforin
Scan ES+
100
%
0
277.2
413.5
537.6
469.6
414.4
275.2
278.2
345.5
355.7
401.6
415.5
467.6
481.3
525.7
553.5
559.4
605.4
644.5
250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700

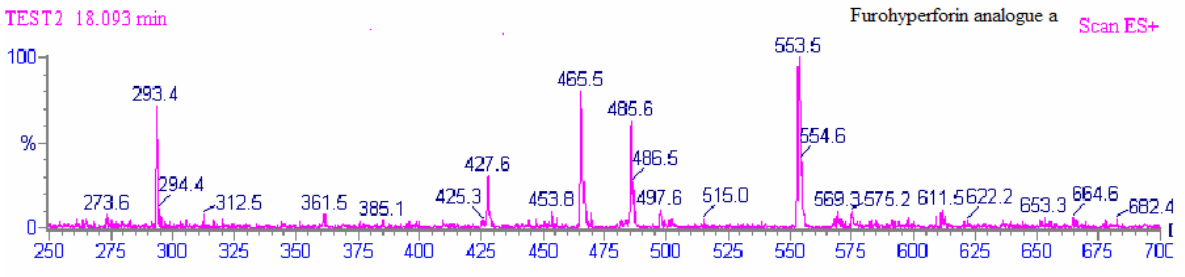
b.


TEST2 21.665 min
Adhyperforin
Scan ES+
100
%
0
411.5
427.4
551.6
291.3
483.3
495.7
292.2
264.0
333.4
359.5
428.8
454.0
521.2
567.5
573.7
620.8
650.3
662.8
685.6
250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700

c.


TEST2 17.518min
Furohyperforin
Scan ES+
100
%
0
553.5
554.6
293.2
349.2
417.5
485.4
664.7
250 275 300 325 350 375 400 425 450 475 500 525 550 575 600 625 650 675 700

d.



e.

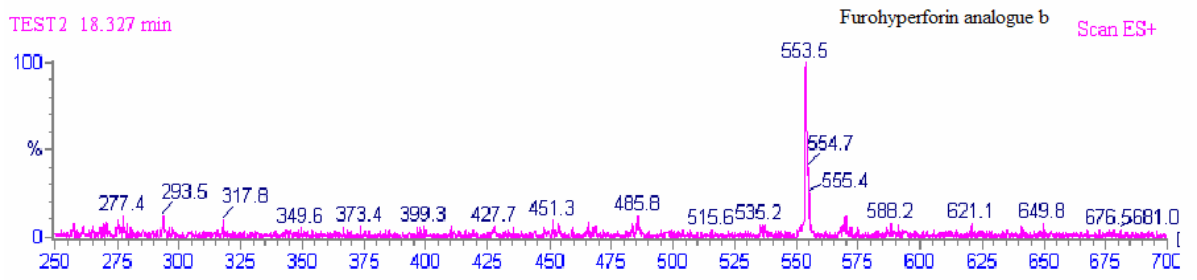


Figure 16. Extracted ion mass spectra of hyperforin, adhyperforin, furohyperforin and its analogues via on-line SFE-LC-MS.

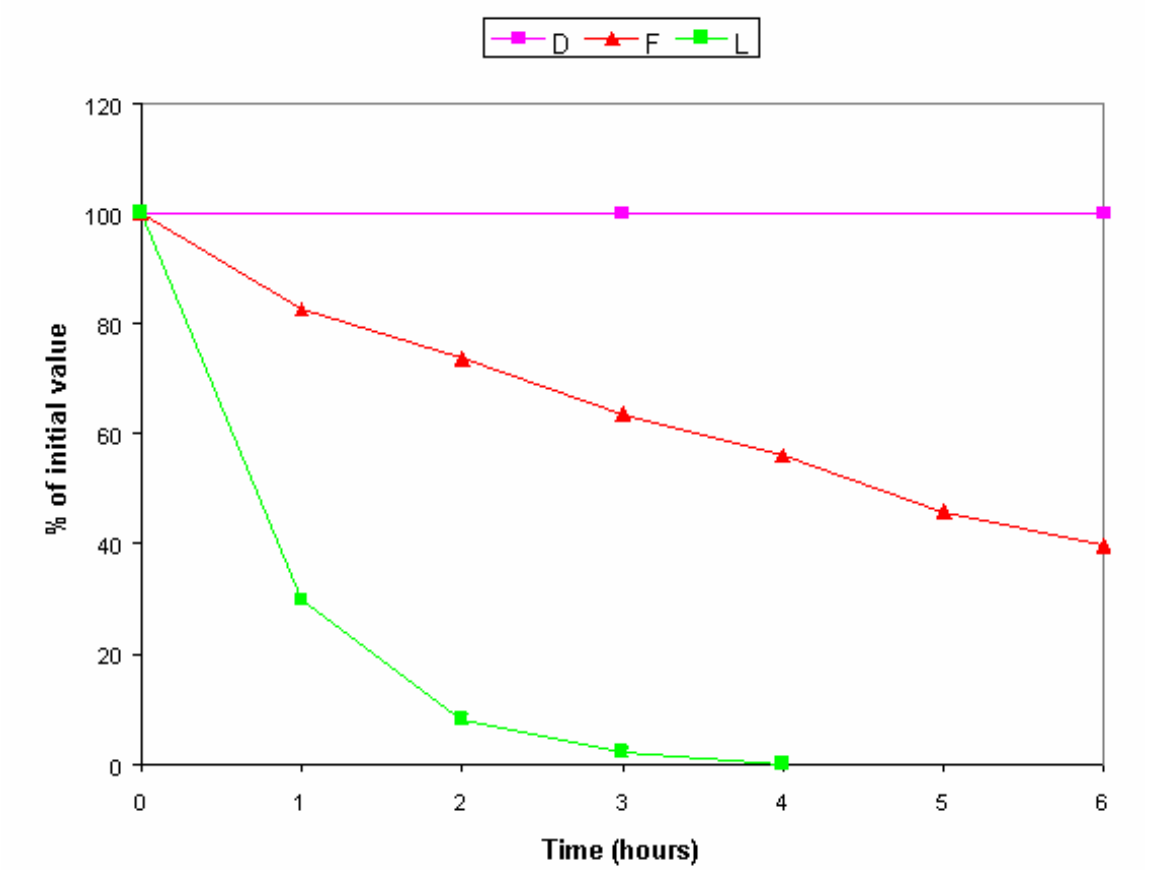


Figure 17. The hyperforin stability profile under natural daylight (L), fluorescent lamp light (F), and dark (D). The hyperforin was extracted from 50 mg of sample by SFE and eluted with methanol. (Average of two measurements. See Figure 14 for LC and SFE conditions.)

was no hyperforin loss (degradation) due to the relatively short exposure to the air between the period of extract collection and injection. The degradation products detected under air/light free conditions (on-line SFE-LC) make us believe that degradation occurred during the plant drying process or further plant storage, instead of during the off-line sample-processing step in the analytical lab. Therefore, the off-line extraction-analysis methods may also achieve reliable results for the analysis of hyperforin, if certain extract handling/storage condition can be noticed.

4. Conclusion

This study has described an on-line supercritical fluid extraction-liquid chromatography-UV absorbance/electrospray ionization mass spectrometry (SFE-LC-UV/ESI-MS) technique for the quantitative analysis of hyperforin in *Hypericum Perforatum*. High recovery (~90%) was achieved under optimized conditions. The hyphenated system made it possible for extraction-separation-detection to be conducted under air/light free conditions. In the on-line design, all the extract is directly transferred to the analytical system. Any possibility for oxidation, thermal degradation, or sample loss during extraction or the extract processing step is avoided. MS data confirmed the presence of hyperforin and its major degradation compound furohyperforin, as well as two furohyperforin analogues in dry *Hypericum Perforatum* plant, which was attributed to pre-analysis degradation in the plant drying or storage.

CHAPTER IV

Quantitative Analysis of Analytes in Aqueous Matrices via On-line Supercritical CO_2 Extraction-Liquid Chromatography

1. Introduction

Most SFE applications are focused on solid samples. SFE of aqueous matrices could give rise to a number of uses such as the isolation of industrial chemicals from waste water; pesticides and herbicides from run-off waters; and contaminants from drinking water. It might also prove useful in the analysis of other liquid matrices such as beverages, biological samples, and pharmaceutical fluids.[42] SFE of aqueous samples for analytical purposes has received less attention compared to solid samples. The major difficulty lies in the confinement of the matrix, therefore, the extraction cell must be of a geometry that retains 100% of the bulk water during the extraction.[43] Otherwise restrictors will suffer from plugging caused by ice formation during supercritical fluid expansion.

Up to now, only three methods have been reported for direct aqueous SFE. These are (a) phase separator method, (b) countercurrent method, and (c) liquid-fluid extraction method. Thiebaut et al. used a novel phase separator to extract 4-chlorophenol and phenol from water with supercritical CO_2.[44] The segmented mixture of CO_2 and water was separated in a phase separator which consisted of an upper hydrophobic membrane (usually polymer) and a lower hydrophilic surface (usually stainless steel, Figure 18). The liquid-fluid mixture passed along the groove in the separator and separation was achieved based on the difference in wettability of the two surfaces. However, sample recovery was less than 60%.

Countercurrent SFE takes advantage of the density difference of supercritical fluid and water.[45] The more dense fluid (water) can be transported in one direction, while the other fluid (supercritical fluid, SF) can be transported in the opposite direction (Figure 19). The two materials meet and mix inside the extractor. Solutes that are soluble in supercritical CO_2 are brought out from the top, while water will elute out from the bottom. This technique allows larger sample volumes to be extracted. Countercurrent SFE of small quantities of water (< 100 mL) is not feasible because of the lack of suitable technology.

Up to now, the most successful design for analytical scale SFE of aqueous matrices is a liquid-fluid extraction configuration, first reported in 1989,[42, 43, 46-54] see Figure 20. Supercritical CO_2 was allowed to enter through a tube thus passing to the bottom of the vessel where it mixed with the aqueous medium to extract the compounds of interest. Due to the lower density of supercritical CO_2 compared to water, the supercritical fluid rose to the top of the extraction vessel and exited through a second tube to either a trap or re-circulation pump. This type of extractor was used to qualitatively extract nitrogenous bases,[42] phenols,[43] phosphonate,[46] active components in drugs,[47] metal chelates,[48] steroids,[49] PAHs,[50] chlorinated pesticides and PCBs [51] from an aqueous matrix.

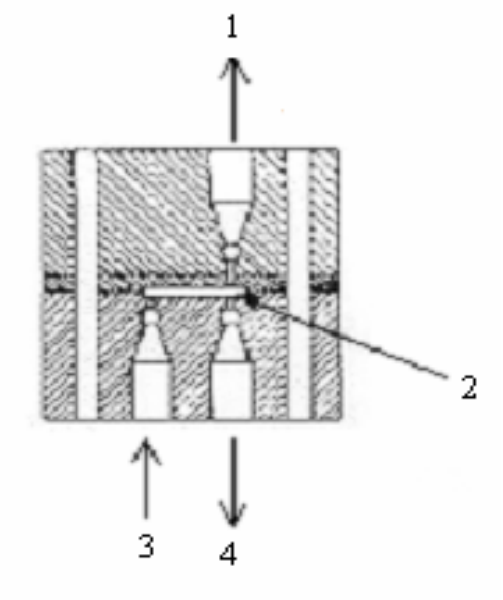


Figure 18. Phase separator design.[44] 1. CO_2 outlet; 2. phase separator; 3. Flow inlet; 4. aqueous waste outlet. (Reproduced with permission from Elsevier Ltd.)

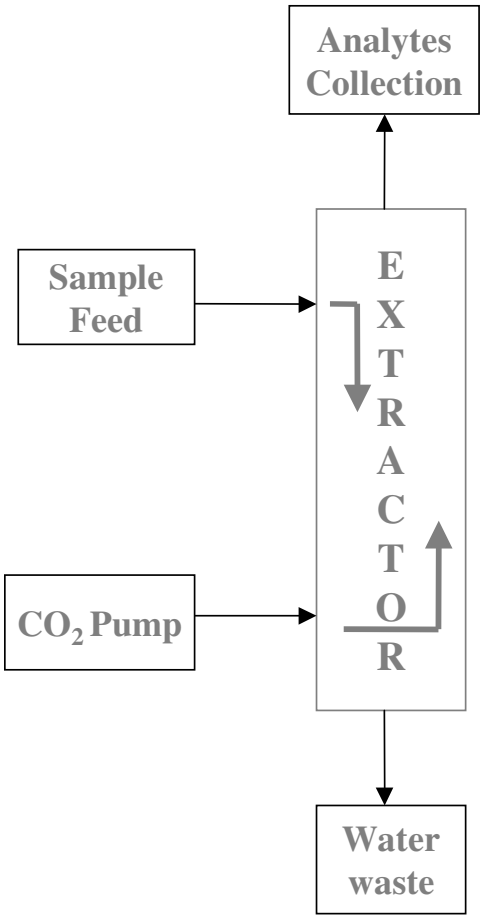


Figure 19. Countercurrent design.

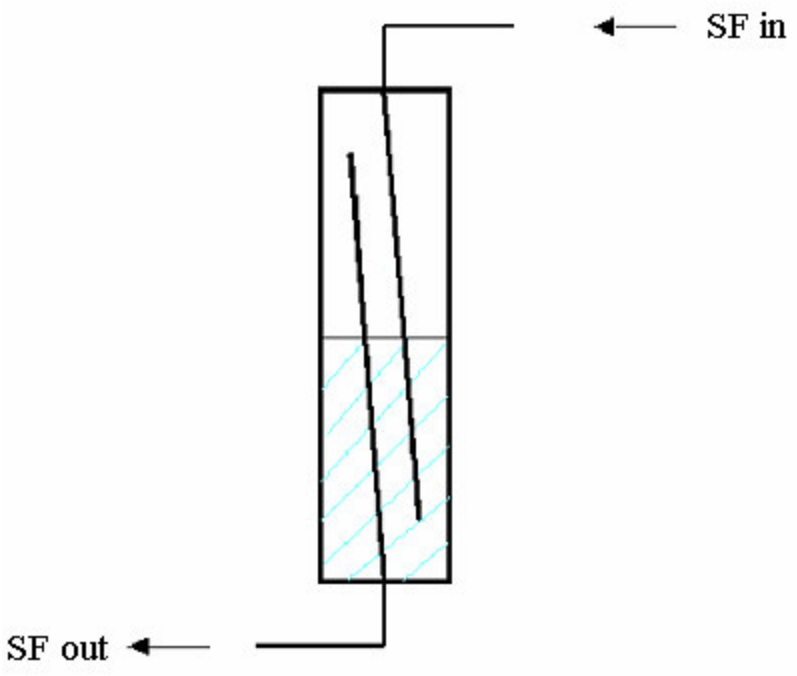


Figure 20. Liquid-fluid extraction vessel.

On line coupling of SFE and chromatographic techniques is beneficial for trace analysis, since all of the extract is transferred to the separation column and to the detector. In addition, this method is far less labor intensive than off-line analysis and the opportunity for the sample to become contaminated, volatilized, or degraded is minimized.[55, 56] Recently, Wenclawiak reported on-line coupling of continuous SFE with HPLC for the analysis of an aqueous pyrethrin solution.[57] It offered extraction of unlimited sample volume, but for a limited sample (several milliliters or less), it was not suitable. In this chapter, we have tested the suitability of the on-line SFE-RPLC method for direct aqueous extraction for ~mL level sample. Two types of compounds, for pharmaceutical interest (caffeine, progesterone) and for environmental interest (phenanthrene, pyrene), were quantitatively extracted and transferred from water and urine as well as a real environmental sample. The pre-spiked matrix method was surprisingly found to be very beneficial for the direct aqueous extraction.

2. Experimental Section

2.1 Apparatus

An Isco-Suprex (Lincoln, NE, USA) Prepmaster supercritical fluid extraction system equipped with Accutrap and modifier pump was used for all parts of the study. SFE/SFC grade carbon dioxide with 2000 p.s.i. helium head pressure was provided by Air Products and Chemical Inc. (Allentown, PA, USA). Extractions were performed using a 12cm × 1.1cm i.d. stainless steel vessel (10 mL volume, specific design can be found in reference 1). The vessel was filled with 5 mL water and homogeneously spiked with 10 μL stock methanol solution by shaking the vessel for ~1 minute. The SFE

variable restrictor was heated to 60 °C for all extractions. A 10 cm × 0.2 cm i.d. stainless steel column filled with C-18 (Isolute Sorbent™, 40 -70 μm particles) was used as the SFE trap. If not noted, the trap temperature was kept at 20 °C for collection and desorption of analytes. In this study the flow rate of liquid CO_2 prior to being heated past the critical temperature was set at 1 mL/min. During analyte desorption, the flow rate of rinse water was 1 mL/min for four minutes.

An Agilent 1050 HPLC system (Wilmington, DE, USA) with programmable multi-wavelength UV detector was used to analyze the extracts. A Phenomenex C-18 column (Torrance, CA, USA), 250 × 4.6 mm with 5 μm particles was used for the separation. The mobile phase was acetonitrile/water (30:70, v/v), then to 80:20 at 10 minutes, to 100% acetonitrile at 20 minutes, kept for 2 minutes. The UV detection wavelength was set at 250 nm. Direct injection of standards into the LC column was accomplished by using a Valco injection valve (Houston, TX, USA) with a 10 μL sample loop.

Experimental details concerning the hyphenated SFE-RPLC interface are given in Chapter 2.

2.2 Chemicals and Real Sample

HPLC grade methanol, acetonitrile, and water (Burdick & Jackson, Muskegon, MI, USA) were used. A mixture of caffeine, progesterone, phenanthrene, and pyrene (Aldrich Chemical Co., Milwaukee, WI, USA) in methanol was prepared as a stock solution, with a concentration of 800, 80, 10, 60 μg/mL, respectively.

Urine and natural water were chosen as two real matrices. Urine was provided by a healthy donor. During the experiment, 1 mL urine, 2.2 mL HPLC grade water and 1.8 mL methanol were mixed in the vessel with 10 μL of 20 μg/mL progesterone stock solution (the absolute concentration in the vessel was 40 ng/mL). The natural water sample was collected at the side water area of Virginia Tech Duck Pond. Natural water (1.0 mL), 2.2 mL HPLC water, and 1.8 mL methanol were mixed in the vessel with 10 μL of 2 μg/mL phenanthrene and 12 μg/mL pyrene stock solution (the absolute concentrations in the vessel were 4 ng/mL and 24 ng/mL for phenanthrene and pyrene, respectively). The extraction was carried out by using 15 g CO_2 as the extraction media.

3. Results and Discussion

3.1 Optimization of Extraction Recovery

3.1.1 Pure Supercritical CO_2 as the Extraction Media

On-line SFE-RPLC of the four analytes spiked into water (i.e. extracted with CO_2, collected on the trap, mobilized from the trap by the mobile phase, and analyzed via LC) yielded the trace shown in Figure 21.

In this design, the extraction mode is similar to liquid-liquid extraction where the upper phase is supercritical fluid and the lower phase is liquid. The extraction process is believed to be partitioning of the analytes between the two phases and the analyte's partition coefficient is the decisive parameter. The coefficient can be roughly estimated from the ratio of the analyte's solubility in these two phases. Among the four target compounds, caffeine is the most polar one and also has the highest water solubility due to

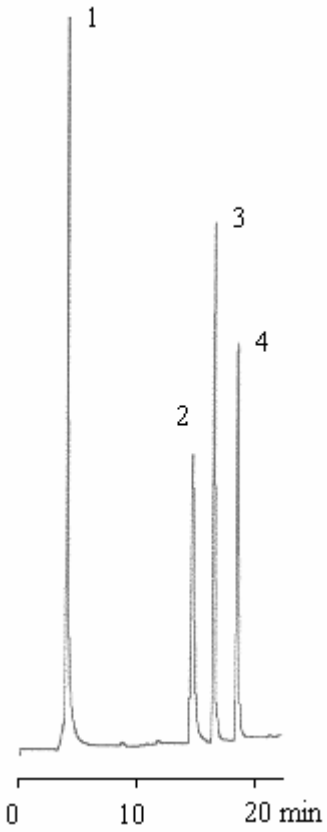


Figure 21. Chromatogram of four spiked compounds via on-line SFE-RPLC. (1) caffeine, (2) progesterone, (3) phenanthrene, and (4) pyrene. C-18 column (250 × 4.6 mm × 5 μm) was used for separation. The mobile phase was acetonitrile/water (30:70, v/v), then to 80:20 at 10 minutes, to 100% methanol at 20 minutes, kept for 2 minutes. Flow rate was 1.0 mL/ min. The UV detection wavelength was set at 250 nm. SFE: 350 atm CO_2 @ 60 °C, 30 g CO_2 consumed for dynamic extraction. C-18 was used as sorbent in the trap. Trap temperature for collection and for desorption both were 20 °C. 4 mL water was delivered to replace residual CO_2 in the trap. 10 μL stock solution was spiked into 5 mL HPLC grade water.

its strong hydrogen bonding with water. The extraction profile shown in Figure 22 reveals that progesterone, phenanthrene, and pyrene can be exhaustively extracted with 60 grams of CO_2; however, for caffeine, the recovery was still only 90% when 90 grams of CO_2 was consumed.

An optimum extraction temperature was explored. As the temperature rises, the analytes become more soluble in water; at the same time, the volatility of those compounds also will increase, which facilitates mass transfer to the upper phase. The density and solvating power of supercritical carbon dioxide also have an effect. They will decrease when temperature increases. The extraction recoveries at three different temperatures are shown in Table 7. The intermediate temperature (60°C) was found to be more efficient than either 40 °C or 80 °C.

The influence of extraction pressure was also investigated by changing the pressure from 250 atm to 450 atm at 100 atm intervals with an extraction temperature of 60 °C (Table 8). The highest recovery was achieved at 350 atm. (e.g. the intermediate pressure). This result may be rationalized in terms of the cohesive energy of supercritical fluid. When pressure increases, the cohesive energy of the supercritical fluid (SF) also increases. In the aqueous matrix, the greater cohesive energy translates into less interaction (or mixing) of the fluid with the matrix because of decreased fluid surface area. Smaller surface area reduces the chance of SF to contact the analytes, so lower efficiency was observed at 450 atm. When the pressure increased from 250 atm to 350 atm, the density of SF increased with higher solvating power, and in this pressure range, surface area is not a major factor, so we observed higher extraction efficiency at 350 atm than at 250 atm due to the increased CO_2 density.

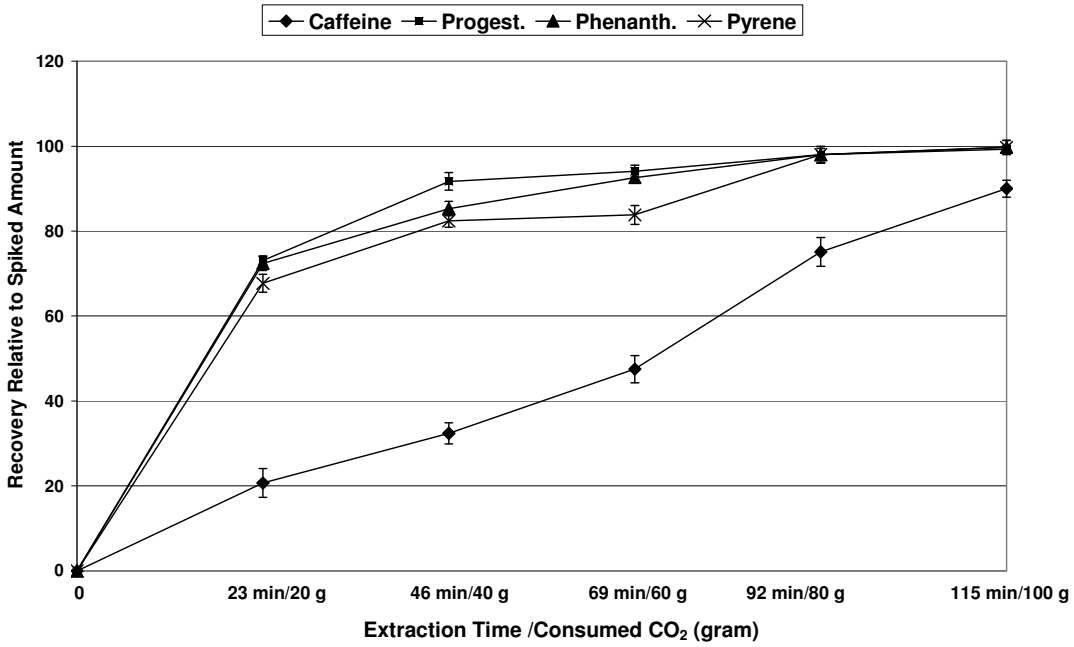


Figure 22. Extraction profiles of caffeine, progesterone, phenanthrene, and pyrene. (See Figure 21 for LC and SFE conditions.)

Table 7. Percent recovery versus extraction temperature. (10 minutes dynamic extraction, other conditions were the same as described in Figure 21)

	350 atm, 40 °C 0.94 g/mL		350 atm, 60 °C 0.87 g/mL		350 atm, 80 °C 0.79 g/mL	
	Rec.	%RSD[a]	Rec.	%RSD[a]	Rec.	%RSD[a]
Caffeine	9.2	7.9	9.8	8.4	7.3	8.6
Progesterone	41.1	4.3	50.6	4.2	32.1	5.4
Phenanthrene	45.3	5.0	56.7	5.3	33.5	6.0
Pyrene	39.2	5.2	48.8	5.7	29.3	5.6

[a.] n = 3

Table 8. Percent recovery versus extraction pressure. (10 minutes dynamic extraction, other conditions were the same as described in Figure 21)

	250 atm, 60 °C 0.79 g/mL		350 atm, 60 °C 0.87 g/mL		450 atm, 60 °C 0.92 g/mL	
	Rec.	%RSD[a]	Rec.	%RSD[a]	Rec.	%RSD[a]
Caffeine	8.0	8.9	9.8	8.4	7.9	8.0
Progesterone	42.4	5.1	50.6	4.2	42.6	4.9
Phenanthrene	47.5	5.2	56.7	5.3	47.9	5.5
Pyrene	39.9	5.6	48.8	5.7	39.3	5.5

[a.] n = 3

"Salting out" is widely used in liquid-liquid extraction.[58] We explored the extraction of our target analytes at 1% and 3% salt (NaCl) concentration (w/w). However, there were no obvious changes in extraction efficiency and extraction kinetics. A similar result was observed when PCBs, OCPs, and pyrethrins were extracted from water (2% NaCl).[57] The probable reason maybe because at low salt concentration, the effect of "salting out" is not significant enough to affect the recovery of the analytes. At higher salt concentration, we may observe the enhancement in the extraction efficiency, but with an SFE instrument, the operation at high salt concentration is adventurous. Salt crystals may deposit and plug the restrictor and tubing, when supercritical fluid is decompressed causing its solvating power to be decreased.

The flow rate of supercritical fluid has an influence on trap efficiency. In our study, the mass of supercritical CO_2 was kept at 15 grams. Under these conditions, recoveries decreased slightly when the measured CO_2 liquid flow rate at the pump was increased from 1 mL/min to 3 mL/min. The trap apparently lost some efficiency at the high flow rate.

3.1.2 Modified Supercritical CO_2 as the Extraction Media

Organic solvent modified supercritical CO_2 is often used for the extraction of polar analytes. Modifier can be dynamically added to the fluid by an additional pump, or it can be pre-spiked into the vessel. For modified SFE experiments, the trap temperature was set at 60 °C to avoid the loss of trapping efficiency.

a. Dynamic Mixing Mode (via Modifier Pump)

Firstly, different percentages of methanol were dynamically mixed with supercritical CO_2 via a modifier pump, to test their influence on the extraction. When 15 g of CO_2 were consumed for dynamic extraction, there was no statistical difference among the data. There was a similar situation when 30 g of CO_2 were consumed. But when more CO_2 (45 g) were used, the modified supercritical fluid gave slightly higher recoveries. In the dynamic modifier mixing mode, there appears to be no obvious enhancement in extraction efficiency when relatively small amounts of CO_2 (or short extraction time) were used. When more CO_2 was employed (or longer extraction time), the modifier begins to show an effect. Such phenomena probably result from the fact that at any given short time period, only a tiny amount of modifier was brought into and mixed with the aqueous matrix. Furthermore, some of dissolved modifier may have been extracted out of the aqueous matrix by supercritical CO_2. When the extraction time is longer, more modifier enters the matrix and the enhancement due to modifier begins to be visible.

b. Pre-spiking Mode (via Spiking Modifier in the Vessel)

Wenclawiak et al. reported that in the continuous SFE mode, the addition of 2 and 5% methanol slightly increased extraction recovery; the addition of 10% methanol decreased the recovery.[57] The pre-spiked matrix modifier mode was then investigated in our design. To equally compare with the dynamic mixing mode (for 15g CO_2 with 2%, 5% and 10% modifier, the total methanol consumed was 0.35, 0.9 and 1.8 mL, respectively), the same amount of methanol was pre-spiked into the vessel that contained water filled to a total volume of 5 mL (15 g CO_2 was the extraction media). When the

extraction was performed, surprisingly the pre-spiked matrix method achieved 10% - 30% enhancement in extraction efficiency (except for caffeine where the enhancement was less than 10% increase), When 1.8 mL of methanol was pre-spiked, an exhaustive quantitative extraction was achieved for progesterone, phenanthrene, and pyrene (Table 9). Their recovery was enhanced as high as 30% -35%, compared to the data obtained with 10% modifier in the dynamic mixing mode. This distinct improvement is probably due to a "co-extraction effect". At the beginning of the extraction, methanol has a relatively high concentration in the vessel and is homogeneously mixed with water and analytes. Due to the high solubility of methanol in supercritical CO_2, it maybe quickly extracted from the water matrix, and at the same time the analytes maybe co-extracted along with the methanol. For caffeine, which has a strong interaction (hydrogen bonding) with water, only a slight increase in recovery was observed. 2-Propanol was also tested as a modifier and it achieved a similar result to methanol.

3.1.3 Linearity

The linear range of C-18 trap capacity (10 cm × 0.2 cm i.d.) was also investigated at optimized conditions (Table 10). The linear range was ~10^2 for caffeine and pyrene, and ~10^3 for progesterone and phenanthrene. All the correlation coefficients were larger than 0.99.

Table 9. Comparison of dynamic mixing modifier mode and pre-spiked modifier mode on percent recovery. (See Fig. 21 for HPLC and SFE conditions, except the trap temperature for collection was 60 °C. Methanol was used as modifier.)

	0.3mL Methanol Spiked in Vessel		2% Methanol dynamic mixing (consumed 0.35mL)		0.9mL Methanol Spiked in Vessel		5% Methanol dynamic mixing (consumed ~ 0.9mL)		1.8mL Methanol Spiked in Vessel		10% Methanol dynamic mixing (consumed ~ 1.8mL)	
	Rec.	%RSD	Rec.	%RSD	Rec.	%RSD	Rec.	%RSD	Rec.	%RSD	Rec.	%RSD
Caffeine	26.5	7.1	17.2	5.5	27.6	2.4	20.4	4.4	28.7	4.6	21.2	1.2
Progesterone	92.9	2.3	71.3	5.3	99.3	0.7	72.9	0.4	100.1	1.0	69.9	1.8
Phenanthrene	82.9	1.1	71.1	7.2	91.4	3.8	69.6	6.2	100.0	0.4	73.8	3.6
Pyrene	75.9	6.4	69.9	7.7	90.8	6.1	65.3	6.3	100.1	1.0	64.4	5.2

Table 10. Linear range, correlation coefficient and precision data.

Compound	Linear range [a]	Correlation coefficient (n = 5)	LOD [b]
Caffeine	3,000 - 300,000 ng	0.9989	50 ng
Progesterone	16 - 16,000 ng	0.9998	1 ng
Phenanthrene	2 - 2,000 ng	0.9997	0.1 ng
Pyrene	2 - 1,000 ng	0.9996	0.5 ng

[a.] Amount of analytes spiked in water.
[b.] Limit of detection (signal to noise = 3).

3.2 SFE-LC Method for the Real Samples

Finally to evaluate this hyphenated system for more complicated matrices, the target analytes in real samples were quantitatively analyzed. Based on the matrix-spiked results, 1.8 mL of methanol as modifier was pre-spiked into the vessel containing the real matrix. Each time 1 mL of the real liquid matrix and 2.2 mL HPLC grade water were added to the vessel (total volume is 5 mL). 10 μL of the stock analyte solution was then added and well mixed. Figure 23a and 24a show the HPLC chromatograms of blank urine and the natural water sample, which were extracted via on-line SFE-LC. Figure 23b and 24b show the chromatographic peaks for 40 ng/mL progesterone in urine as well as 4 ng/mL phenanthrene and 24 ng/mL pyrene in the natural water. Quantitative extraction was achieved in less than 20 minutes for each analyte, with reasonable experimental error (Table 11).

3.3 Comparison of On-line and Off-line Methods for the Analysis of Trace Level Samples

In on-line mode, all of the extracts can be directly transferred to LC, so the sensitivity is improved compared to off-line method, which only partial of the extracts can be sucked by syringe and injected to LC. In order to confirm this advantage of on-line method over off-line method, trace level analytes were used as the target to compare these two methods. The stock solution described in section 2.2 was 10 times diluted. The extraction condition is same as section 3.2. Off-line SFE-LC method was first performed. After extraction, 4 mL methanol was delivered to the solid phase trap to elute the

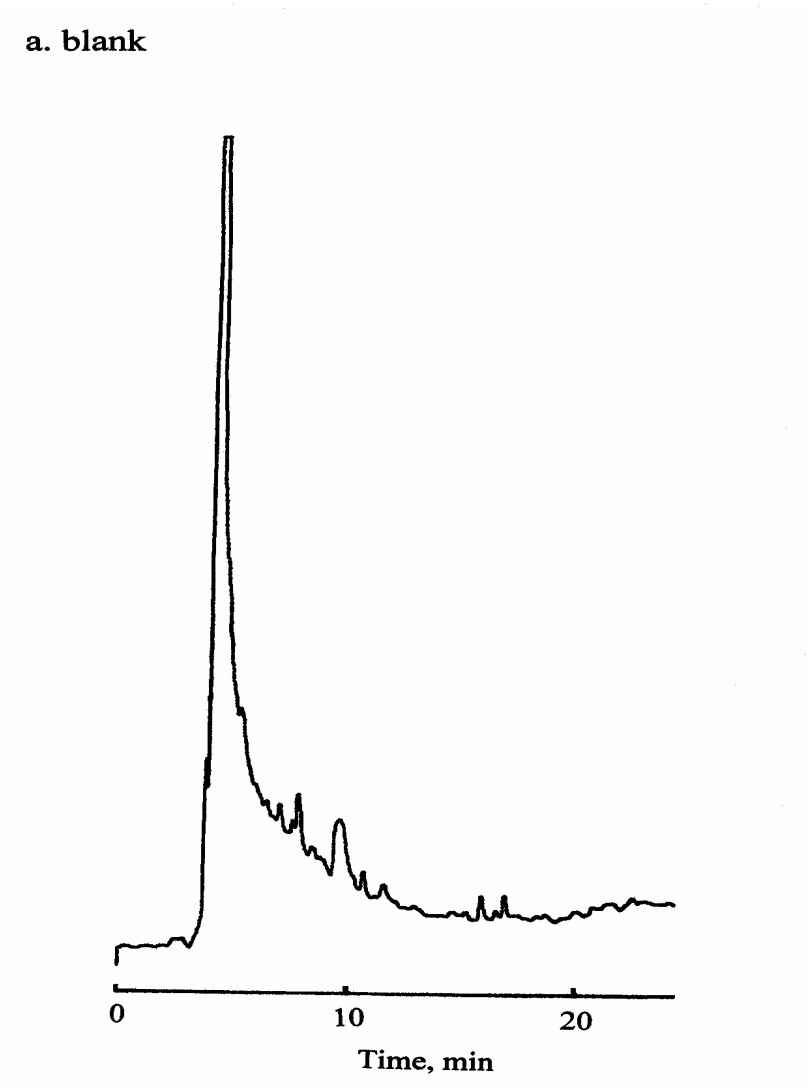


Figure 23a. Analysis of blank urine sample via on-line SFE-RPLC. 1 mL urine, 2.2 mL HPLC grade water and 1.8 mL methanol were well mixed in the vessel with 10 µL 20 µg/mL progesterone stock solution (the absolute concentration in vessel is 40 ng/mL). 15 g CO_2 consumed for dynamic extraction. The trap temperature for collection was 60 °C. Other conditions were same with Figure 21.

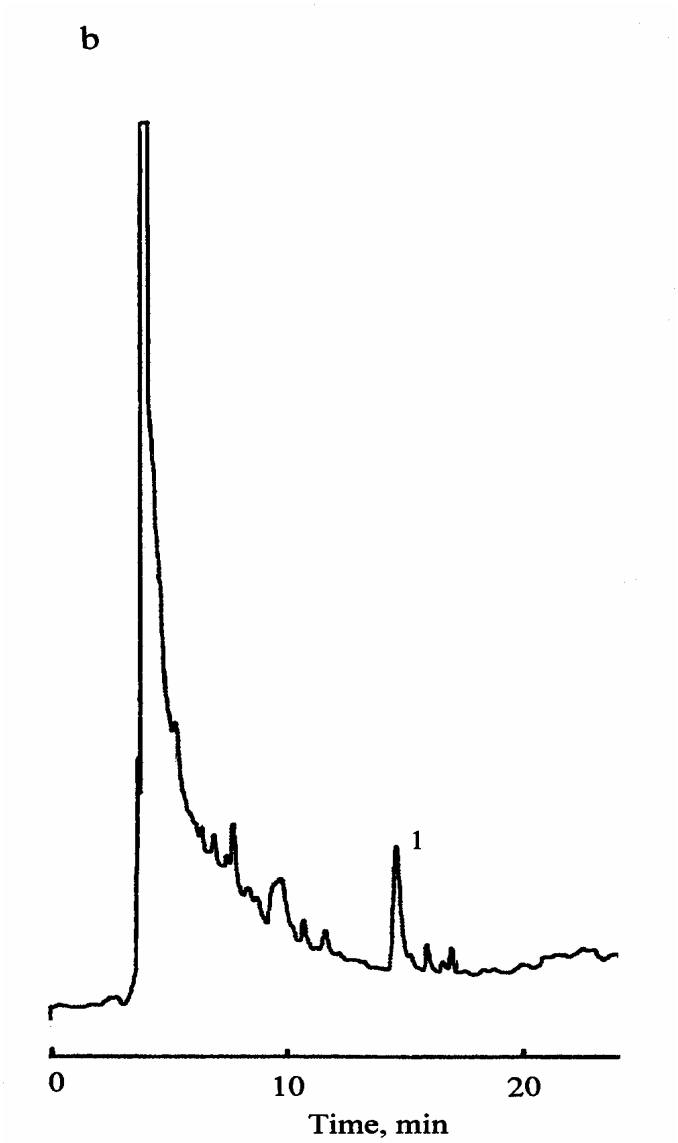


Figure 23b. Analysis of spiked progesterone in urine sample via on-line SFE-RPLC. 1 mL urine, 2.2 mL HPLC grade water and 1.8 mL methanol were well mixed in the vessel with 10 µL 20 µg/mL progesterone stock solution (the absolute concentration in vessel is 40 ng/mL). 15 g CO_2 consumed for dynamic extraction. The trap temperature for collection was 60 °C. Other conditions were same with Figure 21. 1, progesterone.

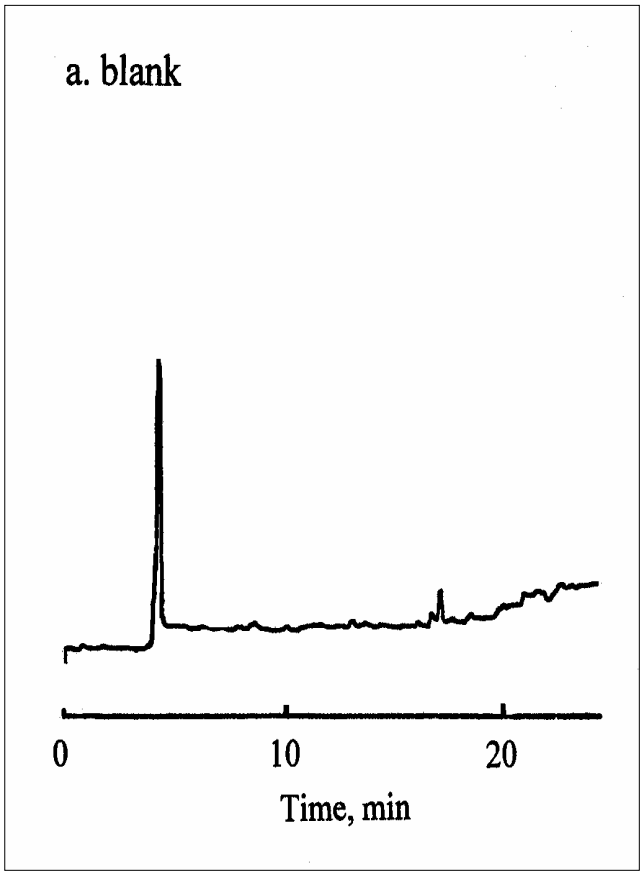


Figure 24a. Analysis of blank natural water sample via on-line SFE-RPLC. 1 mL natural water, 2.2 mL HPLC grade water and 1.8 mL methanol were well mixed in the vessel. Other conditions were same with Fig. 21.

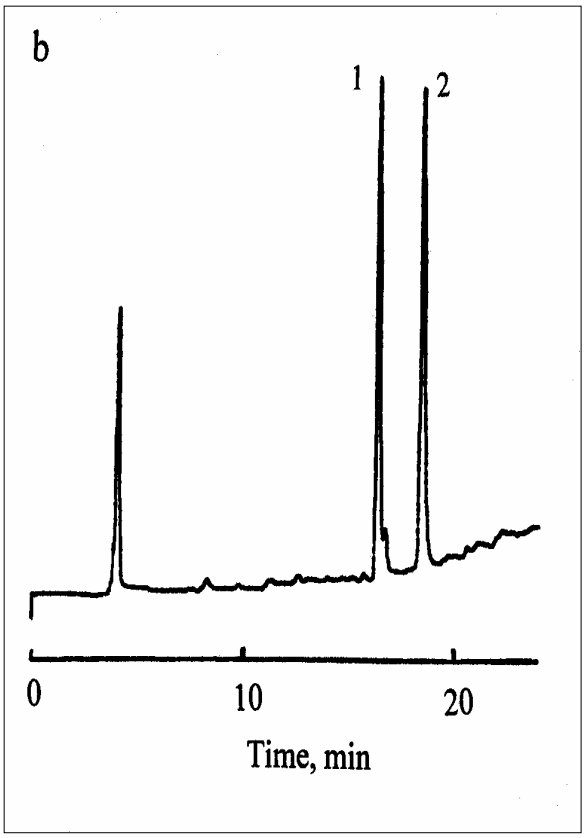


Figure 24b. Analysis of spiked phenanthrene and pyrene in natural water via on-line SFE-RPLC. 1 mL natural water, 2.2 mL HPLC grade water and 1.8 mL methanol were well mixed in the vessel 10 µL 2 µg/mL phenanthrene and 12 µg/mL pyrene stock solution (the absolute concentration in vessel is 4 ng/mL and 24 ng/mL for phenanthrene and pyrene, respectively). Other conditions were same with Fig. 21. 1, phenanthrene; and 2, pyrene.

Table 11 Recovery and precision data for the analysis of real sample. (See Figure 23 and 24 for SFE and LC conditions)

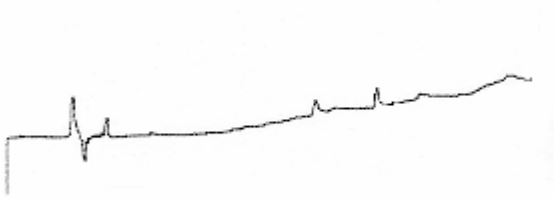
	Urine Sample	Natural water	
	Progesterone	Phenanthrene	Pyrene
Recovery (%)	100.1	100.0	99.8
RSD (%, n =3)	4.1	1.0	2.2

extracts. Then 10 μL of the extracts from the 4 mL methanol collection was injected to LC (injection loop was 10 μL). So it is only 0.25% of the extracted analytes was transferred for analysis. Figure 25a shows that no peak was detectable due to the low quantities of the injected analytes. The SFE was performed once more, and after extraction a concentration step was utilized. The 4 mL methanol collection was concentrated to 100 μL under the stream of nitrogen. Then 10 μL of the concentrated extracts was injected to LC. So this time 10% of the extracted analytes were transferred to LC. Several tiny peaks were observed in the UV trace (Figure 25b). However, the signal was still too weak to be used for quantification. While in on-line SFE-LC mode, after extraction, all the extracted analytes were directly transferred to LC. So it is 100% transfer and four nice peaks were detected in the LC chromatogram (Figure 25c).

Table 12 compares the time usage for these two methods. The extraction time and separation time were identical for both on-line and off-line methods, 20 minutes for extraction and 22 minutes for separation, respectively. While in off-line method, it took about 90 minutes to concentrate the 4 mL solution to 100 μL before injection. The total time usage for each assay is over 2 hours and unfortunately no quantitative result was achieved. For on-line method, after extraction, no extract concentration step is needed except for 4 minutes of rinse step. All of the analytes were directly transferred from extraction system to separation system. The time usage for each assay is about 46 minutes, one third of the off-line method and with quantitative result. In the on-line method, the sensitivity is significantly enhanced and the assay time is greatly diminished.

a.

b.



c.

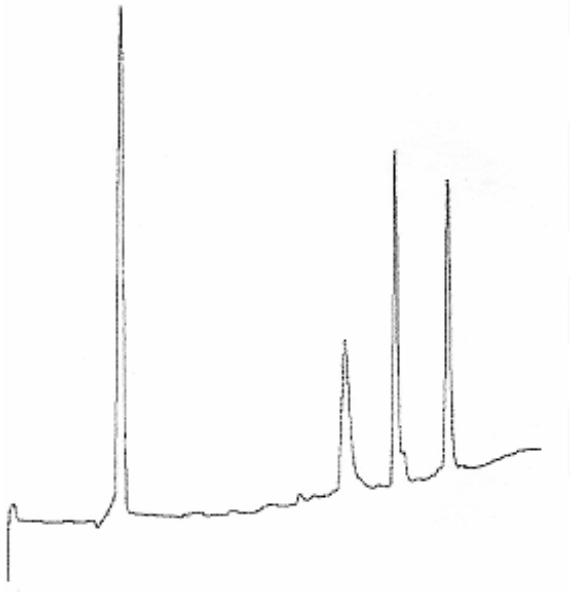


Figure 25. Comparison of on-line and off-line SFE-LC methods for the analysis of trace level analytes. a. off-line SFE-LC without concentration step; b. off-line SFE-LC with concentration step; c. on-line SFE-LC. Concentration of caffeine, progesterone, phenanthrene, and pyrene was 80, 8.0, 1.0, 6.0 μg/mL, respectively. The SFE and LC conditions were the same as described in Figure 21. The LC injection loop was 10 μL.

Table 12. Comparison of time usage (per assay) of on-line and off-line SFE-LC. (See Figure 25 for conditions.)

	Off-line SFE-LC	On-line SFE-LC
Extraction	20 min	20 min
Extract processing	90 min (extracts were concentrated to 100 μL)	4 min (rinse step)
Separation	22 min	22 min
Total time (per assay)	132 min (no quantitative results)	46 min (quantitative results)

4. Conclusion

In this study, on-line SFE-HPLC for the extraction and quantitative analysis of analytes in small volumes of aqueous sample was performed. Experimental parameters were evaluated and optimized. Pre-spiking modifier into the vessel followed by extraction with pure SF CO_2 was found to be very efficient to enhance the extraction recovery due to co-extraction effect. Quantitative extraction and transfer were achieved for the target analytes (progesterone, phenanthrene and pyrene) spiked in water as well as in real samples. Compared to the off-line approach, the on-line method is time and labor saving, since no extract processing or concentrating step is needed. In the on-line mode, all of the extracts can be directly transferred to LC for analysis, which will be beneficial for the quantitative analysis of trace level samples with limited amounts, such as forensic samples. However, for highly polar analytes (such as caffeine), low extraction recovery may be observed due to their high water solubility.

CHAPTER V

Feasibility Study for the Determination of Highly Polar Proanthocyanidins in Grape Seeds

1. Introduction

Grapes are one of the most widely consumed fruits in the world. Grapes are rich in polyphenols, a nutritional supplement that possesses antioxidant activity.[59] Approximately 30% of total grape polyphenols exist in grape skin, while the other 60-70% are contributed from grape seeds.[60] The study of grape seed extracts has been of increasing interests in recent years. Grape seed polyphenols (GSP) have various physiological effects *in vivo*, such as protection against X-ray and ultraviolet rays,[61, 62] anti-cancer effects,[60, 63, 64] and inhibitory effects against hypercholesterolemia.[65]

GSP have a very complicated molecular composition.[66] The main constituents are proanthocyanidins, known as condensed tannin, which include procyanidins and prodelphinidins. Prodelphinidins consist of gallocatechin, epigallocatechin, and their monomeric galloylated derivatives (such as epigallocatechin gallate and gallocatechin gallate, see Figure 26a). Procyanidins which include monomers and oligomers are crucial for the therapeutic activity of grape seed extract. The four monomers of procyanidins are catechin (C), epicatechin (EC), catechin gallate (CG), and epicatechin gallate (ECG) (Figure 26b). The procyanidin oligomers are composed of C and EC units, linked together through C(4)-C(8) inter-flavanoid bonds to form B-type dimer (Figure 26c). The structural variations include A-type dimer, with the formation of a second inter-flavanoid bond by C-O-C bonding (Figure 26d). The phenolic molecular distribution and total

content of polyphenols in grape seed extracts not only depends on the raw materials, but also on the extraction method employed.[59] Extraction, identification, and quantitation of polyphenols pose a challenge because the natural matrix has high enzymatic activity so precautions should be taken to avoid any oxidative, photochemical, and biochemical degradation. Sample preparation is thus a critical step in the analysis of natural polyphenols. Mild extraction conditions are generally believed to be expedient in order to preserve maximum antioxidant capacity.

Supercritical fluid extraction (SFE) with CO_2 affords significant advantages in this regard over conventional solvent extraction techniques. The absence of both air and light during the extraction process can reduce the incidence of degradation, which may easily occur in other extraction techniques. Supercritical CO_2 is an inert extraction medium with a low critical temperature. The analytes, therefore, can be extracted out at very mild conditions (moderate temperature) where the possibility of thermal degradation is reduced. Unfortunately, the solvating power of pure CO_2 is insufficient for removing polyphenols from grape seed. However, pure supercritical CO_2 can be used for the removal of oils from grape seed. Subsequently, methanol-modified CO_2 can be then used for the extraction of the polar components (e.g. principally the polyphenols) from the deoiled seeds.

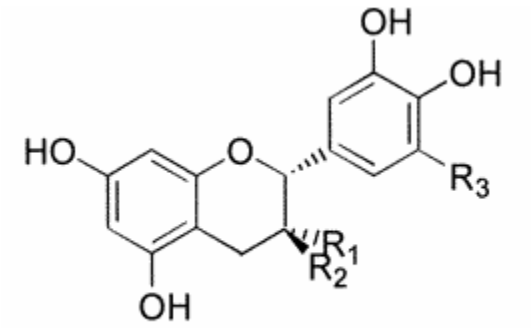
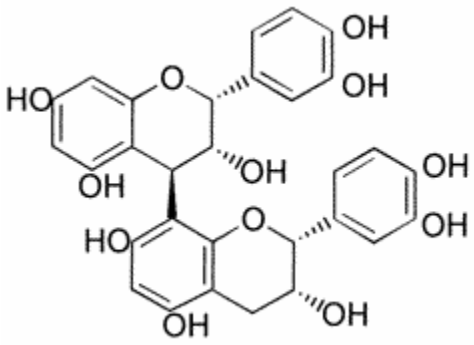
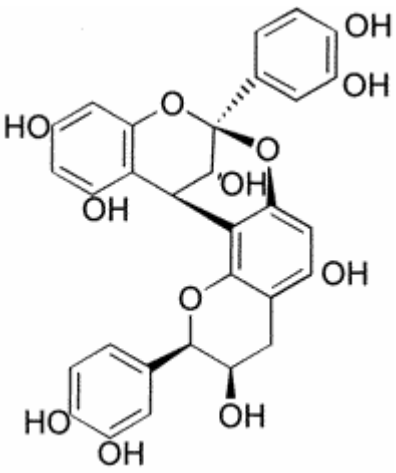


Figure 26. Chemical structures of proanthocyanidins.
a) Prodelphindins:
R_1 = H, R_2 = OH, R_3 = OH: Gallocatechin (MW = 306 amu)
R_1 = OH, R_2 = H, R_3 = OH: Epigallocatechin (MW = 306 amu)
R_1 = H, R_2 = gallic acid ester, R_3 = OH: Gallocatechin gallate (MW = 458 amu)
R_1 = gallic acid ester, R_2 = H , R_3 = OH: Epigallocatechin gallate (MW = 458 amu)

b) Procyanidins:
R_1 = H, R_2 = OH, R_3 = H: Catechin (MW = 290 amu)
R_1 = OH, R_2 = H, R_3 = H: Epicatechin (MW = 290 amu)
R_1 = H, R_2 = gallic acid ester, R_3 = H: Catechin gallate (MW = 442 amu)
R_1 = gallic acid ester, R_2 = H , R_3 = H: Epicatechin gallate (MW = 442 amu)



c) B-type dimer (catechin-catechin)



d) A-type dimer (catechin-catechin)

Palma and Taylor earlier described an off-line extraction method that utilized 35% methanol-modified supercritical fluid CO_2.[67] Three components (i.e. gallic acid, catechin, and epicatechin) were off-line identified and quantified in the de-oiled grape seed extracts via HPLC-UV with standards of the three materials. Mass spectrometric detection was not attempted with these polar extracts.

In this study, we aimed with the addition of electrospray ionization mass spectrometric detection to evaluate the ability of SFE-LC-UV/ESI-MS for the on-line identification and analysis of proanthocyanidins in similar grape seeds. After the removal of all grape oils via pure CO_2, the polar extracts obtained via methanol-modified CO_2 can be directly transferred to a reversed phase LC analytical scale packed column for identification and quantitation by both ultraviolet and mass spectrometric detection. Proanthocyanidins are highly polar compounds, which challenge both SFE extraction and also SPE retention during the water rinse step. This study will assist in finding the limitations of this hyphenated system.

2. Experimental Section

2.1 Apparatus

An Isco-Suprex (Lincoln, NE) Prepmaster supercritical fluid extraction system equipped with solid phase Accutrap and methanol modifier pump was used for all parts of the study. SFE/SFC grade carbon dioxide with 2000 p.s.i. helium head pressure was purchased from Air Products and Chemical Inc. (Allentown, PA). Extractions were performed using a 2.5 mL stainless steel extraction vessel (Keystone Scientific, Bellefonte, PA). Grape seed, previously ground (50 mg) was mixed with enough Ottawa

sand (Fisher Scientific, Fair Lawn, NJ) to fill the vessel. The SFE variable electronically controlled restrictor was heated to 60 °C for all extractions. A 10 cm × 0.2 cm i.d. stainless steel column filled with Discovery® DSC-18 (particle size: ~50μm, Supelco, Bellefonte, PA) was used as the SFE trap. Other trapping materials were also evaluated, including Isolute Sorbent™ C_{18} (particle size: 40-70μm), Discovery® Cyano (~50μm) and DPA-6S (polyamide resin, 50-160μm) from Supelco, Bellefonte, PA, and Oasis® HLB (hydrophilic-lipophilic balance materials, ~60μm) from Waters Co., Milford, MA. The optimized supercritical extraction procedure is shown in Table 13. In the off-line SFE mode, a tandem trap was employed by inserting the outlet tubing of the solid trap into a collection vial half-filled with methanol. A tandem trap arrangement was not possible in the on-line case. The rinse solvent in off-line SFE was methanol. The extraction vessel was 10 mL and each time 5 grams of crushed seeds were extracted in the off-line experiment.

An Agilent 1050 HPLC system (Wilmington, DE) with programmable multi-wavelength UV detector was used to quantitatively analyze the chromatographically separated extracts. A polar embedded alkyl phase Discovery® RP Amide C16 column (Supelco, Bellefonte, PA) with dimensions of 250 × 4.6 mm with 5 μm particles was used for the separation. The mobile phase was acetonitrile and water (each containing 0.5% formic acid, v/v) with a flow rate of 1.0 mL/min. The chromatographic gradient program was begun at 5% acetonitrile for 1 minute, then linearly increased to 15% at 20 minutes (around 0.5% per minute), then linearly increased to 30% at the 30 minutes (1.5% per minute), to 40% at the 40 minutes (1% per minute), and then to 100%

Table 13. SFE conditions for the extraction of grape seeds.

Step 1: De-oil via pure CO_2	
Oven temperature:	80 °C
Pressure:	400 atm
Static time:	30 min
Dynamic time:	120 min
Flow rate:	2.0 mL/min
Modifier:	none
Collection temperature:	70 °C
Trap rinse solvent:	10 mL DCM/Methanol
Extract destination:	to waste

Step 2: Polyphenol extraction via modified CO_2	
Oven temperature:	80 °C
Pressure:	400 atm
Static time:	0 min
Dynamic time:	120 min
Flow rate:	1.0 mL/min
Modifier:	40% methanol
Collection temperature:	70 °C
Trap rinse solvent to remove CO_2:	4 mL water
Trap rinse solvent to remove analytes:	LC mobile phase
Extract destination:	to LC on-line analysis

acetonitrile at the 50 minutes (6% per minute) and held there for 10 minutes, with a total analysis time of 60 minutes. The UV detection wavelength was set at 280 nm.

LC-MS was performed (to afford identification information) with an Agilent 1100 HPLC system (Wilmington, DE), which was interfaced to a Finnigan TSQ Quantum mass spectrometer (ThermoFinnigan, San Jose, CA). In order to operate at low flow rate so that the electrospray (ESI) interface has a high ionization efficiency, the LC column for on-line SFE-LC/MS study was a small dimension Zorbax Eclipse XDB-C18 column (2.1 x 150 mm, 5 μm, Wilmington, DE). The mobile phase was acetonitrile and water (each containing 0.5% formic acid, v/v) with a flow rate of 0.2 mL/min. The chromatographic gradient program was begun at 0% acetonitrile for 3 minutes, then increased to 25% at 45 minutes (0.6% per minute), then increased to 40% at the 55 minutes (1.5% per minute), to 100% acetonitrile at the 60 minutes (12% per minute). The mass spectrometer was operated in the negative electrospray mode. ESI conditions: electrospray voltage 3.0 kV, capillary temperature 300°C, source CID off, scan range *m/z* 200 to 1500.

Experimental details concerning the SFE-LC interface are given in Chapter 2.

2.2 Chemicals and Real Sample

Grape seeds were provided by Synthon Inc. (Blacksburg, VA). They were cultivated in Washington State and hand-picked during the harvest of 1997. Seeds were ground using a coffee grinder. The grinding was halted for ~15 sec at periodic intervals to prevent heating of the sample. The crushed seeds were stored at room temperature prior to extraction. Gallic acid (G), catechin (C), epicatechin (EC) standards and formic acid

were purchased from Sigma-Aldrich Chemical Co., (Milwaukee, WI). HPLC grade methanol, acetone, acetonitrile, dichloromethane, and water (Burdick & Jackson, Muskegon, MI) were used.

3. Results and Discussion

3.1 Optimization of Extraction Recovery

3.1.1 Extract Trapping Materials

Polyphenols have relatively wide polarities and molecule weights, including very polar compounds such as gallic acid, intermediate polar compounds such as catechin and epicatechin, and less polar compounds which are higher molecular mass oligomers. To achieve quantitative results, the extract trapping system must be highly efficient. In our on-line SFE-LC design, the trap, i.e. solid phase extraction (SPE) cartridge should have the ability, not only to hold the extracted analytes during dynamic extraction and CO_2 decompression, but also to hold the adsorbed analytes during water rinsing of the trap. The latter step is necessary in order to displace residual CO_2 gas that appears in the trap and connection line. Otherwise, the gas may enter LC and interfere with UV detection.

Five types of SPE products were tested as on-line SFE trapping materials. Three representative grape seed polyphenolic components (e.g. gallic acid, catechin and epicatechin) were chosen as probes to evaluate these materials. Each time, 10μL of an aqueous solution composed of the three standards was spiked into the SPE cartridge. After rinsing the trap with 4mL of water, the aqueous eluent was analyzed by LC to determine if any of the phenols had been removed from the trap (Figure 27). The cyano phase failed to show good retention for the three highly polar phenolic compounds during

the water rinsing step. All three representative components were detected in the rinse water employed for residual CO_2 removal. Similar results were found when Isolute Sorbent^{TM} C_{18} was used as the trapping material. Discovery® DSC-18, which has high carbon loading (18%), offered better retention during water elution. Only gallic acid was detected in the rinse water with the latter trap material.

Two novel type SPE materials, Discovery® DPA-6S (polyamide resin, designed for retaining polar compounds with an hydroxyl group), and Oasis® HLB (N-vinylpyrrolidone and divinylbenzene co-polymer, designed for retaining both polar and non polar compounds) were also evaluated. With these two phases, catechin and epicatechin remained on the trap during water rinsing, while only 1% (w/w) of gallic acid was removed. Unfortunately, when on-line SFE-LC experiment was performed, the chromatogram showed serious band broadening (Figure 28). One possible reason for the poor peak shape is that a high percentage of mobile phase water (95%) was initially used in the reversed-phase LC gradient in order to obtain sufficient retention and separation of the three polar phenolic compounds. In other words, both solid phases have strong retention for polar compounds so that when the LC mobile phase with low organic content (5%ACN) enters the trap, the analytes cannot be efficiently eluted from the trap to the LC column. This phenomenon was not observed with the other three phases. As a compromise consideration, Discovery® DSC-18 was selected as the trapping material in our research. Therefore, in the on-line method, we were unable to obtain quantitative results on gallic acid and more polar compounds than gallic acid due to less retention by the trap during the preliminary water rinse.

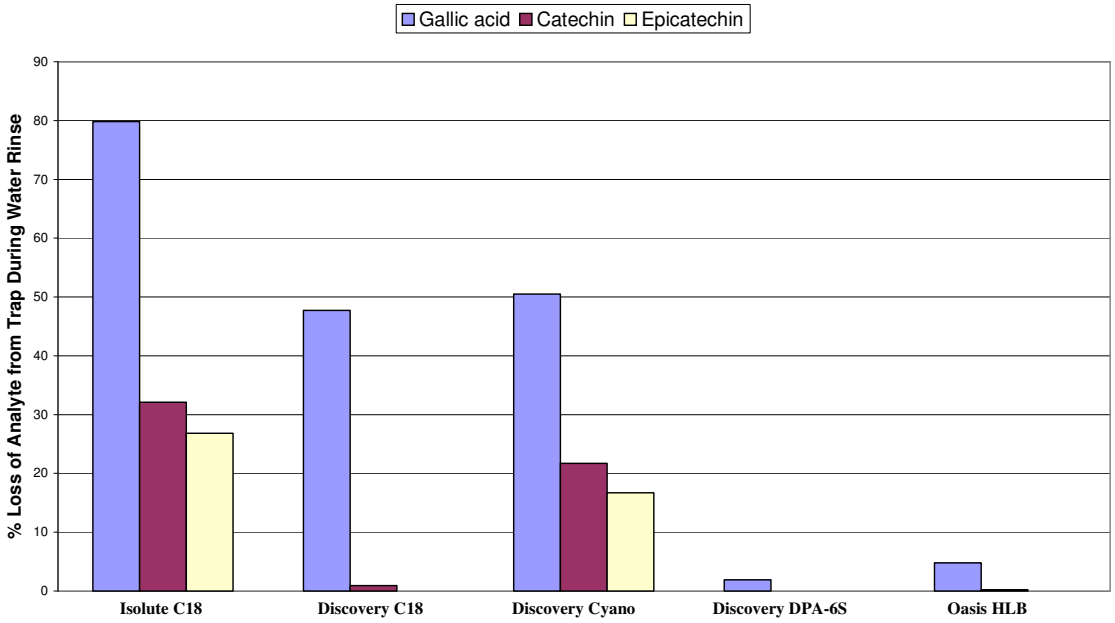


Figure 27. Comparison of loss of analytes from five trapping materials during water rinse. (10 μL of aqueous solution spiked on trap and 4 mL water was used to rinse, each compound has the concentration of 100 μg/mL, average value of two measurements.)

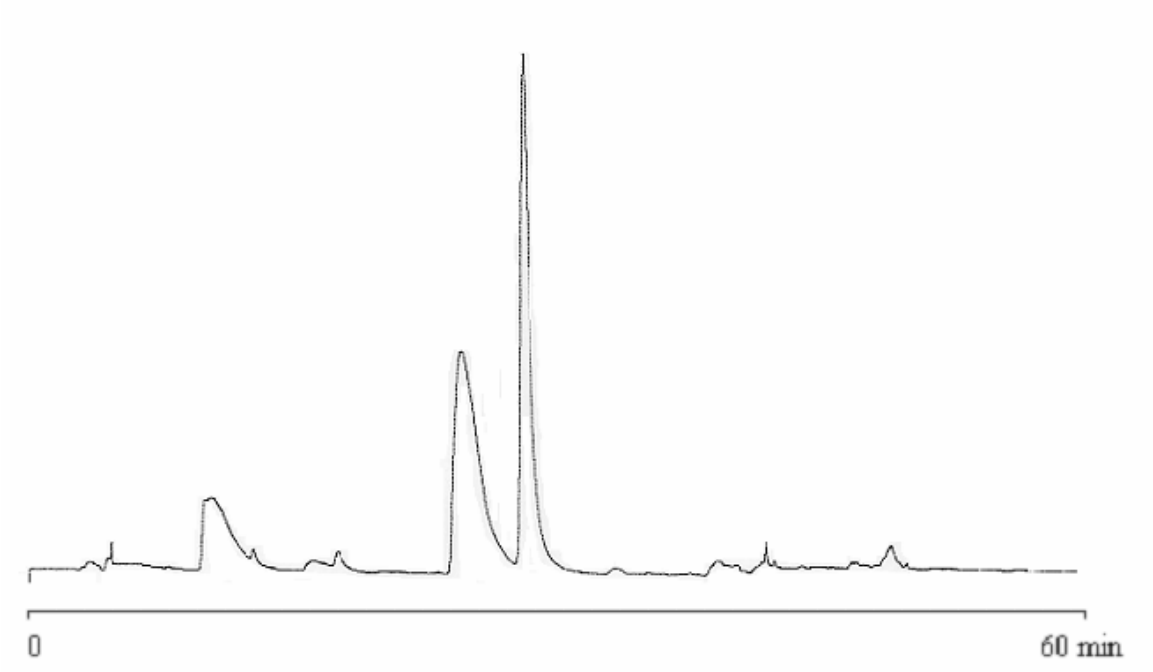


Figure 28. Chromatogram of on-line SFE-LC, Discovery DPA-6S as the trapping materials. See Table 13 for SFE conditions, 120 min dynamic extraction; LC conditions: Discovery® RP Amide C16 column (250 × 4.6 mm, 5 μm). Mobile phase: acetonitrile/water (0.5% formic acid, v/v). Gradient elution: 5% acetonitrile for 1 minute, then increased to 15% at 20 minutes (around 0.5% per minute), then linearly increased to 30% at 30 minutes (1.5% per minute), to 40% at 40 minutes (1% per minute), and then to 100% acetonitrile at 50 minutes (6% per minute) and held for 10 minutes. Flow rate: 1.0 mL/min. UV detection: 280 nm.

3.1.2 On-line Extraction Conditions

Previously, it has been shown that pure CO_2 can be successfully used for the removal of oils from grape seed.[67] For our on-line design, it was essential that all of the oils in the first step be removed. If any oil was left in the seed and extracted by methanol-modified CO_2 during the second step, it may enter the LC column with the extracted polyphenols and plug or damage the LC column. During the de-oiling step, pure CO_2 (80°C, 400 atm) was employed at 2 mL/min for 120 min in order to fully remove oil. A mixture of dichloromethane/methanol (1:1, v/v, room temperature) was used to elute the extracted oil from the solid phase C_{18} trap to waste.

To obtain the highest extraction efficiency of the polyphenols, modifier percentage, extraction temperature, and CO_2 pressure were evaluated. The combined amounts of extracted catechin and epicatechin were used as the criteria to evaluate extraction efficiency at different conditions. Higher extraction pressure (400 atm compared to 200 atm) and higher extraction temperature (80°C compared to 50°C) seemed more favorable for the extraction of catechin and epicatechin from the grape seed (see Table 14). For these polar phenolic compounds, the percentage of modifier had a significant effect on extraction efficiency. The polarity of the extraction medium increases when more polar modifier is added. A former study has demonstrated that methanol is a better CO_2 modifier than ethanol to extract polyphenols.[67] Thus, different percentages (v/v) of methanol as modifier were investigated. When methanol was increased from 30% to 40%, a nearly 80% increase in recovery (catechin 20.2 μg to 36.6 μg, epicatechin 22.0 μg to 38.7 μg.) was observed. Extraction time was another important parameter evaluated. Under optimized conditions (400 atm, 80°C and 40% modifier), 360

Table 14. The influence of modifier, extraction temperature, and pressure on extraction efficiency.[a]

Experiment	CO_2 Density (g/mL)	Modifier (Methanol) Percentage	Extraction Temperature	Extraction Pressure (atm)	Catechin extracted (μg)	Epicatechin extracted (μg)
1	0.79	30%	50°C	200	11.4	13.6
2	0.93	30%	50°C	400	15.1	16.8
3	0.83	30%	80°C	400	20.2	22.0
4	0.83	40%	80°C	400	36.6	38.7

[a.] Crushed grape seeds (50 mg), dynamic extraction for 120 min, liquid CO_2 flow rate: 1 mL/min.

min (triple extraction, 120 min each) were utilized to obtain an exhaustive extraction (Figure 29). Such a long extraction time is probably dictated by the characteristics of the sample. With natural products, analytes have much stronger interactions with matrices, compared to the interaction with an inert support. When 30% modifier was used, a longer extraction time was necessary in order to achieve an exhaustive extraction. The overall results from the two methods (30% and 40% modifier) are comparable. The total extractable catechin in a 50 mg de-oiled sample was 55.5 μg (1.1 mg per gram seed). The total extractable epicatechin in the same 50 mg sample was 62.0 μg (1.2 mg per gram seed).

3.2 Comparison of on-line and off-line SFE-LC

The UV-trace of on-line SFE-LC is shown in Figure 30a. Compared to the LC chromatogram via off-line SFE/LC (Figure 30b), the on-line approach provided less peak quantity and with a slight increase in band broadening. The on-line design introduced extra column dead volume (e.g. trapping cartridge and connection tubing) to the LC system. In addition, some polar compounds were undetectable in the on-line mode due to: (a) less trapping efficiency when a high percentage of methanol was used as the modifier and (b) some loss of trapped extract was introduced during the water rinse step. These disadvantages can be avoided in the off-line mode since (1) a tandem solid-liquid trap was utilized and (2) residual trapped CO_2 can be removed during the trap rinse/recovery step. Table 15 compares the two methods concerning time usage, sample amount, and quantitative results. The on-line method needed less time in that no extract processing step was needed and all the extracts were directly transferred to the LC for analysis. Also

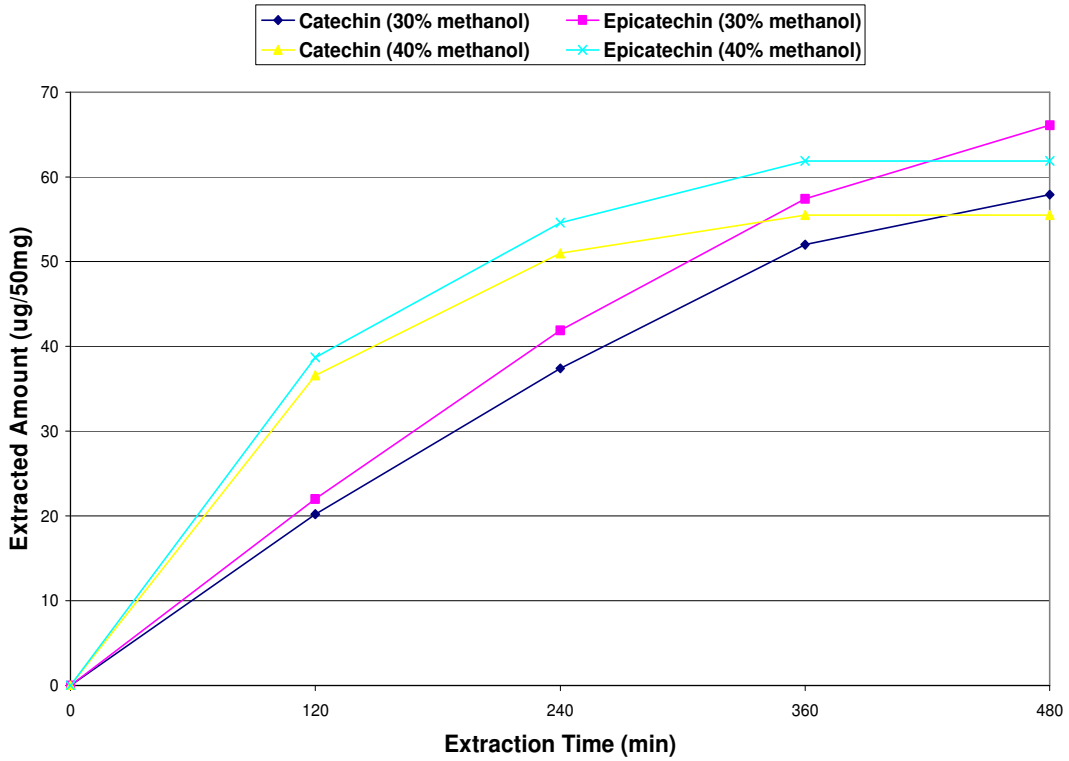


Figure 29. Extraction profile of catechin and epicatechin at diffferent modifier percentages. (See Table 13 for extraction conditions. Average value of two measurements)

a

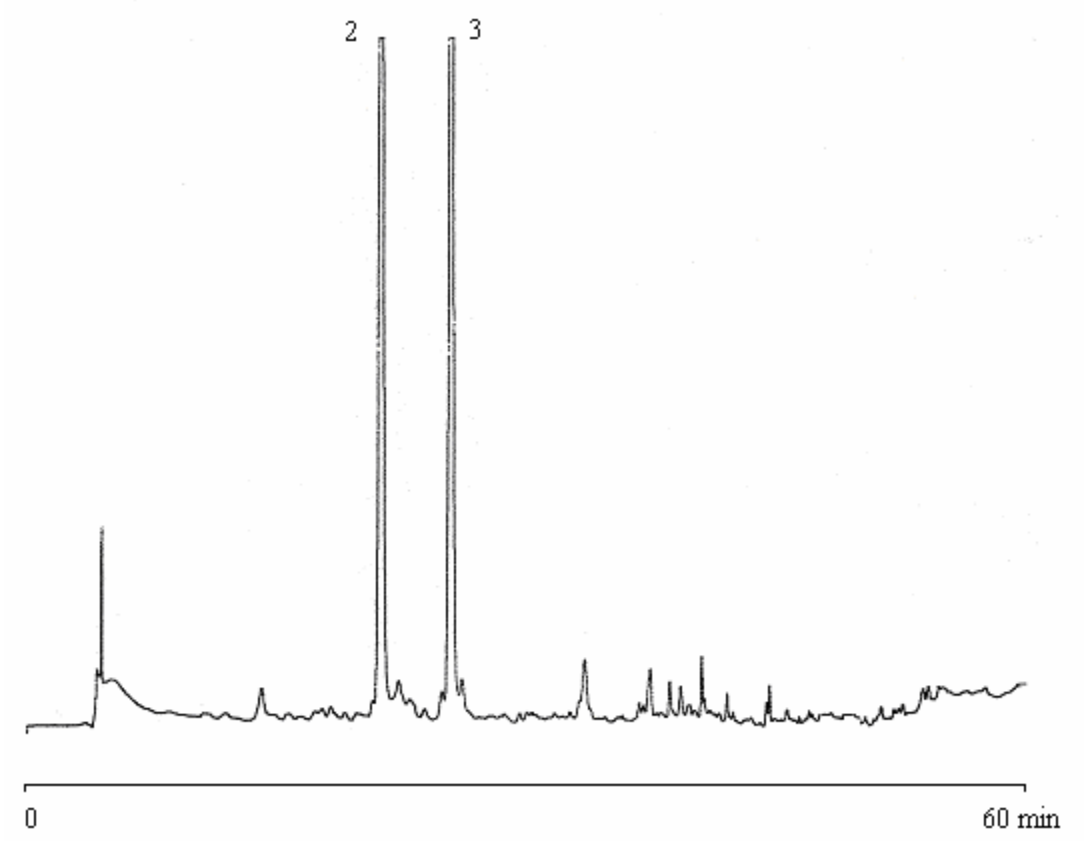


Figure 30a. Chromatogram of on-line SFE-LC grape seed extract. See Table 13 for SFE conditions, 120 min dynamic extraction, trapping material: Discovery C18; LC conditions: Discovery® RP Amide C16 column (250 × 4.6 mm, 5 μm). Mobile phase: acetonitrile/water (0.5% formic acid, v/v). Gradient elution: 5% acetonitrile for 1 minute, then increased to 15% at 20 minutes (around 0.5% per minute), then linearly increased to 30% at 30 minutes (1.5% per minute), to 40% at 40 minutes (1% per minute), and then to 100% acetonitrile at 50 minutes (6% per minute) and held for 10 minutes. Flow rate: 1.0 mL/min. UV detection: 280 nm.

b

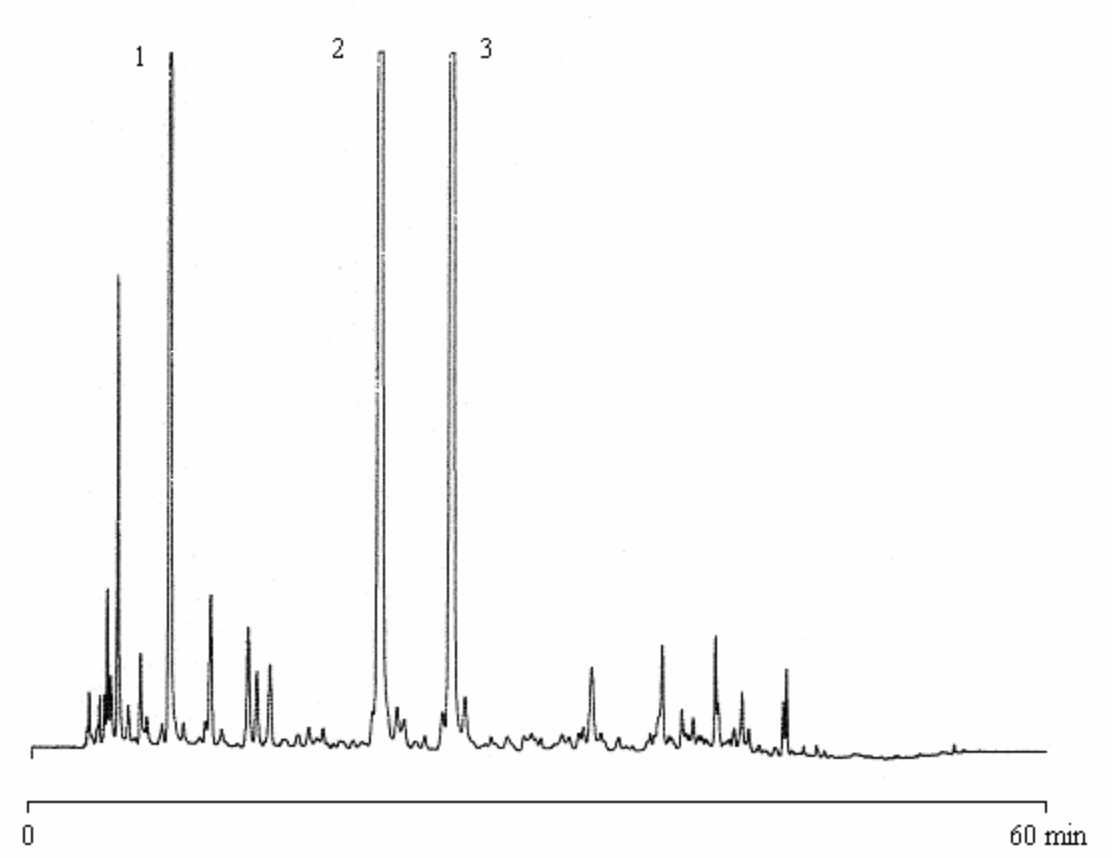


Figure 30b. Chromatogram of off-line SFE-LC grape seed extract. (See Table 14 for SFE conditions, 120 min dynamic extraction, 5 g of sample and 10 mL vessel for extraction. Tandem trap: C_{18} solid trap plus methanol liquid trap. Rinse solvent:4 mL methanol. Collection was concentrated to 1 mL and 10 μL injected for separation. LC conditions see Figure 30a. 1. gallic acid, 2. catechin, 3. epicatechin.

Table 15. Comparison of on-line and off-line methods for deoiled seeds.

	On-line SFE-LC	Off-line SFE-LC
Sample amount	50 mg	5 g
Extraction time	de-oil step: 120 min Extraction 360 min (triple extraction, 120 min each)	de-oil step: 120 min Extraction 360 min (triple extraction, 120 min each)
Extract concentration time	None	30 min Concentrate from 4 mL to 1 mL
Separation time	60 min	60 min
Total time usage for each assey	540 min	570 min
Quantitative results	Only possible for intermediate polar extracts	Possible for intermediate polar to polar extracts

it required less sample (50 mg) to be extracted. While with the off-line method, in order to achieve similar sensitivity as the on-line method, 100 times more sample (5g : 50mg) was needed to be extracted, and the extracts had to be concentrated from 4 mL to 1 mL. Finally, only 10 μL of the 1 mL was able to be off-line injected to the LC for analysis. However, in the off-line method, there is no sacrifice of LC separation efficiency due to extra column dead volume, and most of the extracts are quantifiable. The extractable catechin and epicatechin amounts via the off-line method are 1.7 mg/g, and 1.9 mg/g. The increase in extractables via the off-line method is probably due to the use of a tandem trap. A single solid phase trap (as in on-line mode) may not be efficient enough to retain all the extracts when a high percentage of methanol is used as modifier. Adding a liquid trap also assisted in retaining the analytes that might have eluted from the solid trap.

3.3 On-line SFE-LC-MS study

Off-line SFE/LC-MS was performed in a previous study.[59] Catechin, epicatechin, singly linked procyanidin dimers, and galloylated procyanidin dimers were identified in the supercritical CO_2-methanol extracts. Here, MS was coupled with on-line SFE-LC to provide specific extract compound information. Mass spectral data showed mainly the presence of catechin and epicatechin (Figure 31a). Several singly linked dimers (B-type) were also observed with *m/z* 577 $[M–H]^{-1}$ (Figure 31b), but no higher oligomer was detected. The doubly linked procyanidins (A-type) are produced via the formation of a second interflavanoid bond (C-O oxidative coupling). Due to the complexity of the structure, A-type procyanidins are not as frequently encountered in nature compared to B-type procyanidins.[68] However, several A-type dimers (Figure 32a) and one trimer

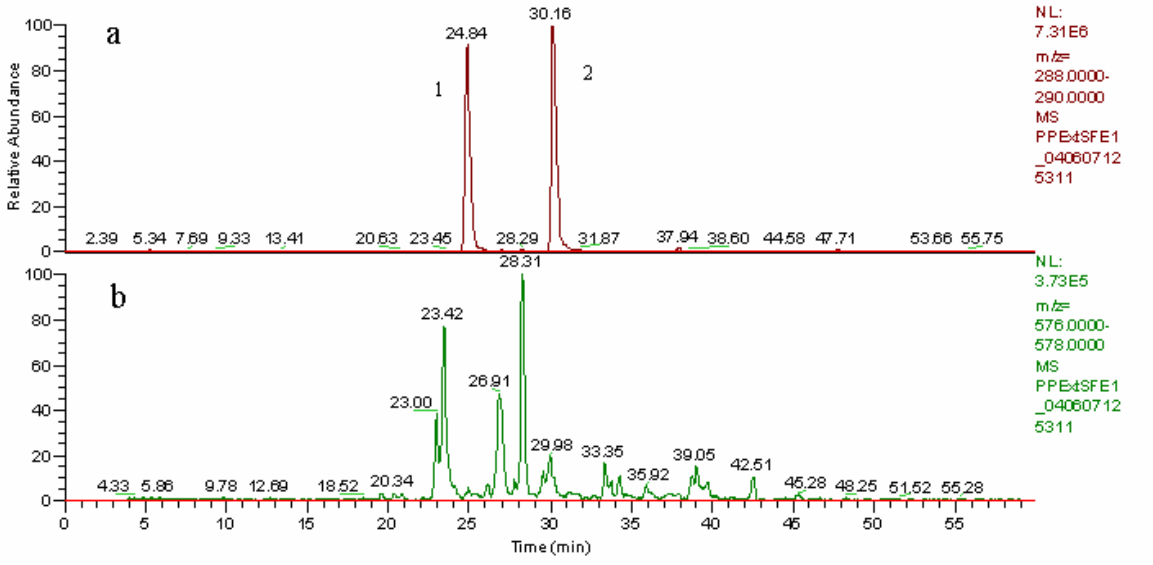


Figure 31. Extracted ions (288-289, 576-578 amu) from on-line SFE-LC/MS of deoiled grape seeds. a: 1, catechin, 2. epicatechin; b: singly linked (B-type) dimers.

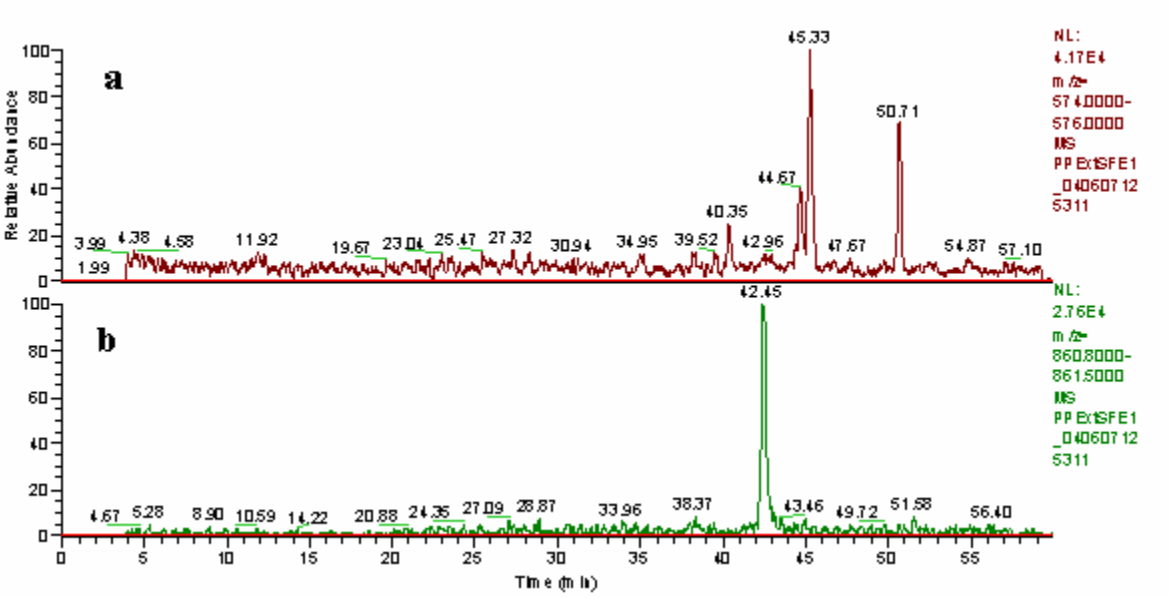


Figure 32. Extracted ions (574-576, 860.8-861.5 amu) from on-line SFE-LC/MS of deoiled grape seeds. a: doubly linked (A-type) dimers; b: doubly linked (A-type) trimer.

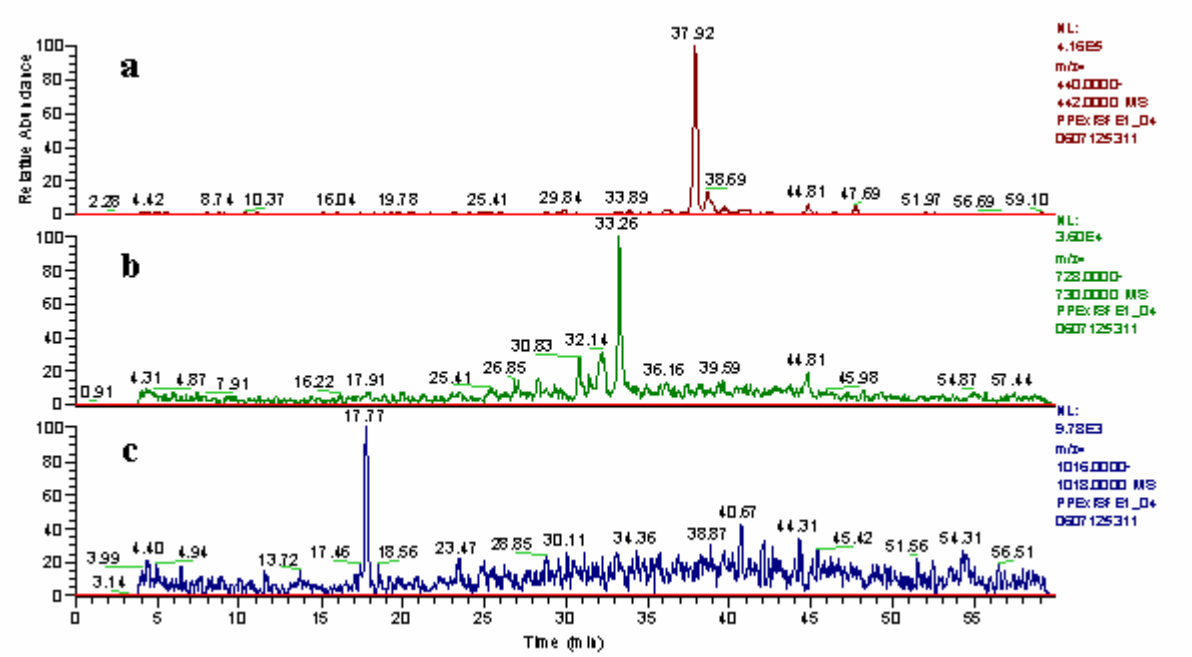


Figure 33. Extracted ions (440-442, 728-730, 1016-1018 amu) from on-line SFE-LC/MS of deoiled grape seeds. a: catechin gallate; b: dimer gallate; c: trimer gallate.

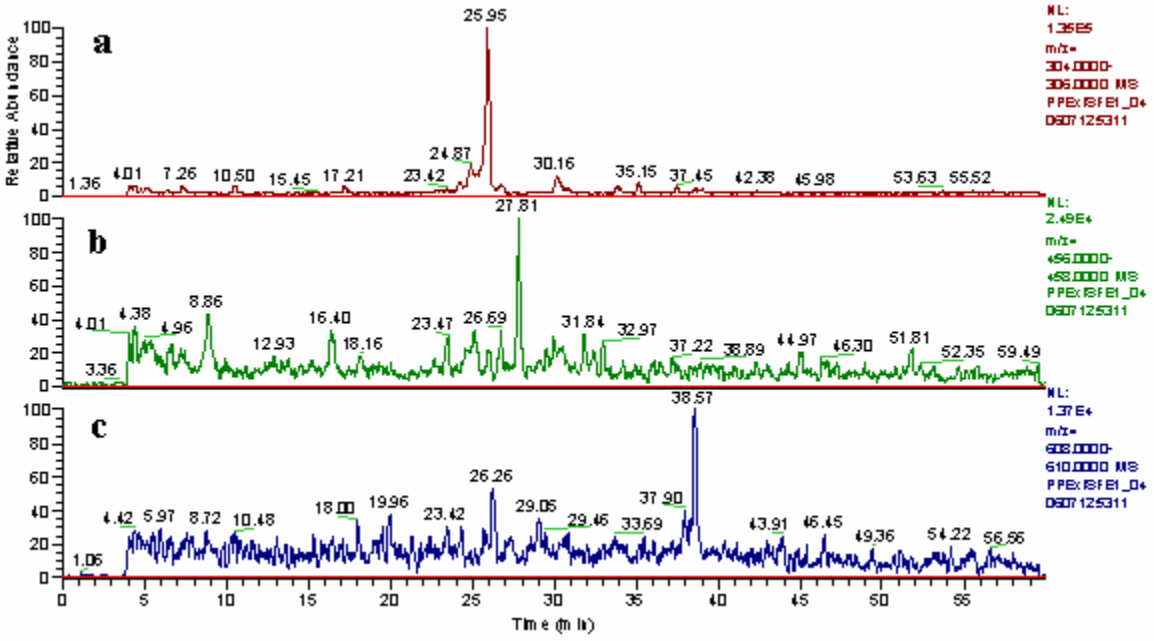


Figure 34. Extracted ions (304-306, 456-458, 608-610 amu) from on-line SFE-LC/MS of deoiled grape seeds. a: gallocatechin; b: gallocatechin gallate; c: gallocatechin dimer.

(Figure 32b) were found in the grape seed SF extracts with *m/z* 575 $[M–H]^{-1}$ and *m/z* 861 $[M–H]^{-1}$. The galloylated procyanidins were found at *m/z* 441, 729, and 1017 $[M–H]^{-1}$, corresponding to catechin gallate (monomer, Figure 33a), dimer gallate (Figure 33b) and trimer gallate (Figure 33c), respectively. No higher oligomer gallates and higher gallate derivatives (e.g. oligomer digallate and oligomer trigallate) were detected. In addition, ions were observed in the mass spectral trace of grape seed SF extracts which suggested the presence of prodelphinidins and their galloylated derivatives (Figure 34). Deprotonated molecular ion *m/z* 305 corresponded to gallocatechin (Figure 34a), while *m/z* 457 and 609 $[M–H]^{-1}$ were related to gallocatechin gallate (Figure 34b) and gallocatechin dimer (Figure 34c). No higher oligomer was found.

4. Conclusion

In this study, we explored the feasibility of extraction and analysis of proanthocyanidins in grape seeds via on-line SFE-HPLC-UV/MS. The hyphenated design avoided possible analyte oxidation, degradation, or contamination which may occur in the extract processing step in a traditional off-line method. Catechin and epicatechin were successfully quantified after optimizing the solid phase trap. Mass spectral data confirmed the existence of four types of proanthocyanidins, e.g. singly linked and doubly linked procyanidins, galloylated procyanidins, and prodelphinidins. Only low molecular oligomers (up to trimer) were found in the supercritical CO_2 extracts. Compare to the off-line SFE-LC approach, much less sample was necessary in the on-line extraction method, since all the extracted compounds can be directly transferred to the LC. Also, no extract processing/concentration step was needed in the on-line method. However, in the on-line

mode, some highly polar compounds were lost during the collection step (lower trapping efficiency of single solid trap when high percentage modifier was used) and during the water rinsing step (less retention of polar compounds on C18 trap).

CHAPTER VI

Conclusions

The purpose of this research was to develop a simple, novel interface for on-line coupling of SFE with RPLC, and to explore its ability for quantitative analysis of trace level compounds in different matrices. The hyphenated system we designed has several advantages:

1) Simple interface

As we know, for hyphenated techniques used in sample preparation and separation, the key point is: the interface should be as simple as possible. The more valves and connection tubes that are used to create the hyphenation, the more extra-column dead volume is introduced into the system. A complicated design will usually sacrifice the separation efficiency and make the hyphenated system less desirable. In our design, only one six port injection valve was used to connect two commercial systems (SFE and LC). A solvent displacement method was employed to eliminate residual decompressed CO_2 gas in the SFE solid phase trap. No CO_2 gas entered the LC system so that stable LC and UV detection was obtained. The simple interface introduced minimal extra column dead volume thus providing less sacrifice in separation efficiency. Based on this system, quantitative analysis of polynuclear aromatic hydrocarbons (PAHs) from both a standard sand matrix and naturally contaminated soil was achieved. Compared to the existing EPA method (Soxhlet extraction following by GC-MS), on-line SFE-LC gave precise results in a much shorter time (Chapter II).

2) Improved sensitivity

On-line SFE-LC design provided several unique advantages over the off-line SFE/LC approach. In the on-line mode, all of the extract can be directly transferred to the LC, so that the sensitivity is improved, compared to the off-line method, where only a fraction of the extract can be injected into the LC. During the analysis of trace levels of caffeine, progesterone, phenanthrene, and pyrene in water, we observed that our on-line SFE-LC achieved quantitative results. While in the off-line approach, for the same analytes, the signal was too weak to be used for quantification, even after concentrating the extracts (Chapter IV).

3) Saved time

In the on-line method, no extract concentration step is needed. All of the analytes were directly transferred from the extraction system to the separation system. However, in the off-line approach, since only a part of the extract can be injected to the LC, we had to concentrate the extracts before LC injection in order to obtain enough detection sensitivity. This concentration step takes additional time and brings possible errors to the whole analysis. The on-line method took only about one-third of the time for the analysis of trace level compounds, compared to that of the off-line method (Table 12 in Chapter IV).

4) Air/light free analysis

On-line coupling of SFE with LC-UV/MS provided an air/light free extraction-separation-detection system. Supercritical CO_2 is an inert extraction media with a low critical temperature (31˚C). The anayltes, therefore, can be extracted at mild conditions. On line coupling of SFE and liquid chromatography is consequently highly beneficial for the trace analysis of air/light-sensitive, thermal liable compounds since all the extract is

directly transferred to the analytical system in an air/light-free environment. Thus, the opportunity for sample degradation or loss during sample processing is avoided. The successful application was demonstrated for the determination of hyperforin and its degradation products in St. John's Wort. MS data confirmed the presence of furohyperforin, which is the major degradation compound of hyperforin, as well as two furohyperforin analogues in dry St. John's Wort plant. The degradation was attributed to pre-analysis degradation in the plant drying or storage.

However, during the study of highly polar phenolic compounds in grape seeds, a major limitation of on-line SFE-LC was observed. In our SFE-LC design, we used a water displacement method to eliminate the residual CO_2 gas in the solid phase SFE trap, which is an essential step in order to prevent the gas from entering the LC system. Highly polar compounds have high water solubility, and the polar extracts may co-elute with water during this rinse step. Although, we optimized the trapping materials, we still found some polar analytes were lost, after comparison with the off-line LC chromatogram. In the off-line method, such losses can be avoided by utilizing a tandem solid-liquid trap. The polar analytes eluted from the solid trap can be retained in the liquid trap. The tandem trap configuration is not feasible in on-line design. Only solid phase SFE trap can be utilized in our hyphenated system. The highly polar compounds may be lost during the collection step (lower trapping efficiency of single solid trap when high percentage modifier was used) and during the water rinsing step (less retention of polar compounds on C18 trap). This disadvantage made on-line SFE-LC less desirable for the analysis of highly polar compounds. But SFE is an excellent technique for the extraction of non-

polar and intermediate polar compounds. SFE with CO_2 is less used for the extraction for highly polar compounds.

References:

[1] R. E. Majors, *LC-GC*, 1991, **1**, 18

[2] M. L. Lee, K. E. Markides, *Analytical Supercritical Fluid Chromatography and Extraction*, Chromatography Conference Inc., Provo, UT, 1992

[3] B. Wenclawiak Ed*., Analysis with Supercritical Fluid: Extraction and Chromatography*, Springer-Verlag, Berlin, 1992

[4] M. D. Luque de Castro, M. T. Tena, M. Valcarcel*, Analytical Supercritical Fluid Extraction*, Springer-Verlag, Berlin, 1994

[5] M. A. McHugh, V. J. Krukonis*, Supercritical Fluid Extraction: Principles and Practice, Butterworths*, Boston, 1986

[6] S. B. Hawthorne, *Anal. Chem*., 1990, **62**, 633A

[7] M. A. Stone, L. T. Taylor, *J. Chromatogr. A*, 2001, **931**, 53

[8] S. B. Hawthorne, D. J. Miller, *J. Chromatogr. A*, 1987, **403**, 63

[9] S. B. Hawthorne, D. J. Miller, J. J. Langenfeld, *J. Chromatogr. Sci.*, 1990, **28**, 2

[10] S. B. Hawthorne, D. J. Miller, *Anal. Chem*., 1987, **59**, 1705

[11] W. Gmuer, J. W. Bosset, E. Plattuer, *J. Chromatogr. A*, 1987, **388**, 335

[12] M. P. McNally, J. R. Wheeler, *J. Chromatogr. A,* 1988, **435**, 63

[13] S. A. Westwood, Ed., *Supercritical Fluid Extraction and Its Use in Chromatographic Sample Preparation,* CRC Press Inc., Boca Raton, FL, 1993

[14] K. K. Unger, P. Roumeliotis, *J. Chromatogr. A*, 1983, **282**, 519

[15] J. B. Nair, J. W. Huber, *LC-GC*, 1988, **6**, 1071

[16] W. G. Engelhart, A. G. Gargus, *Am. Lab*, 1988, **2**, 30

[17] M. Ashraf-Khorassani, M. Barzegar, Y. Yamini, *J. High Resolut. Chromatogr*., 1995, **18**, 472

[18] M. W. Francis, *J. Phys. Chem.*, 1954, **58**, 1099

[19] S. H. Salleh, Y. Saito, Y. Kiso, K. Jinno, *Anal. Chim. Acta*, 2001, **433**, 207

[20] H. R. Johansen, G. Becher, T. Greibrokk, *Anal. Chem*., 1994, **66**, 4068

[21] M. H. Liu, S. Kapila, K. S. Nam, *J. Chromatogr. A*, 1993, **639**, 151

[22] H. J. Cortes, L. S. Green, R. M. Campbell, *Anal. Chem*., 1991, **63**, 2719

[23] C. Mougin, J. Dubroca, E. Barriuso, *J. High Resolut. Chromatogr*., 1996, **19**, 700

[24] R. Batlle, H. Carlsson, E. Holmgren, A. Colmsjö, C. Crescenzi, *J. Chromatogr. A,* 2002, **963**, 73

[25] E. D. Ramsey, B. Minty, A.T. Rees, *Anal. Commun.*, 1997, **34**, 261

[26] D. Mackay, W. Y. Shiu, *J. Chem. Eng. Data,* 1977, **22**, 399

[27] J. J. Langenfeld, S. B. Hawthorne, D. J. Miller, J. Pawliszyn, *Anal. Chem*., 1993, **65**, 338

[28] A. R. Bilia, S. Gallori, F. F. Vincieri, *Life Sciences,* 2002, **70**, 3077

[29] P. Nathan, *Mol. Psychiatry,* 1999, **4**, 333

[30] B. Gaster, J. Holroyd, *Arch. Intern. Med.,* 2000, **160**, 152

[31] G. Di Carlo, F. Borrelli, E. Ernst, A. A. Izzo, *Trends Pharmacol. Sci.,* 2001, **22**, 292

[32] W. E. Muller, *Pharmacol. Res.,* 2003, **47**, 101

[33] G. Lavie, Y. Mazur, D. Lavie, D. Meruelo, *Med. Res. Rev.,* 1995, **15**, 111

[34] S. S. Chatterjee, S. K. Bhattacharya, M. Wonnemann, A. Singer, W. E. Müller, *Life Sciences,* 1998, **63**, 499

[35] A. G. Jensen, S. H. Hansen, E. O. Nielsen, *Life Sciences,* 2001, **68**, 1593

[36] L. Verotta, G. Appendino, J. Jakupovic, E. Bombardelli, *J. Nat. Prod.,* 2000, **63**, 412

[37] N. Fuzzati, B. Gabetta, I. Strepponi, F. Villa, *J. Chromatogr., A* 2001, **926**, 187

[38] H. C. J. Orth, H. Hauer, C. A. J. Erdelmeier, P. C. Schmidt, *Pharmazie,* 1999, **54**, 76

[39] Y. Cui, C. Y. Ang, *J. Agric. Food Chem.,* 2002, **50**, 2755

[40] L. T. Taylor, *Supercritical Fluid Extraction*, John Wiley & Sons, Inc., New York, 1996.

[41] F. F. Liu, C. Y. W. Ang, T. M. Heinze, J. D. Rankin, R. D. Beger, J. P. Freeman, J. O. Lay, *J. Chromatogr. A,* 2000, **888**, 85

[42] J. L. Hedrick, L. T. Taylor, *J. High Resolut. Chromatogr.,* 1992, **15**, 151

[43] J. L. Hedrick, L. T. Taylor, *J. High Resolut. Chromatogr.,* 1990, **13**, 312

[44] D. Thiebaut, J. P. Chervet, R. W. Vannoort, G. J. Dejong, U. A. Brinkman, R. W. Frei, *J. Chromatogr. A,* 1989, **477**, 151

[45] P. Persson, Z. Barisic, A. Cohen, L. Thorneby, L. Gorton, *Anal. Chim. Acta,* 2002, **460**, 1

[46] J. L. Hedrick, L. T. Taylor, *Anal. Chem.,* 1989, **61**, 1986

[47] L. J. Mulcahey, L. T. Taylor, *Anal. Chem.,* 1992, **64**, 981

[48] M. Ashraf-Khorassani, M. T. Combs, L. T. Taylor, *Talanta,* 1997, **44**, 755

[49] M. Ashraf-Khorassani, L. T. Taylor, *J. Chromatogr. Sci.,* 2000, **38**, 477

[50] I. N. Glazkov, I. A. Revelsky, I. P. Efimov, Y. A. Zolotov, *Chromatographia,* 2000, **52**, 495

[51] I. N. Glazkov, I. A. Revelsky, I. P. Efimov, Y. A. Zolotov, *J. Microcol. Sep.,* 1999, **11**, 729

[52] M. T. Combs, M. Ashraf-Khorassani, L. T. Taylor, *J. Supercrit. Fluids,* 1996, **9**, 1220

[53] L. J. Barnabas, J. R. Dean, S. M. Hitchen, S. P. Owen, *J. Chromatogr. A,* 1994, **665** 307

[54] M. Kane, J. R. Dean, S. M. Hitchen, C. J. Dowle, R. L. Tranter, *Analyst,* 1995, **120**, 355

[55] R. Batlle, H. Carlsson, E. Holmgren, A. Colmsjö, C. Crescenzi, *J. Chromatogr. A,* 2002, **963**, 73

[56] M. A. Stone, L. T. Taylor, *J. Chromatogr. A,* 2001, **931**, 53

[57] J. Pól, B. W. Wenclawiak, *Anal. Chem.,* 2003, **75**, 1430

[58] A. Ault, *Techniques and Experiments for Organic Chemistry*, the Sixth Edition, University Science Books, Sausalito, CA, 1998

[59] M. Ashraf-Khorassani and L. T. Taylor. *J. Agric. Food Chem.,* 2004, **52**, 2440

[60] J. Zhao, J. Wang, Y. Chen, and R. Agarwal. *Carcinogenesis*, 1999, **20**, 1737

[61] J. Castillo, O. Benavente-Garcia, J. Lorente, M. Alcaraz, A. Redondo, A. Ortundo, and J.A. Del Rio. *J. Agric. Food Chem.*, 2000, **48**, 1738

[62] M. Carini, G. Aldini, E. Bombardelli, P. Morazzoni, and R. M. Facino. *Life Science*, 2000, **67**, 1799

[63] J. A. Bomser, K. W. Singletary, M. A. Walling, and M. A. L. Smith. *Cancer Lett.*, 1999, **135**, 151

[64] C. Agarwal, Y. Sharma, and R. Agarwal. *Mol. Carcinog.*, 2000, **28**, 129

[65] K. Tebib, P. Besancon, and J. M. Rouanet, *J. Nutr.*, 1994, **124**, 2451

[66] Y. Nakamura and Y. Tonogai. *J. Agric. Food Chem.,* 2003, **51**, 7215

[67] M. Palma and L. T. Taylor. *J. Chromatogr. A,* 1999, **849**, 117

[68] S. Morimoto, G. I. Nonaka, and I. Nishioka. *Chem. Pharm. Bull.*, 1985, **33**, 4338

VITA

Zhenyu Wang was born on February 21, 1976 in Wuhan, P. R. China. He received his B.Sc. degree in Chemistry from Wuhan University in July, 1998, and M.Sc. degree in Analytical Chemistry from the same university. He began his Ph.D study at Virginia Tech in August, 2001, under the guidance of Dr. Larry T. Taylor, and received his Ph.D in December, 2004. While at Virginia Tech, he worked as an intern for Novartis Pharmaceutical Co. R&D in the summer of 2003. After graduation, he will begin his career at Roche Pharmaceutical Co. in New Jersey.

Publications:

1. "Feasibility Study for the Determination of Highly Polar Proanthocyanidins in Grape Seeds via on-line SFE-LC-UV/MS" Z. Wang, M. Ashraf-Khorassani, L. T. Taylor, ***Journal of Chromatographic Science,*** submitted.

2. "Air/Light-Free Hyphenated Extraction/Analysis System: Supercritical Fluid Extraction On-line Coupled with Liquid Chromatography-UV Absorbance/Electrospray Ionization Mass Spectrometry for the Determination of Hyperforin and its Degradation Products in *Hypericum Perforatum*", Z. Wang, M. Ashraf-Khorassani, L. T. Taylor, ***Analytical Chemistry,*** 2004, ***76***, 6771-6776.

3. "On-line Coupling of Supercritical CO_2 Extraction with Reversed Phase Liquid Chromatography for the Quantitative Analysis of Analytes in Aqueous Matrices", Z. Wang, M. Ashraf-Khorassani, L. T. Taylor, ***Journal of Chromatography A***, 2004, ***1033***, 221-227.

4. "Design for On-line Coupling of Supercritical Fluid Extraction with Liquid Chromatography: Quantitative Analysis of PAHs in Solid Matrix", Z. Wang, M. Ashraf-Khorassani, L. T. Taylor, ***Analytical Chemistry***, 2003, ***75***, 3979-3985.

Presentations/Posters:

1 "On-line Supercritical CO_2 Extraction-Liquid Chromatography for Quantitative Analysis of Analytes in Aqueous Matrices", invited presentation, Z. Wang, M. Ashraf-Khorassani, L. T. Taylor, ***SFC/SFE-2004 International Conference***, Pittsburgh, PA.

2. “Supercritical Fluid Extraction On-line Coupled with Liquid Chromatography-UV Absorbance/Electrospray Ionization Mass Spectrometry for the Determination of Hyperforin and its Degradation Products in *Hypericum Perforatum*”, poster, Z. Wang, M. Ashraf-Khorassani, L. T. Taylor, ***SFC/SFE-2004 International Conference***, Pittsburgh, PA.

3. "Direct Aqueous Extraction and Quantitative Analysis by On-line Supercritical CO_2 Extraction-Liquid Chromatography", oral presentation, Z. Wang, M. Ashraf-Khorassani, L. T. Taylor, ***PittCon 2004***, Chicago, IL.

4. "On-line SFE/HPLC Techniques for the Analysis of PAHs", poster, Z. Wang, M. Ashraf-Khorassani, L. T. Taylor, ***PittCon 2003***, Orlando, FL.