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Analysis of prostaglandins in aqueous solutions by supercritical fluid extraction and chromatography

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Abstract: Trace amounts of prostaglandins (PGs) were selectively analysed without derivatization using supercritical fluid extraction (SFE) and open tubular column supercritical fluid chromatography (SFC). The specific compounds studied were prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$), esters of $PGF_{2\alpha}$, prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) and prostaglandin E_2 (PGE_2). An open tubular column was used with carbon dioxide as the mobile phase and with universal flame ionization detection. Samples were introduced into the column by direct injection using a 1-μl sample loop or by SFE with solute focusing. The 11 standard compounds were effectively separated within 35 min using a density program at constant temperature. The minimum detectable quantity (signal-to-noise ratio = 3) using the direct injection method was 9 ng for 15-propionate $PGF_{2\alpha}$ isopropyl ester. Using the extraction method, the sample size in the extraction cell was increased to 100 μl, which made it possible to analyse compounds that were present in low concentrations. Aqueous PG samples were extracted from adsorbents onto which the samples had been loaded.

Keywords: *Prostaglandins; supercritical fluid extraction; supercritical fluid chromatography.*

Introduction

Prostaglandins (PGs) are biologically active metabolites derived from C_{20} polyunsaturated fatty acids containing a substituted cyclopentane ring. The PGs possess a strongly diversified physiological activity and are potent at sub-ppm levels. The PGs modulate the action of hormones rather than act as hormones themselves. They have been used, for example, to start parturition, in some cases for the purpose of effecting abortion in an early stage of pregnancy. They are also known to lower the intraocular pressure in low concentrations, to increase the intraocular pressure in high concentrations, to cause hypertension, and to inhibit the breakdown of fat in adipose tissue [1]. Different PGs have vastly different biological activities even though they have rather similar isomeric structures.

The need for a sensitive and highly selective analytical method that is also mild for these labile compounds has made PGs one of the most challenging classes of compounds to analyse in biological matrices. For the most accurate results, the sample preparation steps should be minimized, and derivatizations or any chemical modifications should be avoided to prevent the loss and degradation of the PGs. The methods that have been used to analyse trace amounts of PGs on a routine basis are based on gas chromatography (GC), liquid chromatography (LC), or radioimmunoassay (RIA).

Gas chromatography with selective detection [electron capture (ECD) and mass spectrometry (MS)] is a sensitive method with high resolving power, but PGs require multiple derivatizations to make them volatile prior to analysis [2]. Dimethylethyl-, dimethyl-*n*-propyl- and dimethylisopropylsilylethers, and pentafluorobenzylesters are common derivatives of prostaglandins [3–6]. Mai *et al.* [7] reported that the *t*-butyldimethylsilyl (*t*-BDMS) derivative was more stable to hydrolysis than other alkylmethylsilyl derivatives, but water still had to be eliminated prior to derivatization.

The PG carbonyl groups have been converted into the methoxime derivatives prior to esterification to increase their volatility [3, 7]. Rosenfeld *et al.* [2] simplified the PG preparation step by isolating and derivatizing PGE_2 on a solid XAD-2 support. The yields from a buffer solution and from plasma were 73 and 54%, respectively. The minimum detectable quantity (MDQ) using GC with electron capture detection has been reported to be in the range 20–50 pg [4, 8]. In GC–MS analysis, both electron impact (EI) and negative-ion

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chemical ionization (NICI) modes have been used. Weber *et al.* (6) reported that the use of ammonia as an ionization reagent gas gave better sensitivity and linearity than methane in the analysis of brain tissue samples in which the PGs were derivatized to the methoxime pentafluorobenzylester tris(trimethylsilyl)ethers. The MDQ using GC–NICI–MS was 200 fg [4] as compared with 500 fg when using GC–EI–MS [6]. In MS detection, the *t*-BDMS derivative gave more simplified MS spectra than the other alkylmethyl silyl ethers [7].

Liquid chromatography can be used to separate high-molecular-weight labile acids at low temperatures, but some PGs must be derivatized in order to use the available LC detectors. For example, UV-absorbing compounds can be obtained by *p*-bromophenacyl-, *p*-nitrophenacyl-, or α-bromo-2′-acetonaphthone esterification [9–12]. Oxidation of the hydroxyl group to a keto group in position 15 by enzymes or pyridinium dichromate makes PGs (except prostaglandin B) absorb UV-light at 228 nm [12, 13]. Prostaglandin B is detected at 298 nm [12]. In addition, 9-anthryldiazomethane has been used to attach a fluorophore to the PGs [14]. Underivatized PGs are commonly analysed by LC with a refractive index detector (LC–RID), but high detection limits and an incompatability with gradient elution make trace analysis impossible. Doehl and Greibrokk reported MDQs of 30 ng for nonderivatized PGs with UV detection at 192 nm [12]. Stein *et al.* [15] were able to detect 40 pg of PGs with fluorescence detection. The MDQs of 2,4-dimethoxyaniline derivatives of PGs using ECD have been in the same range [16].

The RIA technique compares in sensitivity with GC–MS. However, RIA has limited specificity because of potential cross-reactivity of antibodies with biologically-related compounds [17]. The cross-reactivity can be minimized by using LC to fractionate the sample prior to RIA [18]. However, fractionation is difficult if it is done after the addition of the antibody because of the unstable equilibrium of the antigen–antibody complex [19].

The use of supercritical fluid chromatography (SFC) for qualitative analysis of PGs has been demonstrated previously [20, 21]. In this work, open tubular column SFC was evaluated for the quantitative analysis of PG isomers in trace level amounts. Direct injection and supercritical fluid extraction (SFE) were evaluated. The analysis of aqueous samples without time-consuming preparation steps was of major concern. Reduction of the preparation steps would minimize contamination of the sample and also degradation of thermally labile compounds. Many biological samples are easily oxidized or degraded by light and thus fast analysis after sampling is needed for accurate results.

Experimental

Chemicals

The PG standard compounds were obtained from Cayman Chemicals (Ann Arbor, MI, USA), and the derivatives of the acids were prepared at the Department of Organic Chemistry, Pharmacia (Uppsala, Sweden). The solvents were LC grade acetonitrile from J.T. Baker (Phillipsburgh, NJ, USA) and water from Fisher Scientific (Fair Lawn, NJ, USA). The aqueous samples analysed in this study were taken from an *in vitro* perfusion cell described in detail elsewhere [23]. The Ringer's solution used in the perfusion model was composed of sodium chloride (6.200 g l^{-1}), potassium chloride (0.358 g l^{-1}), monobasic sodium phosphate monohydrate (0.103 g l^{-1}), sodium bicarbonate (2.454 g l^{-1}), calcium chloride dihydrate (0.115 g l^{-1}), magnesium chloride hexahydrate (0.159 g l^{-1}), glucose (0.900 g l^{-1}) and oxidized glutathione (0.090 g l^{-1}). The solution that was applied on the cornea consisted of 0.100 g l^{-1} 15-propionate $PGF_{2\alpha}$ isopropyl ester in Ringer's solution. All samples were stored in a freezer or with dry ice during shipping. The perfusion study was made at Pharmacia Ophthalmics (Sweden).

Instrumentation

Two different SFC systems were used, depending on the sample introduction method desired: one with direct injection and one with SFE. Both SFC systems (Series 601, Lee Scientific, Salt Lake City, UT, USA) were used with carbon dioxide as the mobile phase (Scott Specialty Gases, Plumsteadville, PA, USA) and flame ionization detection. For direct injection, the samples were introduced into the column by a cooled (15°C) 1-μl internal sample loop rotary microvalve injector (Valco Instruments, Houston, TX, USA). The connections were made with two-piece PEEK ferrules (Upchurch Scientific, Oak Harbor, WA, USA). A 50-cm × 50-μm i.d. fused

silica capillary (Polymicro Technologies, Phoenix, AZ, USA) was used on the carbon dioxide inlet side of the injector to reduce backflow of sample during injection. The analytical column was a 3-m × 50-μm i.d. fused silica open tubular column that was deactivated with a cyanopropyl hydrosiloxane reagent and then coated with an oligoethylene oxide-substituted polysiloxane stationary phase [22]. A cyanopropyl deactivated 50-μm i.d. frit restrictor (Lee Scientific) was used to connect the column to the FID that was held at 380°C. Before analysis, the aqueous samples (100–200 μl) were dried with a vacuum centrifuge and then dissolved in 50 μl of acetonitrile.

The extraction system consisted of an extraction cell (Valco Instruments or Upchurch Scientific), a helium actuated rotary microvalve injector with a 200-nl internal sample loop (Valco Instruments), a 10-port valve (Valco Instruments), and a cryogenic solute trap (Scientific Glass Engineering, Austin, TX, USA). A schematic diagram of the instrumentation is shown in Fig. 1. Two ovens were used: the extraction cell and 10-port valve were housed in one oven (5710A GC oven, Hewlett-Packard, Avondale, PA, USA), and the column and solute trap were contained in the other (601 SFC/GC oven, Lee Scientific). A microvalve injector was installed before the extraction cell to make it possible to introduce a modifier during the extraction.

The thawed samples (100 μl of Ringer's solution) were loaded onto SFE-cleaned octadecylsilane (ODS) or onto XAD-2 adsorbent material (Alltech Associates, Deerfield, IL, USA) in a 2.0-mm i.d. by 20-mm long stainless steel extraction cell with stainless steel endfrits. A 15-μm i.d., 155-μm o.d. linear restrictor or a 25-μm i.d., 155-μm o.d. integral restrictor connected the extraction cell to the solute trap (Fig. 2). The end of the restrictor was inside a 183-μm i.d., 291-μm o.d. deactivated precolumn. The sample was initially purged for 5 min with SFC grade carbon dioxide. The centre of the trap was then effectively cooled with dry liquid carbon dioxide and the extraction was performed (0.800 g ml^{-1}) at 35–50°C. The extracted solutes were focused in the solute trap. The inner diameter of the trap was minimized (350-μm) to obtain maximum cooling.

After extraction, the density of the carbon dioxide was lowered to the starting density of

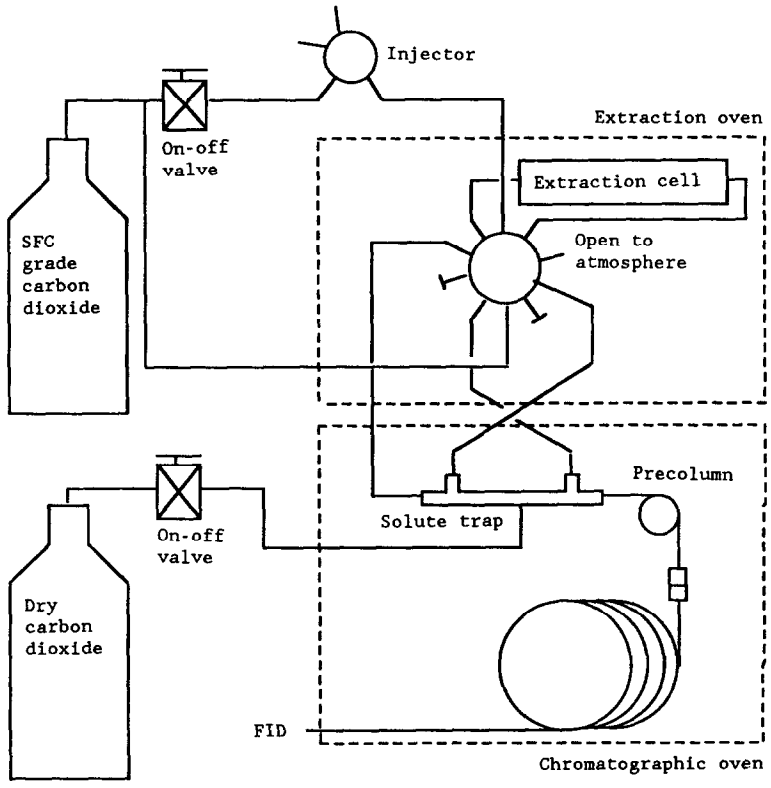


Figure 1
Schematic diagram of the SFE–SFC system (not to scale).

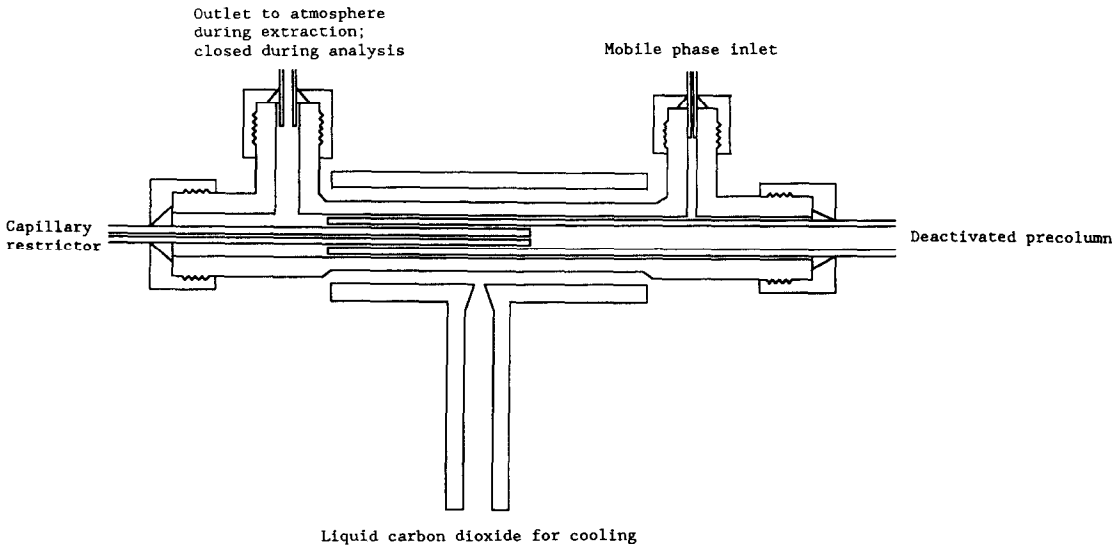


Figure 2
Schematic diagram of the solute trap used in the SFE experiments.

the chromatographic run and the valve was switched to the column position. The mobile phase was programmed from 0.150 g ml^{-1} to 0.760 g ml^{-1} at a rate of 0.020 g ml^{-1} min^{-1} after a 5-min isopycnic period. The column used in the SFE–SFC work was either a 6.5-m × 50-μm i.d. oligoethylene oxide polysiloxane [22] or a 9.0-m polymethylsiloxane column. Other run parameters and conditions were the same as in the direct injection system.

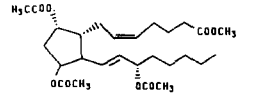
Results and Discussion

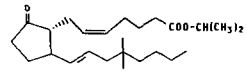
Carbon dioxide was used as mobile phase because it has a low critical temperature and therefore sample degradation due to high temperature was prevented. Carbon dioxide is also inert towards acidic compounds since it is slightly acidic itself. The compounds evaluated in this study are listed in Table 1 and their structures are shown in Fig. 3. These compounds were chosen because they penetrated the cornea better than free acids due to their hydrophobic character. A chromatogram of a mixture of the standards is shown in Fig. 4. All 11 PGs were effectively separated within 35 min. The PG standards varied widely in polarity. The first eluting compound was 9,11,15-triacetoxy $PGF_{2\alpha}$ methyl ester (i.e. all of the hydroxyl and carboxyl groups had been derivatized), while the last three compounds contained three to four hydroxyl groups, and $PGF_{2\alpha}$ contained a free carboxylic acid group. The oligoethylene oxide polysiloxane stationary phase was selective and inert towards all of these compounds. Peaks 3 and 5 (15-deoxy-13,14-dihydro $PGF_{1\alpha}$ isopropyl ester and 13,14-dihydro $PGF_{1\alpha}$ isopropyl ester, respec-

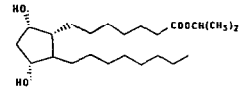
Table 1
Prostaglandin standard compounds

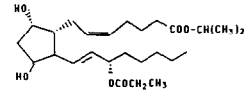
Peak no.*	Compound
1	9,11,15-Triacetoxy $PGF_{2\alpha}$ methyl ester
2	11-Deoxy-16-dimethyl PGE_2 isopropyl ester
3	15-Deoxy-13,14-dihydro $PGF_{1\alpha}$ isopropyl ester
4	15-Propionate $PGF_{2\alpha}$ isopropyl ester
5	13,14-Dihydro $PGF_{1\alpha}$ isopropyl ester
6	$PGF_{2\alpha}$ isopropyl ester
7	$PGF_{2\alpha}$ methyl ester
8	15-Pivaloate $PGF_{2\alpha}$ benzyl ester
9	$PGF_{2\alpha}$ alcohol
10	$PGF_{2\alpha}$ benzyl ester
11	$PGF_{2\alpha}$

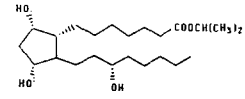
* Peak numbers refer to labelled peaks in Fig. 4.

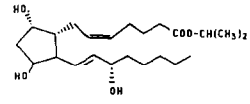

9,11,15-triacetoxy $PGF_{2\alpha}$ methyl ester

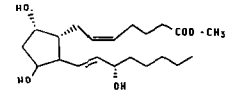

11-deoxy-16-dimethyl PGE_2 isopropyl ester

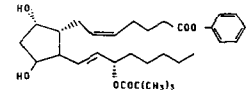

15-deoxy-13,14-dihydro $PGF_{1\alpha}$ isopropyl ester

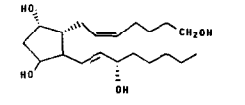

15-propionate $PGF_{2\alpha}$ isopropyl ester

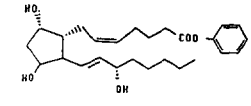

13,14-dihydro $PGF_{1\alpha}$ isopropyl ester


$PGF_{2\alpha}$ isopropyl ester


$PGF_{2\alpha}$ methyl ester


15-pivaloate $PGF_{2\alpha}$ benzyl ester


$PGF_{2\alpha}$ alcohol


$PGF_{2\alpha}$ benzyl ester

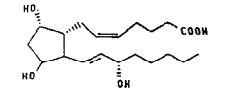

prostaglandin $F_{2\alpha}$

Figure 3
Molecular structures of the analysed PG compounds.

tively) cannot be analysed with LC because they cannot be detected using a UV-detector, even at low wavelengths (<193 mm).

The low solute concentrations in the sample matrices and the characteristics of the detector dictated the minimum allowable injection volume for adequate detection of the PGs. The split injection method did not transfer enough sample to the column; therefore, two other injection methods were evaluated: direct injection and on-line SFE. The largest commercially available sample loop for the rotary microvalve injector was 1 μl. Calibration curves were constructed that were linear in the

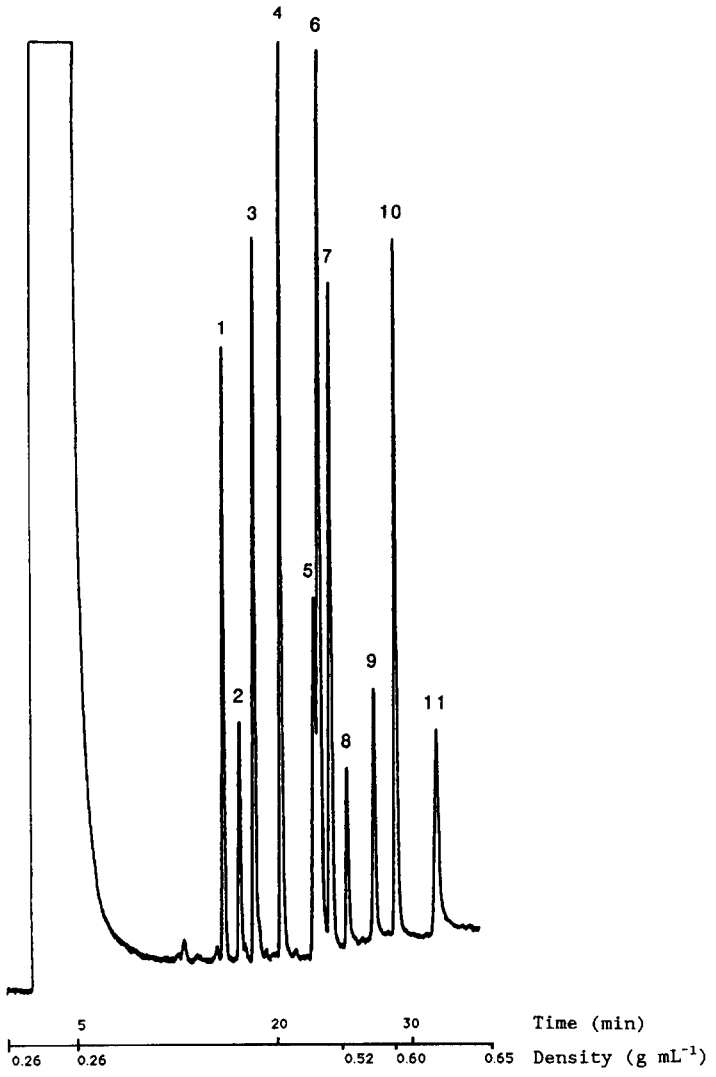


Figure 4
Capillary column supercritical fluid chromatogram of PG standards. Conditions: 3-m × 50-μm i.d. oligoethylene oxide-substituted stationary phase (d_f = 0.15 μm), carbon dioxide mobile phase at 100°C; density programmed from 0.260 to 0.525 g ml^{-1} at 0.013 g ml^{-1} min^{-1}, from 0.525 to 0.600 g ml^{-1} at 0.020 g ml^{-1} min^{-1}, and from 0.600 to 0.760 g ml^{-1} at 0.010 g ml^{-1} min^{-1} after a 5-min isopycnic period. Compounds are listed in Table 1.

range studied (5–125 μg ml^{-1}). The MDQs for PGs using the direct injection method varied from 9 to 60 ng (Table 2) with relative standard deviations (RSDs) from 1.5 to 5.6%. The highest MDQ was obtained for $PGF_{2\alpha}$, which was the most polar compound in the sample. The peak broadening and tailing of the $PGF_{2\alpha}$ peak made quantification more difficult, as evidenced by the RSDs for $PGF_{2\alpha}$ that ranged between 9.0 and 19.1%. The levels of PGs in some samples were below the detection limit. Further concentration steps were not possible because of the limited available sample volume of only 100 μl that had already been concentrated to 50 μl. This was the smallest practical sample volume that allowed replicate analyses. For increased detectability, sample volumes of 1 μl or larger must be introduced into the column. Therefore, a SFE method was chosen as an alternative sample introduction method because the limited detectability could be overcome by increasing the sample volume to 100 μl or greater in the extraction cell.

The extraction cell and 10-port valve were located in a separate oven from the column and the solute trap. This allowed efficient cooling of the trap, even if the extraction oven were heated above 60°C. The capillary restrictor

Table 2
Minimum detectable quantities (S/N = 3) of eight PG standards using an FID and the direct injection method with a 1-μl sample loop*

Compound	MDG (ng)
9,11,15-Triacetoxy $PGF_{2\alpha}$ methyl ester	27
15-Deoxy-13,14-dihydro $PGF_{1\alpha}$ isopropylester	16
15-Propionate $PGF_{2\alpha}$ isopropyl ester	9
13, 14-Dihydro $PGF_{2\alpha}$ isopropyl ester	28
$PGF_{2\alpha}$ isopropyl ester	22
$PGF_{2\alpha}$ methyl ester	18
$PGF_{2\alpha}$ benzyl ester	18
$PGF_{2\alpha}$	60

*Injection time, 5–7 s.

from the extraction cell to the solute trap was approximately 40 cm in length with the restrictor positioned precisely at the exit end. This restrictor capillary was contained in the extraction oven, with the exception of approximately 5 cm that was in the column oven and connected to the solute trap. The column oven was held at 30°C during extraction. At this temperature, the formation of ice in the solute trap was minimal; however, the trap was still cold enough to quantitatively focus the PG solutes. An observation was made that at the beginning of the chromatographic analysis the solute trap had to be heated as fast as possible to the analysis temperature in order to achieve good peak shape. This fast heating was not possible if the trap were coated with a layer of ice during solute trapping.

A microvalve attached to the extraction cell was used to introduce an organic modifier into the carbon dioxide stream. Modifiers were used to increase the polarity of the extraction fluid in order to improve the extraction efficiency. Several research groups have demonstrated the effectiveness of organic modifiers in SFE [24–26]. The configuration described here made it possible to (a) add modifiers without contaminating the pump, (b) easily produce a gradient in the mobile phase, and (c) change the modifier even during extraction.

In the SFE of liquid samples, the solvent must be eliminated prior to analysis to prevent overloading of the column. Additionally, excess solvents affect the selectivity of the column and act as a mobile phase modifier. Volatile organic solvents are easily eliminated by evaporation from an open extraction cell. Xie *et al.* [27] used nitrogen gas to purge ethanol from an extraction cell containing an adsorbent.

Several studies have been undertaken to evaluate the use of SFE in water purification processes [28, 29]. Roop *et al.* [28] removed toxic organic compounds, including creosote, from water. Ehntholt *et al.* [29] extracted various compounds that would naturally be present in drinking water. Generally, 0–46% of spiked compounds were left in the water samples after the extraction; however, as much as 81% of caffeine was not extracted [29]. Several additional aspects must be taken into consideration when using on-line SFE–SFC. Since water is slightly soluble in supercritical carbon dioxide (i.e. 0.3–1.4 mol%) [30], it can easily be deposited in the solute trap and disrupt the flow of carbon dioxide. Excess amounts of water can freeze in or at the end of the restrictor to prevent successful extraction. Water can also act as a modifier by changing the retention times of the compounds of interest and making detection less reproducible.

Several different methods have been used to reduce the amount of aqueous solvents entering the column. Hedrick and Taylor [31] used a 10-cm long extraction cell that was held vertically and half-filled with aqueous sample. The extraction fluid was introduced at the bottom of the cell and the extracted material was collected from the "headspace" in the top of the cell. Thiebaut *et al.* [32] took advantage of the low solubility of water in supercritical carbon dioxide. They used an inverted phase separator designed for liquid–liquid extraction to separate phenol and supercritical carbon dioxide from urea. With low carbon dioxide flow through the column, the separation was good but some of the sample was lost through the waste exit line. If the column flow were increased, an increase in water entering the column was observed [32].

In this study, the liquid sample was spread as

a thin film on an inert support to maximize the surface area for facilitating vaporization of the solvent and extraction of the sample. The aqueous samples were loaded onto the packing materials using vacuum suction. The most effective removal of water was obtained by brief vacuum aspiration at ambient temperature combined with a 5-min carbon dioxide gas purge at 60°C. The carbon dioxide gas purge was used instead of a nitrogen gas purge to prevent sample contamination. If the purging gas is not of high purity, contaminants will accumulate during extraction, seriously affecting solute detectability.

Octadecylsilane (ODS) modified silica particles were tested as the packing material in the extraction cell. Nanogram levels of $PGF_{2\alpha}$ isopropyl ester were almost quantitatively extracted in 45 min. When the same sample was extracted again for another 45 min, the $PGF_{2\alpha}$ isopropyl ester peak area was 2% of the peak area obtained from the first extraction. Luderer *et al.* [33] used an ODS-packed column to eliminate the liquid–liquid extraction step for PGs. The samples were eluted from the cartridge and subsequently analysed by LC. The recoveries of PGF_2 and PGE_2 were 88 and 82%, respectively [33]. A polystyrene divinyl benzene resin, XAD-2, performed better than ODS in this application, yielding higher recoveries for the extraction of acidic compounds. Fifteen microlitres of an aqueous PG standard solution were loaded on the XAD-2 and then extracted with supercritical carbon dioxide for 45 min at 0.800 g ml^{-1} and at 45°C (see Fig. 5). Figure 6 shows a chromatogram of a 200-μl sample from an *in vitro* perfusion model. The extraction conditions are listed in the figure legends. A disadvantage of commercial polymeric packing materials is that they contain impurities that are released during extraction to give a higher than acceptable background when using the universal flame ionization detector. Yonker *et al.* [34] also reported that many aromatic impurities were released from purified XAD-2 after contact with water, but the packing material could be purified with repeated liquid–liquid or SFE.

The configuration of the solute trap reported here made it possible to extract and analyse moderately polar compounds without severe peak tailing. A similar trap was reported by Xie *et al.* [27], however, several improvements have been made in the design. The expanding carbon dioxide mobile phase from the extraction cell was released through a different outlet than the incoming carbon dioxide, and thus contamination of the system from the previous analysis was minimized. Additionally, extracted solutes were only in contact with deactivated fused silica surfaces after leaving the stainless steel extraction cell. Trace level solutes from small samples could be analysed because the extracted material was deposited directly in the precolumn, dead volumes were minimized, and chromatography was optimized. In SFE–GC, the samples can be deposited in the precolumn in the same way

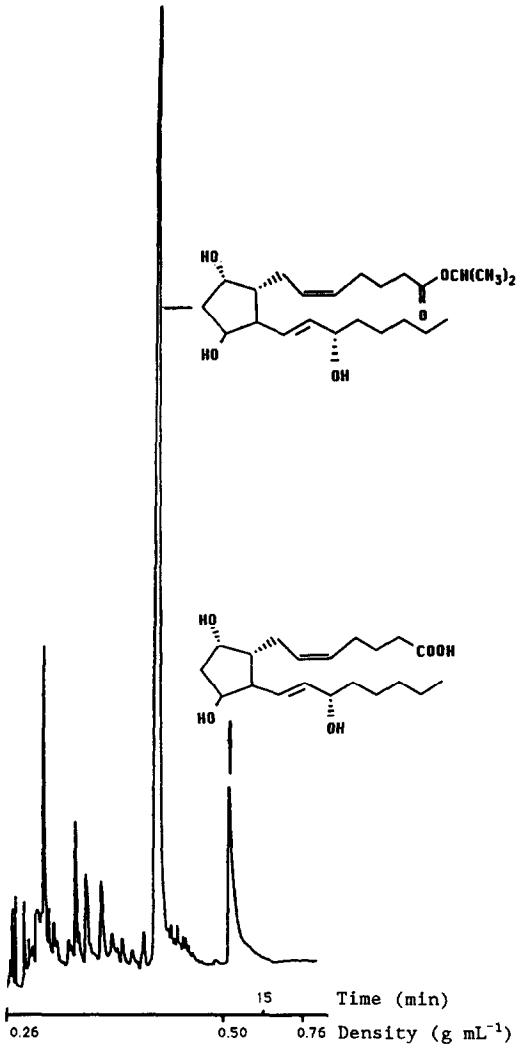


Figure 5
SFE–SFC of an aqueous PG standard solution. Conditions: 15 μl of 13.3–20.0 ppm aqueous standards loaded on XAD-2; extraction at 0.800 g ml^{-1} and 45°C for 45 min; 6.5-m × 50-μm i.d. oligoethylene oxide-substituted stationary phase (d_f = 0.15 μm), carbon dioxide mobile phase at 100°C; density programmed from 0.150 to 0.760 g ml^{-1} at 0.020 g ml^{-1} min^{-1} after a 5-min isopycnic period.

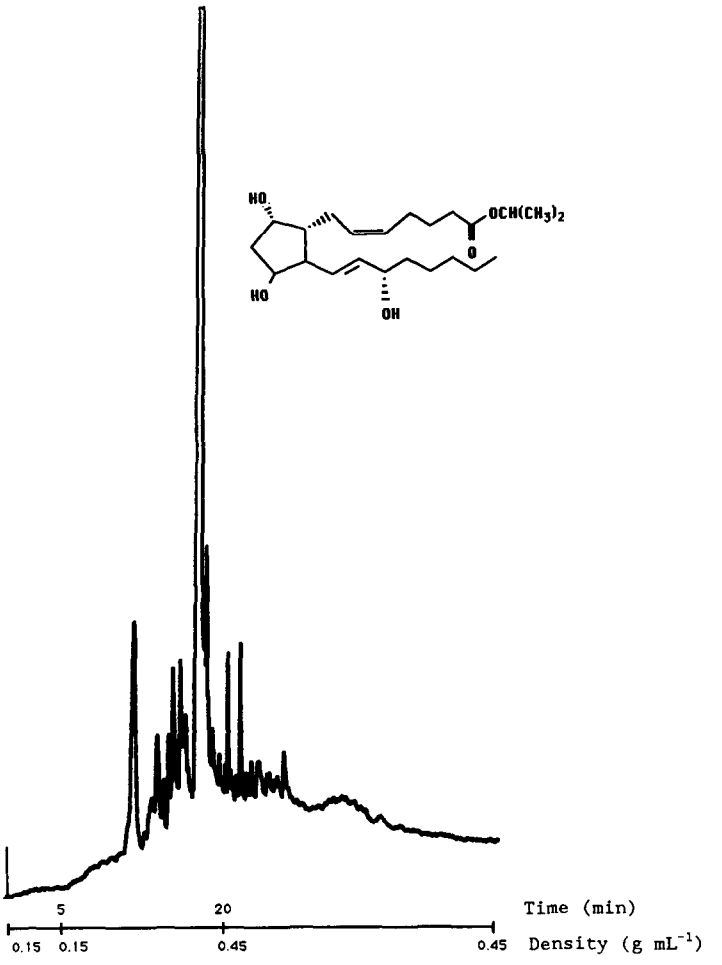


Figure 6
SFE–SFC of a PG extract from an *in vitro* perfusion cell. Conditions: 200-μl sample loaded on XAD-2; extraction at 0.400 g ml^{-1} and 50°C for 120 min; 9-m × 50-μm i.d. polymethylsiloxane coated fused silica column (d_f = 0.25 μm), carbon dioxide mobile phase at 100°C, density programmed from 0.150 to 0.760 g ml^{-1} at 0.015 g ml^{-1} min^{-1} after a 5-min isopycnic period.

[35, 36]. There is a risk, however, that non-volatile compounds can be introduced into the GC column, thus shortening its lifetime and decreasing its efficiency. In SFE–SFC, this is not a problem because the same solvating power that is achieved during extraction is attained during the analysis.

These investigations have shown that trace levels of PGs could be analysed with SFE–SFC. There was no need for sample preconcentration or derivatization, as would have been the case for GC or LC analysis. A wide concentration range can be analysed with this analytical method because the amount of sample in the extraction cell can be adjusted to give a reasonable signal at the detector. Work is underway to optimize the extraction, reduce the total analysis time, increase the reproducibility of the extraction, and automate the system.

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