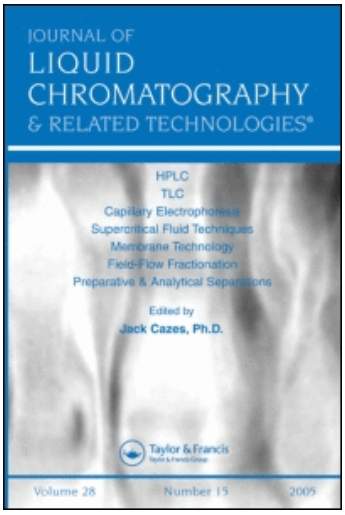

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Solid-Phase Extraction Device Coupled to a Microcolumn Liquid Chromatograph with a UV Detector for Determining Estrogens in Water Samples

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Abstract: A solid-phase extraction (SPE) device in conjunction with a microcolumn liquid chromatograph with a UV absorbance detector was developed for determination of estrogens in water samples. The SPE device consisted of a syringe filter unit containing 3 mg of triacontyl (C_{30})-bonded phase (15–30 μm particle diameter) and an LC syringe needle. Analytes were extracted onto the sorbent by passing the sample from a syringe (tens mL volume) to the device. To perform analyte desorption, the syringe was replaced by a microliter syringe that contained ethanol serving as the desorption solvent. After the device was installed in the needle port of an LC injection valve equipped with an external sample loop (200 μL volume), by passing the desorption solvent through the SPE device, the analytes were desorbed from the sorbent into the sample loop. The contents of the sample loop were transferred into the column by turning the valve from load to injection position. Separations were performed on a C_{30} column under isocratic conditions using an aqueous ethanol solution as the mobile phase. Bandbroadening problems resulting from such a large sample loading on the microcolumn were circumvented by use of water plugs placed before and behind the ethanol extract in the sample loop. Recoveries of 92.6–96.3 and preconcentration (enrichment) factors of 1852–1926 were achieved for the

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estrogens studied (estradiol, estriol, estron, and ethynyl estradiol). Limits of detection for the estrogens were 3.8–12.6 ng/L.

Keywords: Solid-phase extraction, Microcolumn LC, Triacontyl, Estrogen, Water analysis, On-column focusing

INTRODUCTION

The modern trend in analytical chemistry is toward the miniaturization of analytical methods and devices currently used in laboratories, aiming at the increased performance and the development of an environment-friendly system. Liquid chromatography (LC) is the most versatile method for separating environmentally, biologically, and clinically important materials. Progress in microcolumn LC is still continuing since its inception twenty years ago. (The term microcolumn is used here to refer to small i.d. (<1.0 mm) columns.) In recent years there has been a strong interest in further miniaturization of LC, that is, implementing LC on a microchip, although the technologies are at present still far from their routinely usable stages.

The most obvious advantage of using microcolumn LC is the decreased solvent consumption. Since the volumetric flow rate of mobile phase required to attain the same linear velocity is proportional to the square of the column radius, the utilization of microcolumn LC results in a significantly reduced cost of operating a liquid chromatograph and fewer problems with solvent waste disposable. In this context, reduced flow rates make a microcolumn an attractive means of achieving the coupling of LC with electrospray ionization mass spectrometry (ESI-MS). Moreover, microcolumn LC has the potential advantage that sensitivity may be enhanced with concentration-sensitive detectors, such as a UV absorption detector (the most common LC detector). The smaller peak volumes in small-diameter columns lead to much less dilution of eluate: the concentration of solutes eluted from a 1 mm i.d. column is higher than that from a 4.6 mm i.d. column by a factor of 20. Thus, using the same optical pathlength, the detector sensitivity can be proportionally increased.

However, when determining analytes in dilute samples such as environmental samples, there is often a lack of detection sensitivity even with microcolumn LC. A variety of approaches have been investigated to enhance the low concentration sensitivity. Liquid-liquid extraction has traditionally been applied to environmental samples; however, this extraction method requires large volumes of organic solvents and long preparation times. In the on-column focusing (or peak compression sampling) technique, analytes are dissolved in a large volume of solvent whose elution strength is weaker than that of the mobile phase. Another approach is a column switching

technique, where an additional pump and injection valves are used for sample loading and subsequent flushing.

In the last few years, solid-phase microextraction (SPME) has emerged as a valuable alternative for sample preparation in LC analysis.[1] In SPME, a polymer coated fiber is employed to extract analytes from samples. Desorption is performed in a specially designed interface consisting of a six-port switching valve and a desorption chamber (90 μL internal volume). An organic solvent (in static desorption mode) or the mobile phase (in dynamic desorption mode) is used to desorb the analytes from the fiber. Afterward an internally coated, open tubular capillary was used for SPME,[2] allowing the automation of the entire SPME-LC system equipped with a commercial LC autosampler. In this system, a finite portion of sample is drawn into the capillary tube and then is ejected to the sample vial. The draw/eject cycle is repeated to enhance the recovery (typically 10–20 cycles). The automated SPE-LC system generally provides improved accuracy and precision, but needs more complex instrumentation compared with the manual technique. The problem common to both the manual and automated SPME-LC is the absence of commercially available fibers whose coatings withstand various solvents, while providing high extraction coefficients. The automated SPME system was recently coupled to microcolumn LC with a 0.3 mm i.d. column.[3]

In order to increase the extraction efficiency, polymeric filament yarns were packed into a PTFE tube to be used as an external sample loop of an LC injection valve: this approach is called fiber-in-tube solid phase-extraction (FIT-SPE).[4] Although, this approach allows a simultaneous operation of analyte extraction/LC analysis in spite of the simple design; however, it needs some accessories, such as a switching six-port valve and two additional LC pumps for delivering a sample solution and a rinsing solvent. The FIT-SPE was coupled to microcolumn HPLC for the analysis of phthalates[5] and drugs.[6] A further miniaturization of the extraction process was achieved by inserting a much shorter piece of yarn-filled tube into the sample hole of the valve rotor.[7–9]

Recently, we have designed an SPE device that is usable with standard liquid chromatographs without any modification.[10] The device consisted of an LC syringe needle and a filter unit packed with a small amount of sorbent. After a sample solution was loaded onto the sorbent from a syringe mounted on a syringe pump, the syringe was replaced by another syringe containing a desorption solvent, and then the needle of the device was inserted into the needle port of an injection valve. By forcing the solvent through the packed sorbent, the analytes were transferred to the sample loop, after which sample injection into a conventional LC column was performed in a usual way.

In the present work, we evaluated the possibility of using the SPE device in the coupling to microcolumn LC for the analysis of estrogens in water samples. Estrogens examined are the natural estrogen estradiol (E2), its main metabolites estriol (E3) and estron (E1), and the synthetic estrogen

ethynyl estradiol (EE). Analysis of these estrogens presents a challenging task, because among the wide range of water pollutants they are of strong interest, due to their high estrogenic effects at very low levels in water. In microcolumn LC, the injection volume and the way in which sample is transferred to the column significantly affect band broadening and resolution. Because the sample volume that can be placed on a microcolumn is greatly reduced, desorption from the SPE device has to be done with as small a volume as possible of solvent. An on-column focusing technique was applied to solve the problem. Triacontyl (C_{30})-bonded phase was used as the SPE sorbent and the LC stationary phase. The C_{30} phase has desirable properties for extraction and separation under highly aqueous conditions, because in a water-rich environment the alkyl-chains do not undergo phase collapse, and hence, keep the retentivity even after a large amount of water is passed through the phase. An additional feature of this work is that ethanol is used as the mobile phase for LC as well as the desorption solvent for SPE. Although methanol and acetonitrile are typical organic solvents in reversed-phase LC, ethanol is preferable to other organic solvents on account of the fact that it does less harm to human health and to the environment.

EXPERIMENTAL

Chemicals and Materials

Estrogenic compounds are all purchased from Wako Pure Chemical Industries (Osaka, Japan). HPLC grade ethanol was obtained from Kanto Kagaku (Tokyo, Japan). Water used for LC analysis and sample preparation was obtained on a Nihon Millipore (Yonezawa, Japan) Milli-Q SP water purification system. The C_{30} phase used for SPE was Develosil C30-UG from Nomura Chemical (Seto, Japan). The physical properties of the sorbent are as follows: particle diameter, 15–30 μm with an average diameter of 20 μm; mean pore size, 14 nm; pore volume, 1.15 mL/g; surface area, 300 m^2/g; carbon content, 18%. Stock solutions were prepared by dissolving the estrogens in ethanol at a concentration of 1.0 g/L for each compound. Standard solutions for the experiments were freshly made by dilution of the stock solutions with water.

LC Instrumentation

The liquid chromatograph consisted of a Jasco (Tokyo, Japan) 880-PU pump, a Jasco 870 UV detector equipped with a flow cell of 5 mm pathlength and 1.0 μL cell volume, a Rheodyne (Cotati, CA, USA) 7125 six-port injection valve, and a Jasco 860 CO column oven. The injection valve was fitted with a sample collection loop made from a 1 mm i.d. stainless steel tubing, the

volume of which had been calibrated. Separations were carried out on a Nomura Chemical Develosil C30-UG column (1.0 mm i.d. × 15 cm, 5 μm particle size) with a mixture of ethanol and water (30/70, v/v) at a flow rate of 50 μL/min. The temperature of the column oven was set at 39°C. Detection was performed at 207 nm. The signal from the UV detector was digitized by a 16-bit A/D converter in an iDX4 (100 MHz)-based personal computer. Data was acquired with a program written in Visual-BASIC, developed by one of the authors, Y. Hirata.

SPE Device

The previously developed device[10] was used for SPE with minor modifications. The SPE device was fabricated by utilizing a disposable filter unit, Prep-LCR4-LH (Nihon Millipore), which had a female Luer-Lok inlet and a male slip outlet. The housing was made of polypropylene and the membrane filter was hydrophilic PTFE membrane filter with 4 mm diameter and 0.5 μm pore size (Figure 1). The membrane filter lodged in the bottom of the filter unit served to retain sorbents. A smaller amount (3.0 mg) of the C_{30} phase than that used in the previous study[10] was packed in the filter

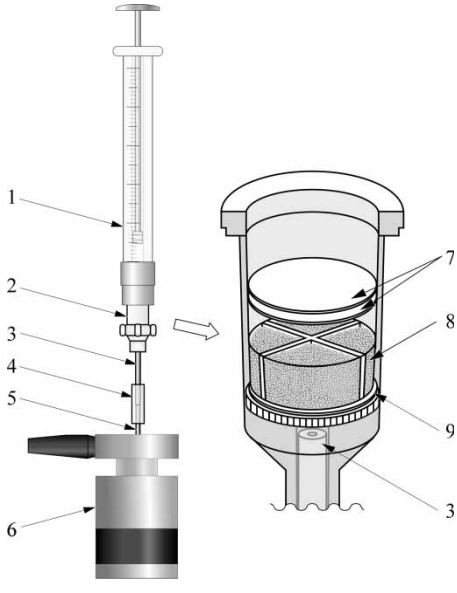

Figure 1. Schematic diagram of the SPE device serving as an LC injector: 1, Luer-Lok syringe; 2, filter unit; 3, SS tube; 4, PTFE tube; 5, 22-gauge syringe needle; 6, Rheodyne 7125 injection valve; 7, cellulose paper filter; 8, C_{30}-silica packings; 9, hydrophilic PTFE membrane filter with a support (originally attached to the filter unit).

unit. Two pieces of cellulose paper filters (Advantec Toyo, Tokyo, Japan) were cut out to match the inside diameter of the filter unit and were placed on the sorbent. When an LC injection syringe was attached to the filter unit via Luer-Lok connection, no free space was generated between the packed bed and the barrel tip.

The male slip outlet of the filter unit was shortened to reduce extracolumn dispersion. A stainless steel tube of 0.15 mm i.d., 0.81 mm o.d., and 14 mm length was pressed into the male slip outlet to provide a tight fitting connection. This SS tube was then connected butt-to-butt to a stainless steel tube of 0.13 mm i.d., 0.71 mm o.d., and 55 mm length (manufactured in-house) with a short section of PTFE tubing. Note that the o.d. of the latter SS tube matches that of a 22-gause needle, which the needle port of the injection valve used can accommodate. The entire assembly was thoroughly rinsed with ethanol and water and it was confirmed that no analyte peaks appeared in the chromatogram of a blank ethanol eluate.

Procedure

The SPE device was attached to a sample-containing syringe (60 mL volume) via the Luer-Lok connection. The SPE-syringe assembly was mounted on an Azumadenki Kogyo (Tokyo, Japan) MF-2 syringe pump and the sample was pumped to the device for a certain period of time. The syringe was then replaced with an air-containing syringe to expel water left in the device. Subsequently, a 50 μL microsyringe filled with a desorption solvent was attached to the device. The needle of the device was inserted into the needle port of the injection valve in the load position: the device, as such, acts as a sample injection syringe. As the syringe plunger was pressed manually, thereby forcing the desorption solvent through the SPE device, analytes were desorbed and transferred into the loop of the valve. By switching the injection valve to the injection position, the contents of the loop were carried to the column and LC separation was started. After every extraction, the SPE device was washed with 1.5 mL of ethanol and 0.5 mL of water.

On-column focusing was performed by sandwiching the desorbed analyte solution between two equal lengths of water plug within the confines of the sample loop. For this purpose, the sample loop was at first filled with water. A chosen volume of desorption solvent was dispensed through the device into the loop by the displacement of the syringe plunger, followed by injection of another water plug.

RESULTS AND DISCUSSION

In preliminary experiments, large volumes of ethanol that exceed the maximum sample volume acceptable with the microcolumn were found to

be required for analyte desorption. The injection of large sample volumes can be tolerated by using the on-column focusing technique, where either a weaker solvent than the mobile phase is used as a sample solvent or a non-eluting solvent is introduced just prior to the injection. According to Šlais and Kouřilová, up to 1 mL of sample could be introduced into a 1 mm i.d. microcolumn without any deleterious effect.[11] In our study, a sample loop of 200 μL volume was filled in turn with a water plug, the ethanol extract, and another water plug having the same length as the leading water plug. This method permitted the introduction of a large volume of the desorption solvent into a microcolumn, with a consequent improvement in the system sensitivity.

Effects of Sorption Parameters

In SPE, it is desirable to shorten the total analysis time while attaining high analyte recoveries. Therefore, the flow rate used for sample loading is an important parameter that affects the performance of the SPE procedure. Holding the sample volume constant (5 mL), standard solutions (containing 5 μg/L of each analyte) were passed through the SPE device at different flow rates ranging from 0.074 to 0.60 mL/min. No significant differences were observed in the recovery of each analyte in the flow rate range studied. In view of the low flow resistance, the maximum flow rate allowed by the SPE device seemed to be beyond the highest flow rate (0.60 mL/min), which is the limit of the syringe pump equipped with a 60 mL syringe. All further studies were based on the loading flow rate of 0.60 mL/min.

The breakthrough volume of the SPE device was investigated with various volumes of standard solutions (containing each analyte at 5 μg/L), ranging from 1.0 to 60 mL. It was found that the peak area of each analyte increased linearly with the sample volume in the measured range ($r^2 > 0.9982$). This indicates that no losses of these compounds by breakthrough occur for sample volumes up to 60 mL. Consequently, subsequent experiments were done with sample volumes of 60 mL unless otherwise noticed.

Effects of Desorption Parameters

To find the required volume of ethanol to elute the estrogens from the C_{30} sorbent, the standard solution was passed through the SPE device, and then the sorbed analytes were eluted by 10 μL of ethanol into a sample loop (200 μL volume) that had been filled with water. After every desorption, the contents of the loop were chromatographed. The desorption and subsequent LC separation were repeated until no detectable peaks appeared on the chromatogram. All the peak areas for each analyte were summed up and plotted as a function of the total amount of ethanol used for desorption

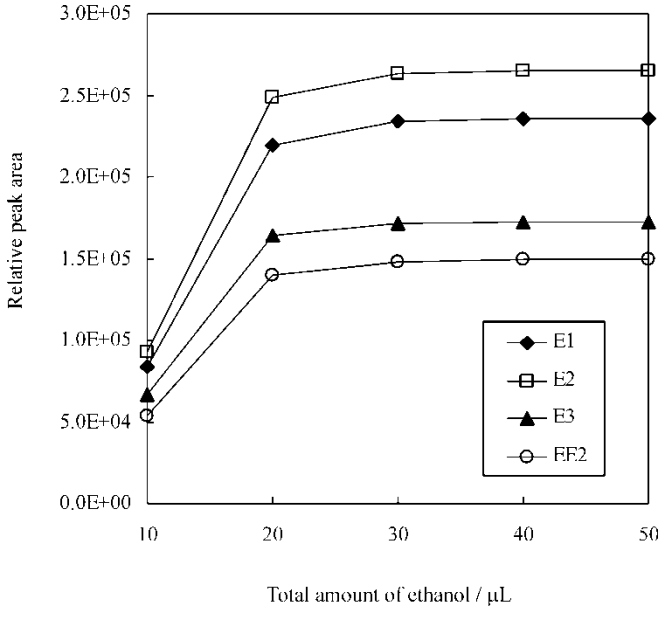

Figure 2. Peak area obtained for selected estrogens from the replicate desorption with ethanol.

(Figure 2). The peak area of the analytes increased with the volume of the extraction solvent up to approximately 30 μL, above which the peak areas remained virtually invariant.

The effect of the amount of the desorption solvent on the separation of the analytes is shown in Figure 3, where a single injection of 10–50 μL of ethanol was carried out to elute the analytes into a 200 μL loop containing water plugs. Clearly, desorption by 30 μL of ethanol provides the highest peak area and good peak resolution.

Next, the water content was varied while the volume of the desorption solvent was held at 30 μL. For this purpose, sample loops having volumes of 50, 100, 200 μL were employed. As can be seen in Figure 4, the strongest peak with good symmetry is obtained when 85 μL of water was loaded on either side of the extracted portion. The fronting of the peaks is remarkable in the chromatograms obtained with lower volumes of water in the loop: it usually occurs when the sample is made up in a strong solvent. It is also noted that analyte elution is delayed as the volume of water contained in the loop is increased; this is most likely due to the decrease in the elution strength of the mobile phase as a consequence of the introduction of a large amount of water into the column.

Finally, the combination of 30 μL of ethanol and 170 μL (total volume) of water was adopted in the following studies.

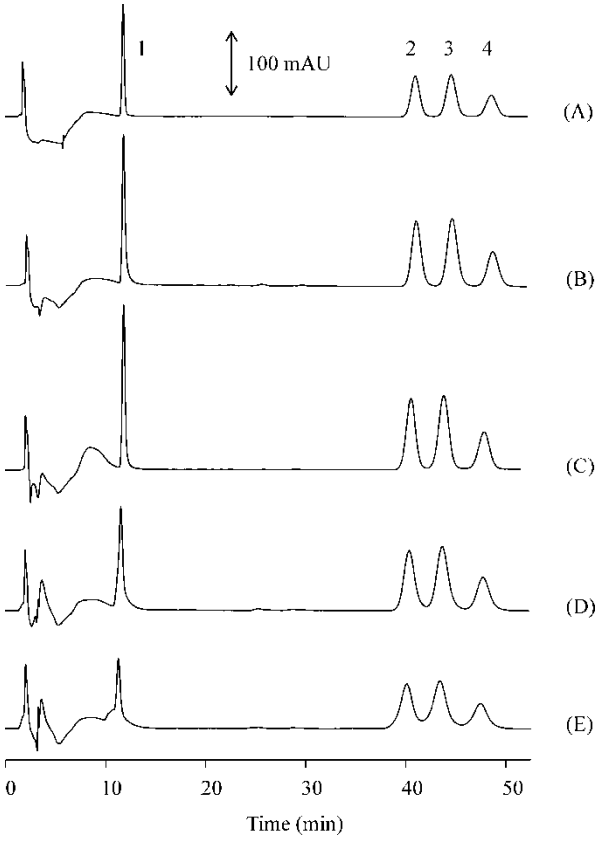

Figure 3. Chromatograms obtained from the injection of different volumes of the ethanol extract and water into a 1 mm i.d. microcolumn: (A) 10 μL ethanol, 190 μL water; (B) 20 μL ethanol, 180 μL water; (C) 30 μL ethanol, 170 μL water; (D) 40 μL ethanol, 160 μL water; (E) 50 μL ethanol, 150 μL water. Peaks: (1) E3; (2) E1; (3) E2; (4) EE2. Individual chromatograms were obtained by the loading of 0.20 μg of each analyte on the device.

Method Performance

The preconcentration (enrichment) factor, the recovery, and the limit of detection (LOD) were evaluated for 60 mL of water sample containing each compound at 5.0 μg/L. The results are shown in Table 1. In all instances, the preconcentration factors were calculated from the ratio of the peak area obtained with the SPE device to that obtained by the direct injection of a 30 μL portion of the water sample (with water plugs on either side of the sample in a 200 μL loop); the recovery percentages were calculated by dividing the peak area obtained from the extracted samples with the product of the peak area obtained by the direct injection method and the ratio of the

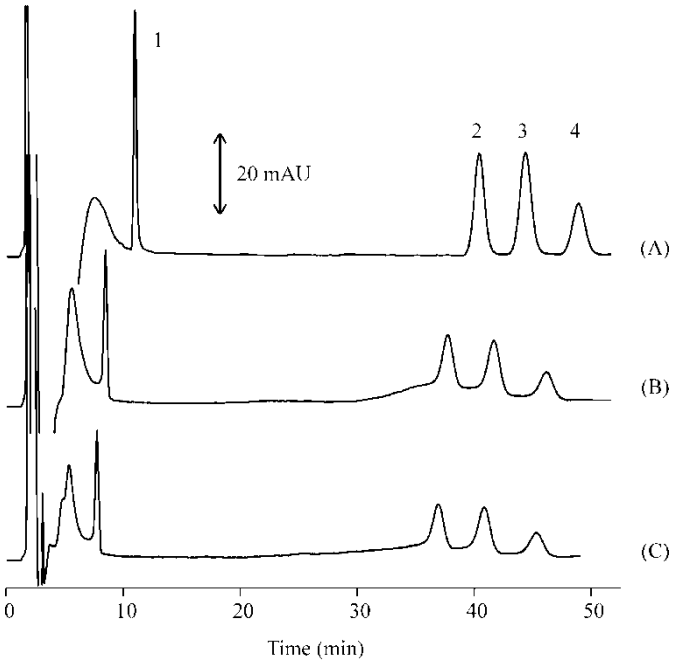

Figure 4. Chromatograms obtained from the injection of different volumes of water at a constant volume (30 μL) of ethanol : water of 170 μL (A), 70 μL (B), and 20 μL (C) was injected using the injection valve equipped with a sample loop of 200 (A), 100 (B), and 50 μL (C) volume depending on the total volume of the ethanol and water used. Individual chromatograms were obtained by loading of 0.025 μg of each analyte on the device.

loop volume (200 μL) to the sample volume (60 mL); the LODs were calculated as the concentration required to give a signal-to-noise ratio of 3. The obtained preconcentration factors are >1850, meaning that the sensitivity of LC analysis is proportionately increased. Satisfactory recoveries ($>92\%$) were obtained for all compounds. The LOD values ranged from 3.8 to

Table 1. Recoveries, preconcentration factors, and LODs obtained with the SPE device

Compound	Preconcentration factor	Recovery (%)	LOD (ng/L)
E1	1880	94.0	8.9
E2	1892	94.6	11.7
E3	1926	96.3	3.8
EE2	1852	92.6	12.6

12.6 ng/L; the values are much lower than those obtained using UV detection for SPE-LC[12,13] and SPME-LC,[14] although the LODs of an order of magnitude lower than these values were obtained with ESI-MS detection.[15–17]

Calibration plots of peak area versus concentration were constructed for individual analytes in the concentration range from 0.025 to 20 μg/L. The linearity was good with determination coefficients, r^2 of 0.9985–0.9998. The reproducibility of the method, expressed as relative standard deviation (RSD), was determined for the triplicate analysis of water (20 mL) spiked at 0.25 μg/mL of each compound. The RSD values were 3.5, 5.8, 4.3, and 5.4% for E3, E1, E2, and EE2, respectively.

CONCLUSION

An SPE device developed in our laboratory was successfully used in conjunction with a microcolumn LC. On-column focusing was used to concentrate the analytes prior to the LC analysis. One of the advantages of the method is that there are no needs for modification of existing equipments. Also, the use of the device eliminates the intensive manual labor needed in conventional SPE methods. Although the sensitivity of the method is much higher than those for SPE coupled to LC with UV detection thus far reported in the literature, a further decrease in LOD should be possible by the use of mass spectrometric detection. In addition, the LOD would be decreased by performing a gradient elution.

Another important feature of the method is that ethanol is employed as both a desorption solvent and an organic modifier of mobile phase: obviously the solvent is the least harmful among organic modifiers used in reversed-phase LC. The use of the mixture of ethanol and water as the mobile phase in microcolumn LC is favorable for coupling with ESI-MS, in view of the high volatility and low surface tension of the organic modifier to be introduced at a low volumetric flow rate into the ion source.[18] The method here described is currently being applied to the evaluation of estrogenic compounds in real samples and it will be the subject of future publications.

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