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Current Analytical Chemistry, 2013, *?*, 00-00

Molecularly Imprinted Solid Phase Extraction Before Capillary Electrophoresis for the Analysis of Estrogens in Serum Samples

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Abstract: An estrogen selected molecular imprinted polymer (MIP) has been developed to be used as an alternative sorbent in solid phase extraction of serum samples before capillary electrophoresis analysis. Following a heat polymerization, MIP has been synthesized using methacrylic acid (MAA) as functional monomer, ethyleneglycol dimethacrylate (EGDMA) as crosslinker, benzoyl peroxide as radical initiator and 17β- estradiol as template, and toluene as porogen. The optimized molecular imprinted solid phase extraction (MISPE) was compared to traditional C18 solid phase extraction to purify estrogens from complex matrices. It was concluded that the MISPE system developed is useful for the selective extraction of 17β- estradiol and its major natural related estrogens, estriol and estrone, from serum samples with minimal interferences and high recoveries.

Keywords: Estrogens, Molecular imprinting, Solid phase extraction, Serum, Capillary electrophoresis.

INTRODUCTION

Estrogens are an important group of C18 sex steroids connected with physiological functions and pathological effects. They are linked to fertility, pregnancy, menopause, metabolic disorders, cancer and bone disease. Additionally, they are also used as therapeutic agents in the hormonal replacement, like contraceptives or enhancement like illegal anabolic drugs [1-4]. Therefore, their determination is very important in the diagnostic field, treatment monitoring, pharmaceutical quality control, environmental studies and doping control [5-9].

To achieve a reliable determination of estrogens in complex matrices, it is necessary to apply effective analytical procedures capable to quantify these analytes with high sensitivity and selectivity together with the simplicity needed to be used in routine analysis.

Currently, there are two groups of methods for the effective monitoring and detection of estrogens: immunological and chromatographic methods.

The first group is highly selective due to the antibody-antigen specificity interaction. However, it presents many disadvantages such as high cross-reactivity with a remarkable overestimation, high grade lot-to-lot variation and instability of antibodies, false positive and negative data and, additionally, the disadvantage of the necessity to analyze hormones individually [5, 10].

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The chromatographic methods (GC, LC and CE) which combine different sample pretreatments such as solid phase extraction (SPE), liquid-liquid extraction (LLE), supercritical fluid extraction (SFE), etc, are the most commonly used systems in confirmatory estrogen testing.

SPE is the most popular procedure used for preconcentration and clean-up of complex matrices due to the advantages of simplicity, speed and little consumption of organic solvents [10]. Despite these attractive features, the classical SPE hydrophobic sorbents (C8, C18) retain analytes by non-selective hydrophobic interaction which lead to high amounts of matrix interferences where are co-extracted. This factor decreases separation and enrichment efficiency of the analytical method [10-12].

In order to enhance the selectivity of the extraction, new selective materials involving mechanisms of molecular recognition such as immunosorbents and molecular imprinting polymers (MIP), were recently developed [10].

Due to the drawbacks of immunosorbents like being highly expensive and time consuming, MIPs techniques have become popular in the last few years. MIPs are synthetic polymers with specific binding sites (cavities) with complementary size, shape and functional groups designed to interact with a template molecule [11]. The advantages of MIPs over antibodies include stability, easy preparation, low cost and reusability [10] leading to an increase in using as sorbent for SPE, the so-called molecularly imprinted solid phase extraction (MISPE).

Although different polymers have been developed for estrogen extraction [7, 11-14], the use of MISPE in serum samples has not previously been reported.

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In a previous work we have developed a simple and rapid CE method for the quantitative by simultaneous analysis of nine steroids with good precision and accuracy [15]. The aim of this study was to synthesize a non-covalent molecularly imprinted polymer using 17β- estradiol as template molecule to be used as MISPE prior to the analysis by the CE method previously developed to selectively extract the most biologically active estrogens: 17β- estradiol, estriol and estrone from serum samples.

EXPERIMENTAL

1. Chemicals and Reagents

17β-estradiol (E_2), estriol (E_3), estrone (E_1), androstenedione (Δ_4), testosterone (To), β-cyclodextrin sulfate sodium salt, sodium dodecyl sulfate (SDS) and sodium cholate hydrate (CA), were purchased from Sigma (St. Louis, MO, USA). Tetronic® 1107 was a gift from BASF Corporation (Florham Park, NJ, USA). Sodium monohydrogen phosphate, sodium borate 10-hydrate, tetrahydrofuran, acetonitrile and methanol were of HPLC grade and supplied by E. Merck (Darmstadt, Germany) and C18 SPE cartridges were from Enviro clean®, USA. Ammoniun sulphate was purchased by J.T. Baker, Mexico. Ultrapure water was obtained from an EASY pure ™ RF equipment (Barnstead, Dubuque, IA, USA). All solutions were filtered through a 0.45 µm nylon membrane (Micron Separations Inc., Westboro, MA, USA) and degassed before use. Methacrylic acid (MAA), ethylene glycol dimethacrylate (EGDMA) and benzoil peroxide were purchased from Aldrich, Germany. Toluene and acetic acid were of analytical grades and were supplied by Sintorgan, Argentine.

2. Instrumentation and Analysis

CE Analysis

Analysis was carried out with a P/ACE ™ MDQ capillary electrophoresis system (Beckman, Fullerton, CA, USA). Uncoated fused silica capillaries (Microsolv technology, Eatontown, NJ, USA) of 50 cm (40 cm length to the detector) x 75 µm i.d., were used. A detailed description of the analytical method has already been described [15]. Briefly, the separation was performed by a MEKC system consisting of 44 mM cholic acid, 10 mM SDS, 0.05% w/v tetronic ® 1107, 2.5% v/v methanol, 2.5% v/v tetrahydrofuran and 5 mM borate- 5 mM phosphate buffer (pH=8.0) as background electrolyte.

The capillary temperature was maintained at 25 °C, and UV detection was set at 210 nm. Samples were injected under 0.5 psi pressure for 3s and electrophoretic system was operated under positive polarity and a constant voltage of 18 kV.

Stock standard solutions of E_1, E_2, E_3, Δ_4 and To were prepared in methanol at 1mg/mL and stored at -20 °C. Working solutions were prepared daily by the appropriate dilution of the stock solutions.

HPLC Analysis

Analysis was performed using an HPLC Thermo Scientific, equipped with a quaternary pump (P 4000), temperature control, a vacuum degasser (SCM 1000), a dual UV detector (UV 2000), an automatic injector (AS 3000) and ChromQuest 5.0 software controlling instrumental parameters. The analytical column was an ultremex C18 column (Phenomenex, USA) 250 x 4.6 mm and 5 µm particle size. The chromatographic conditions were based on the system developed by Gadzala-Kopciuch et al. [16] and only the elution profile was modified changing from methanol: water (60:40, v/v) during 6.5 min. to methanol: water (70:30, v/v).

Complementary Instrumentation

A diode array spectrophotometer (Agilent 8452), ultrasonic bath (Transsonic Digitals, ELMA), rotary evaporator RE47 (Yamato Co Limited), shaker (Minitherm-Shaker, Adolf Kühner AG Schweiz) and Soxhlet (IVA S: A, Argentine) were used for the optimization studies and template remotion.

3. Polymer Preparation

The estrogen imprinted polymer was prepared by bulk polymerization according to the non-covalent approach, dissolving the imprint template (E_2; 0.05 mmol), in the functional monomer (MAA, 4 mmol). Then toluene (80 % w/w of the total reagents) as the porogen solvent was added and the mixture was sonicated for 40 min. Finally, the cross-linking monomer (EGDMA, 10 mmol) and the initiator benzoyl peroxide (45 mg; 2 % w/w of the total reagents) were added and the polymerization was induced by heat in glycerin bath at 60 °C with permanent stirring during 24 hrs.

Removal of the template was carried out with methanol/acetic acid (9:1, v/v) using Soxhlet apparatus for 24 hs or until template was not detectable by HPLC UV at 280 nm. As a control, a non-imprinted polymer (NIP) was synthesized simultaneously under the same procedure in the absence of template molecule.

4 Preparation of MISPE Cartridges

An amount of 140 mg of dry particles of MIP or NIP was packed with methanol into 3 mL SPE empty cartridges with two glass-wool frits at each end. The cartridges were air dried, washed with methanol (4mL) and conditioned with water (5 mL) before use.

5. Uptake and Binding Experiments

To evaluate efficacy in the uptake and the binding capacity of estrogens by the MIP, 100 milligrams of the sorbents (MIP and NIP) were added to 5 mL of a standard mixture of E_1, E_2 and E_3 (200 µg/mL) in acetonitrile (ACN) and the mixture was mechanically shaken at 120 rpm for 24 hs at room temperature and then separated by centrifugation at 5000 rpm for 15 min. The supernatant (free estrogen) was analyzed by HPLC at 280 nm [16]. The adsorption quantity (B) was calculated by subtracting the free concentrations from the initial concentrations of each estrogen. This experiment was also used to evaluate the porogen to be used in the polymer synthesis.

6. Molecular Imprinted Solid Phase Extraction (MISPE) Procedure in Serum Samples

2 mL of serum samples were spiked with E_1, E_2, E_3, Δ_4 and To to a final concentration of 0.1 µg/mL of each one in

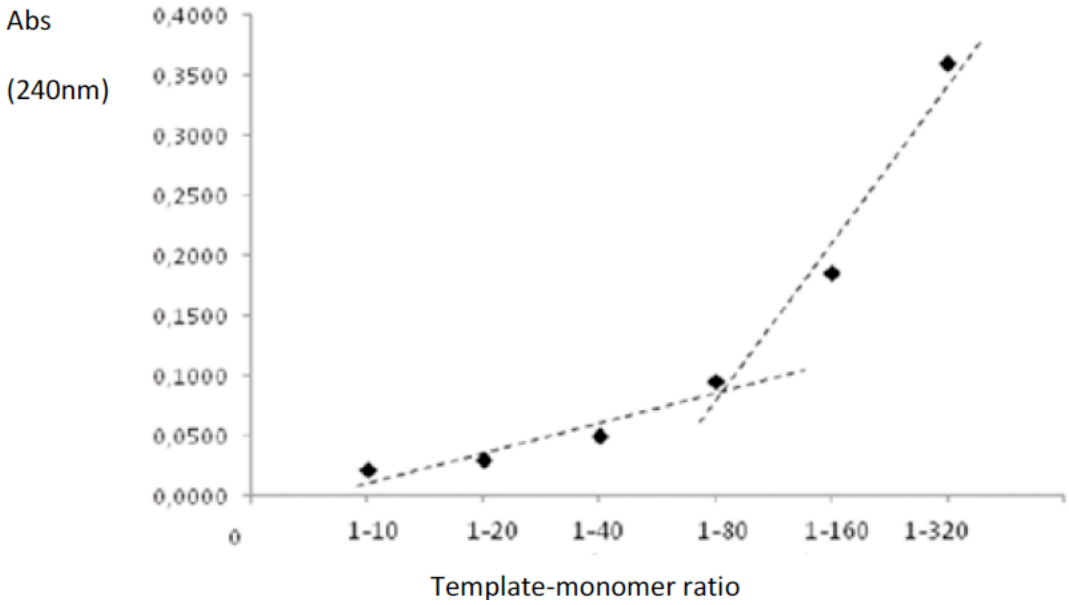

Fig. (1). Selection of the appropriate template: monomer ratio. For details see the text.

methanol and precipitated with 3 mL of cold ACN. Afterwards, 1 g of ammonium sulphate was added to the supernatant and the organic phase was evaporated and completely redissolved in 0.7 mL of ACN, then 6.3 mL of water was added and, finally, loaded to MIP, NIP and C18 cartridges previously conditioned with 4 mL of methanol and 5 mL of water. The cartridges were washed with 5 mL of water to eliminate molecules retained by non-specific adsorption of the sorbent. Elution step was then performed using 4 mL of methanol and the eluates were finally evaporated to dryness and redissolved in 40 µL of diluent, a mixture of sulfate-β-cyclodextrin 3% w/v was adjusted to pH 8.0 in 5 mM borate-5 mM phosphate, and then 20 % of methanol was finally added; allowing a 50-fold increment in concentration before injecting the sample into the CE equipment.

Androstenedione and testosterone were selected to evaluate the selectivity of MIP cartridge.

RESULTS AND DISCUSSION

1. Optimization of MIP Synthesis Procedure

The key problem connected with MIPs is to find out the preparation procedure for the development of useful and effective polymers with highly selective recognition sites [12]. An initial series of polymers was prepared to determine the best condition to obtain an appropriate MIP for the estrogens extraction. Evaluation of template: monomer ratio, monomer: cross-linker ratio and porogen solvent was taken into account.

1.1. Template: Monomer Ratio

The amount of formed complex was affected by the molar ratios between template and monomer in the synthesis and evidenced by spectroscopy. The study of UV spectra was used for evaluating the complexes and optimizing the template: monomer ratio. This approach is used to limit the number of polymers to synthesize and evaluate, time consumption and expensiveness [11]. Thus, six molar ratios between template and monomer of 1:10, 1:20, 1:40, 1:80, 1:160 and 1: 320 were tested by UV spectra. A wavelength of 240 nm was selected because only the complex absorbs at this value. Fig. (**1**) shows that in accordance to bibliography [7, 16] 1:80 ratio produces the maximum bound without compromising specific bounds at the final polymer. Theoretically, lower molar ratios can induce less binding sites in polymers and higher molar ratios can induce non-specific binding [11].

1.2. Monomer: Cross-Linker Ratio

The amount of crosslinker affects the quality of MIP. Fixing the template amount (0.05 mmol) different monomer were tested: the cross-linker molar ratios were (1:0.5, 1:2.5, 1:5.0) and a macroscopic evaluation was also carried out. Low amounts (1:0.5) of crosslinker produced soft, gelled and unstable polymers which are inadequate to be used as sorbent. High amounts of crosslinker (1:5.0) produced polymers which are too compact and hard that make the liquid passage through the sorbent very difficult. A 1:2.5 ratio showed to be adequate to produce the desired polymer.

1.3. Choice of Porogen

The porogen solvent is one of the most important factor, determining effective molecular recognition because the accuracy of the assembly between the template and the monomer is related to the physical and chemical characteristics of the porogen [11]. From the literature, MIPs are generally prepared using MAA as functional monomer and EGDMA as cross-linker. In this study, toluene and acetonitrile were evaluated as porogens. The uptake experiment determined by HPLC, choosing E_2 as analyte, was performed to evaluate the effectivity of MIPs recognition. Each porogen was evaluated using the template: monomer: cross-linker ratio determined previously. The better E_2 uptake was obtained using toluene as porogen. In Fig. (**2**) it was observed that after incubation with MIP synthesized with toluene, E_2 free concentration measured by HPLC was lower than after incubation with NIP synthesized with the same porogen. However, MIP and NIP showed a similar retention when synthesized with ACN. Thus, toluene

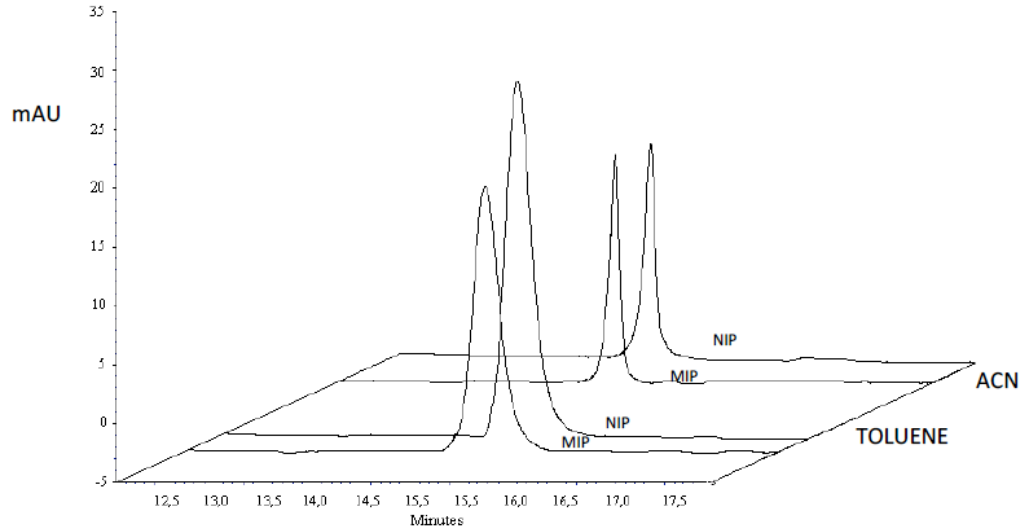

Fig. (2). HPLC Evaluation of free E_2 using different porogens during polymerization. Experimental condition, see in the text.

was selected as porogen solvent because it shows that there are different E_2 uptake between MIP and NIP.

2. Evaluation of MIP Binding and Uptake

After MIP synthesis the imprinting effect was initially evaluated by performing binding experiments in which fixed amounts of imprinted and non-imprinted polymers were incubated with a solution of E_1, E_2 and E_3 in ACN. Fig. (**3**) shows that the binding capacity of imprinted polymer increased with respect to non-imprinted polymer for each estrogen.

HPLC method [16] was used to control uptake efficacy in standard samples because it is simple and useful to resolve E_1, E_2 and E_3. However, it was not the chosen technique for analysis of serum samples because it cannot resolve estrogens from endogens interferences in serum. Therefore, previously developed CE method for the analysis of estrogens in the sample was employed.

3. MISPE in Serum Samples and Extraction Selectivity

Although many studies were concerned with the development of MIPs for one target only, this work points to the development of an MIP for the extraction of a group of structural analogs (E_1, E_2 and E_3) because they are the most representative hormones to evaluate estrogenic condition in different physiological and pathophysiological states. 17β-estradiol as template was selected because it produces cavities able to present affinity for the whole group. Although CE analytical method can resolve many of the structural related steroid hormones, it was necessary to evaluate the selectivity of the sorbent to be used as the best sample pretreatment procedure.

For selectivity, Δ_4 and To where chosen as the most suitable structurally related steroid hormones. A serum sample was spiked with E_1, E_2, E_3, Δ_4 and To (0.1 µg/mL in methanol for each one), MISPE, NISPE and C18 SPE procedures, were compared. Fig. (**4**) shows that using MISPE for a quantitative extraction an excellent selectivity was obtained with E_1, E_2 and E_3 while the recovery of Δ_4 and To is null. The recovery of in MIP cartridge was 93 % for E_3, 42 % for E_1 and 100 % for E_2, higher than that of NIP cartridge (12 %, 30 %, and 65 %, for E_3, E_1 and E_2 respectively) and C18 cartridge (75%, 0% and 27%, for E_3, E_1 and E_2 respectively). Moreover, if C18 cartridges are employed additional peaks

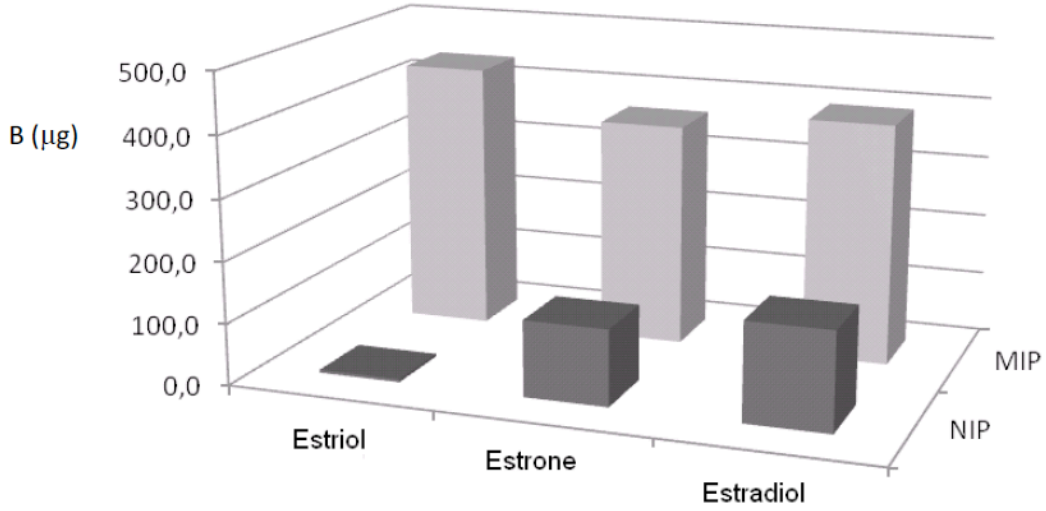

Fig. (3). Binding experiments of MIP and NIP for E_1, E_2 and E_3.

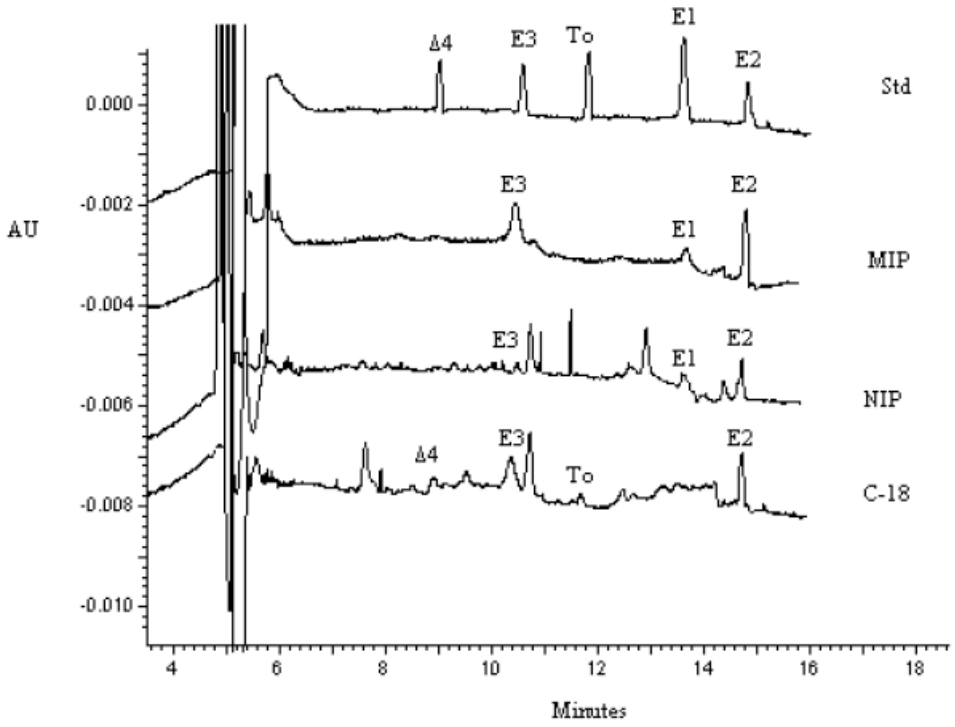

Fig. (4). Electropherogram of spiked serum sample comparing MISPE (**a**), NISPE (**b**), C18 SPE (**c**) and a mixture of standard.

corresponding to Δ_4 and To together with a co-elution of serum interferences appear in the chromatogram. Thus, it was observed that better recovery and adequate selectivity could be obtained using MISPE procedure without the presence of any interference.

CONCLUSION

This study demonstrated that a successful clean –up of a serum sample was achieved using the developed MISPE system followed by CE method with good selectivity and low interferences compared to C18 SPE for the analysis of E1, E2 and E3 which are the most important estrogens in clinical studies.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflicts of interest.

ACKNOWLEDGEMENTS

We wish to thank the assistance provided by Pharmacognosy and Pharmaceutical Technology chairs, Faculty of Pharmacy and Biochemistry, University of Buenos Aires, Argentine.

ABBREVIATIONS

Δ_4	=	androstenedione
CE	=	capillary electrophoresis
EGDMA	=	ethylene glycol dimethacrylate
E_2	=	17β- estradiol
E_3	=	estriol
E_1	=	estrone
GC	=	gas chromatography
LC	=	liquid chromatography
MAA	=	methacrylic acid
LLE	=	liquid-liquid extraction
MIP	=	molecular imprinting polymer
MISPE	=	molecular imprinted solid phase extraction
NISPE	=	non-imprinted solid phase extraction
SDS	=	sodium dodecylsulphate
SFE	=	supercritical fluid extraction
SPE	=	solid phase extraction
To	=	testosterone.

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Received: ???? 20, 2011 Revised: ?????? 02, 2012 Accepted: ??????? ??, 2012