

## **Sensitive determination of isoprostanes in exhaled breath condensate samples with use of liquid chromatography – tandem mass spectrometry.**

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## **ABSTRACT**

A fast analytical procedure for determination of four isomers from group of F2-isoprostanes in exhaled breath condensate sample (EBC) based on application of liquid chromatography tandem mass spectrometry has been developed. Because of limited volume of EBC sample and very low concentrations of biomarkers we choose lyophilization as a technique for preconcentration. Determined diastereoisomers show similar fragmentation patterns, that is why complete chromatographic separation with excellent peak shape was essential for accurate quantitation. Separation of isoprostanes was achieved with using narrow bore column with isocratic elution mode what reduce time of analysis, amount of used solvents and increased sensitivity of isoprostane determination. The recoveries for all isoprostanes were in the range of 96.7÷101.7, with relative standard deviation <7%. The limits of determination and quantitation values for the whole procedure of determination of four isoprostanes in samples of EBC ranged from 1 to 3 pg/ml. Stability of isoprostanes in different temperature conditions was measured as well. The aim was to develop rapid measurement of F2-isoprostanes in EBC samples by equipment which is nowadays available and routinely exploited in analytical laboratories.

### ***Keywords***

Exhaled Breath Condensates, EBC, Biomarkers, Isoprostanes, Liquid Chromatography, Tandem Mass Spectrometry



## 1. Introduction

Oxidative stress (OS) is a state of imbalance between continuously generating reactive oxygen species (ROS) during normal cell metabolism and mechanism of detoxifying oxygen radicals through a network of antioxidative enzymes. High concentration of ROS enhances lipid and protein peroxidation in the membrane bilayer thus inducing a variety of cellular dysfunctions that ultimately lead to tissue damage. Biochemical pathways, by which oxidative stress may cause cellular damage, include changes in intracellular redox state, overexpression of multiple genes in vascular cells and altered signal transduction pathways [1].

Products of ROS activity are measured as a biomarkers of oxidative stress. Peroxidation of unsaturated fatty acids like arachidonic acid and eicosapentanoic acid leads to formation of prostaglandins, tromboxanes and leukotrienes, all called eicosanoids. Prostaglandins are formed nonenzymatically, while tromboxanes are products of enzymatic cyclooxygenase activity,. Abnormal levels of OS biomarkers are observed in various acute and chronic diseases e.g. cancer [2], cardiovascular disease [3] neurodegenerative disease [4], lung disease [5] and even normal aging process [6].

One of the most studied marker is 8-iso-PGF<sub>2α</sub> due to its sensitivity, chemical stability and reliability as index of lipid peroxidation [7; 8]. This substance and its isomers are measured in different biological specimen like urine [9], plasma [10], cerebrospinal fluid (CSF) [11], liver [12], kidneys [12], brain tissue [13], bronchopulmonary lavage (BAL) [14], induced sputum [15] and exhaled breath condensates (EBC)[16].

To evaluate inflammation process in lungs BAL, induced sputum and EBC samples are analyzed. Traditionally, the measurement of lung disease biomarkers has involved invasive procedures to procure the samples or to examine the affected compartments which caused the patient discomfort. As a consequence, there is a need for less or non-invasive approaches to measure oxidative stress. Some of biomarkers found in expired breath can be detectable in the

liquid obtained by cooling it. The collection of exhaled breath condensate (EBC) has recently emerged as a non-invasive sampling method for real-time analysis and evaluation of oxidative stress biomarkers in the lower respiratory tract airways.

The main advantages of this method are non-invasive, convenient, and possibility to be carried out on mechanically ventilated patients as well as on children. Limitations of EBC samples analysis are as follow: small amount of a sample (usually 1ml for 15 min of breathing), high levels of interfering substances, relatively low concentrations of biomarkers and lack of standard method of collection.

Quantification of isoprostanes is used as a reliable marker of lipid peroxidation *in vivo* [17-19] and several techniques are currently used [20-23] including: gas chromatography (GC)–mass spectrometry (MS), which might be associated with an immunoaffinity extraction; GC–tandem MS; and liquid chromatography–tandem MS. Immunoassays have been developed to measure levels of isoprostanes, however the used antibodies have not been tested for cross-reactivity with the numerous F2-isoprostane isomers and their metabolites. Results obtained with immunoassays may differ from those obtained using GC–MS assays, therefore immunoassays are to be considered as semi-quantitative indices of F2-isoprostanes.

In this study, we have investigated potential usefulness of HPLC-MS/MS procedure for accurate qualitative and quantitative analysis of four isoprostanes in exhaled breath condensate samples. Radioimmunoassay (RIA) or enzyme immunoassay (EIA) techniques offer easiness of performance, possibility of automation and low cost of analysis. In most published studies, F2-isoprostanes have been measured with the use of commercially available enzyme immunoassays [24-28]. However, the available ELISA kits were originally validated for matrices that differ from EBC, such as urine, plasma or serum. Quantification by ELISA-kits may be influenced by the matrix containing dissolved analytes. Due to extremely high dilution of the epithelial lining fluid in EBC (resulting low concentrations), EBC is considered to contain only little matrix with comparison to other highly concentrated and protein-rich matrices like plasma or



urine. The results obtained by comprehensive comparison study, performed with two unrelated analytical techniques for quantitation of three most commonly analyzed eicosanoids, showed overestimation of analytes levels in EBC.

A new procedure has been developed for determination of content of four isoprostanes in samples of exhaled breath condensates. Sample preparation step is based on lyophilization of collected samples of EBC and dissolution of emerged dry residue prior to analysis using LC-MS/MS.

Proposed procedure is specific due to its ability to work in multiple reaction monitoring mode (MRM), where a specific set of precursor ions are selected and their transition from the selected precursor ion to a specific product ion is monitored. MRM mode reduces chemical noise leading to higher mass spectral sensitivity and selectivity, which is used for quantitative determination. To obtain high precision of quantification by compensation of detector response a deuterated internal standard is used. Application of lyophilization for sample preparation resulted in enrichment of analytes of interest and high sample throughput. Since the majority of laboratories is equipped with HPLC-MS/MS system, hence both procedures can be used routinely for determination of isoprostanes in EBC samples.

## 2. Materials and methods

### 2.1 Chemicals

HPLC-MS grade methanol and acetonitrile were obtained from Witko (Łódź, Poland). Formic acid was purchased from P.O.Ch. (Gliwice, Poland). Ultrapure water was obtained from an HPLC5 system (Hydrolab, Poland).

Authentic standards of isoprostanes including 8-iso-PGF<sub>2α</sub>, 8-iso-15(R) PGF<sub>2α</sub>, 11-PGF<sub>2α</sub>, 15(R)-PGF<sub>2α</sub>, were obtained from SPI-BIO (Montigny le Bretonneux, France) as well as the deuterated internal standard 8-iso-PGF<sub>2α</sub>-d<sub>4</sub>.

### 2.2 Standard solutions

The stock solutions of 8-iso-PGF<sub>2α</sub> (8-isoP), 8-iso-15(R)PGF<sub>2α</sub> (8,15-isoP), 11-PGF<sub>2α</sub> (11-isoP) and 15(R)-PGF<sub>2α</sub> (15-isoP) 1ng/mL of each, were prepared by diluting standards in methanol and obtained solutions were stored at -80 °C.

A series of calibration solutions were prepared by dilution of the intermediate solutions with methanol:acetonitrile:water (25:25:50 v/v/v) in a concentration range of 1-200 pg/mL.

The chemical structures of four isoprostanes and internal standard are shown in Fig. 1.

<insert Fig. 1.>

### 2.3 Sample preparation

EBC samples were collected from students of Gdańsk University of Technology (GUT). 1ml of EBC sample was spiked with internal standard and stored in -80°C. Frozen samples were lyophilized to dryness, reconstituted in 200μl mobile phase (H<sub>2</sub>O:ACN/MeOH 1:1) and analyzed (Fig. 2).

<insert Fig. 2.>

### 2.4 HPLC-MS/MS analysis

HPLC analyses were performed on Agilent 1200 HPLC system. The chromatographic system consisted of G1367C autosampler, with the injection volume set to 100  $\mu$ l, G1312B binary pump and G1316B thermostated column. The separation of analytes was done by using Agilent Extend C-18 (50mm $\times$ 2.1mm; 1,8 $\mu$ m). The mobile phase A (methanol/acetonitrile 1:1) and B (ultrapure water) contained 0,01% (v/v) formic acid at flow rate set to 0.35 ml/min. The column was maintained at 40°C.

The HPLC system was directly coupled to a triple quadrupole mass spectrometer (API 4000; Applied Biosystems, Darmstadt, Germany). The mass spectrometer was optimized in the MRM mode by diffusing 50 ng/ml of each individual isoprostane standard solution. The instrument was operated in the negative ion mode with declustering potential at 115 eV, focusing potential at 200 eV, entrance potential at 10 eV, collision energy at 33 eV, collision cell exit potential at 15 eV, ion spray voltage at 4500V and source temperature at 550°C. The most abundant product ion of all analytes was found at  $m/z$  193, the mass transition of [M-H<sup>-</sup>]  $m/z$  353 in Q1 to  $m/z$  193 in Q3 was selected for the quantitative analysis during LC-MS/MS and the corresponding transition of  $m/z$  357 to 197 was monitored for the deuterated internal standard. Instrument control, data acquisition and data analysis were carried out with Analyst Software 1.5.2 (Applied Biosystem).

### 3. Results and discussion

Figure 3 shows a mass spectrum of 8,15-isoP, because 8-isoP, 8,15-isoP, 11-isoP, 15-isoP are diastereoisomers and they depict similar fragmentation patterns. Due to this fact, complete chromatographic separation with excellent peak shape is essential for accurate quantitation. Selectivity of the method was achieved by combination of retention time, precursor ion and product ions.

<insert Fig. 3A, B.>

The proposed HPLC procedures enable the quantification of 8-isoP, 8,15-isoP, 11-isoP, 15-isoP in EBC samples with tandem MS as a system of detection. Using the chromatographic conditions mentioned above for narrow-bore column the retention times for I.S. and four isoprostanes are 4.77 and 4.47 min, 4.77 min, 5.15 min, 6.01 min respectively. Figure 4 demonstrates a chromatogram of EBC sample spiked with isoprostanes.

<insert Fig. 4.>

### 3.1 Method validation

The stock solutions were used for the preparation of eight calibration solutions at concentration 5, 10, 25, 50, 75, 100, 150 and 200pg/ml. The prepared calibration solutions were spiked with the IS (8-iso-PGF<sub>2α</sub>-d<sub>4</sub>). The calibration curves were generated using the dependence of the ratio of the peak area of the particular substance (8-isoP, 8,15-isoP, 11-isoP, 15-isoP) to the peak area of the IS (8-iso-PGF<sub>2α</sub>-d<sub>4</sub>) on the particular biomarker concentration. Analysis of each calibration solution was repeated three times. Limits of detection (LODs) and limits of quantitation (LOQs) were determined by serial dilution of standard solutions. LODs and LOQs were evaluated on the basis of a signal-to-noise ratio of 3 and 10, respectively. Calibration data including calibration line equations, coefficients of determination, limits of detection and quantitation are listed in Table 1.

<insert Table 1.>

Calibration curves were linear within the studied range of concentrations with coefficients of determination over 0.998 for all isoprostanes.

The trueness, repeatability and reproducibility of the method were tested with fortified samples of exhaled breath condensates.

To determine trueness, sample of EBC were spiked at a different concentration levels (25, 100, 250pg/mL accordingly). Every sample was analyzed triply according to procedure described above. The results were obtained by comparison of the peak areas of spiked and





processed EBC samples with the corresponding standard solutions (matrix free) analyzed without lyophilization. Collected data are presented in Table 2.

<insert Table 2.>

Satisfactory recoveries (96.7÷101.7%), with relative standard deviations (RSDs) below 7%, were obtained for all isoprostanes regardless of the spiking level.

The repeatability and reproducibility of the method were assessed by replicate analyses of EBC samples at chosen spiking level (200pg/mL). The samples of EBC were analyzed three times a day (nine samples of EBC) to determine the intra-day repeatability expressed as RSD within-day averages. The analyses were repeated for three consecutive days to calculate inter-day reproducibility expressed as RSD between-days averages. The results of these studies are presented in Table 3.

<insert Table 3.>

The intra-day repeatability was between 6.4÷9.4%. The inter-day reproducibility was between 4.5÷5.8%.

### *3.4 Sample stability*

Stability of oxidative stress markers is closely connected with matrix composition of sample. Due to the fact that most samples are usually stored before final analysis, it is necessary to examine the stability of biomarkers of interest in different conditions. Samples like blood plasma or solid tissues frozen in liquid nitrogen and stored at -80°C do not undergo auto-oxidation up to eight months. [29]. Changing conditions of blood plasma storage to +4°C resulted in increased levels of isoprostanes [30]. The reason of this is undoubtedly auto-oxidation of plasma lipids due to artificial generation of isoprostanes.



Matrix of EBC samples is relatively simple and autooxidation processes should generate less autooxidation products than from samples containing high amounts of lipids, proteins and salts.

#### *3.4.1 Stability of EBC samples after one, two and three freeze–thaw cycles*

The variation of isoprostanes concentrations when submitting QC samples to successive three freeze–thaw cycles was checked. The results obtained indicate no significant loss of isoprostanes is to be expected after up to three freeze–thaw cycles.

#### *3.4.2 Stability of water samples in HPLC vials (i.e.ready for HPLC analysis) at $-80^{\circ}\text{C}$ , $-20^{\circ}\text{C}$ , $+4^{\circ}\text{C}$ and $+20^{\circ}\text{C}$*

EBC sample consists of 99.9% of water. The stability studies of water samples spiked with isoprostanes were done. Samples were left at four different temperatures and analyzed during 8 weeks. To compensate changes in response of detector fresh portion of deuterated internal standard was added to each sample before analysis. The variations of the levels of isoprostanes at concentrations of 250 pg/ml of each were lower than 11.1%.

<insert Table 4.>

#### *3.4.3 Stability of EBC samples after six hours of storage at $+20^{\circ}\text{C}$*

In contrast to stability studies made by Syslova [31], we measured concentration of four isoprostanes by LC-MS/MS in EBC samples, which were hold at room temperature until processing remained essentially unchanged (Fig. 5).

<insert Fig. 5.>

#### *3.4.4 Stability of EBC samples after two weeks of storage at $-80^{\circ}\text{C}$ , $+4^{\circ}\text{C}$ and $+20^{\circ}\text{C}$*

Stability studies made during two weeks show no significant loss of isoprostanes. At  $-80^{\circ}\text{C}$ , the substances were stable for the experimentally evaluated period. Comparing the matrix



effect of stability of isoprostanes in water and EBC matrix under  $-80^{\circ}\text{C}$  was negligible (Fig. 6).

<insert Fig. 6.>

This study shows that multiple EBC samples can be analyzed in a safe period of time without relevant loss of isoprostanes.

### *3.5 Analysis of exhaled breath condensate samples*

In this study narrow-bore column was used with 2.1 mm internal diameter and 1.8  $\mu\text{m}$  particle size. Small column ID and particle size allowed to obtain sharper, more intense peaks than those obtained using standard-bore columns, which resulted in lower limit of detection and quantitation. Narrower column width results in lower LC flow rate. This approach allows to use less amounts of reagents and consequently produces less waste to dispose.. Narrow-bore column allows to use isocratic elution program that resulted in excellent resolution of peaks with shorter retention times. There is no need to equilibrate the column and it permits to analyze larger number of samples in shorter time.

<insert Fig. 7.>

A main drawback of narrow-bore columns versus standard-bore columns is that their capacity is reduced. Due to the fewer stationary phase particles are able to physically fit into the narrower column, there is less surface area available for species for bind. As a result, these columns can become overloaded easily. Typically, such reduced capacity is compensated by diluting the samples more or by using smaller injection volumes. This problem does not appear in analysis of EBC samples, because concentrations of biomarkers are on pg/mL levels.



To demonstrate the application of the method for biological sample analysis, a series of samples of EBC collected from volunteers from Gdańsk University of Technology were analyzed.

Obtained results revealed elevated concentrations of isoprostanes in students with asthma and in tobacco smokers (Fig. 7). In samples of healthy volunteers, isoprostanes concentrations were under the LOD level.

#### **4. Conclusions**

The collection of EBC has been proposed as a simple, quick, safe, comfortable, and non-invasive technique to obtain samples from the lower respiratory tract of humans with inflammatory lung disease at any age. A wide range of constituents in EBC have been previously measured. Therefore, the analysis of EBC components may provide a means of monitoring airway inflammation and cellular metabolism of the lung. However, a range of important methodological issues needed to be addressed for any EBC compound. These include repeatability, dilution, environmental contamination, effect of breathing patterns and assay sensitivity. An important issue is internal coating of the collection device may influence levels of some constituents. For some compounds dilution markers may be important, as it has been suggested. Therefore, a direct comparison between different protocols is complicated due to the lack of an adequate standardization of the sample collection and varied specificity of measurements methods.

The study of the stability of isoprostanes in exhaled breath condensates proven, that they can be stored even at room temperature, in a short period of time, without significant impact on their recovery. However, due to the fact that EBC samples from each individual may vary in composition of the matrix and to eliminate undesirable reactions involving isoprostanes, it is recommended to store samples in temperature  $-20^{\circ}\text{C}$  or  $-80^{\circ}\text{C}$ .



A method for the detection and quantification of 8-iso-PGF<sub>2α</sub>, 8-iso-15(R) PGF<sub>2α</sub>, 11-PGF<sub>2α</sub>, 15(R)-PGF<sub>2α</sub>, as oxidative stress markers, in exhaled breath condensate samples by LC–ESI-MS/MS was developed. In order to circumvent various problems in the conventional assay methods such as laborious and time-consuming pretreatments in GC–MS and possible cross-reactions of the target compounds in enzyme immunoassay (EIA), liquid chromatography coupled with tandem mass spectrometry was used. Furthermore, unlike to GC-MS, it does not require the two-step derivatization procedure, meaning improved recovery, a shorter sample pre-treatment time and the absence of incomplete derivatization of by-products.

The excellent accuracy, reproducibility, and high throughput of this method should permit it to be used in large clinical studies and standard clinical laboratories.

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### Figure Captions

**Fig. 1.** Chemical structures of determined isoprostanes and internal standard (8-iso-PGF<sub>2α</sub>-d<sub>4</sub>)

**Fig. 2.** Scheme of collection, preparation and analysis of EBC samples.

**Fig. 3.** Mass spectrum of 8,15-isoP A) full scan with proposed fragmentation pattern and B) spectrum of product ion m/z 353.3 with selected quantitation fragment ion m/z 193.3.

**Fig. 4.** LC-MS/MS chromatogram of standards (100pg/ml) and internal standard showing chromatographic separation (1. 8-iso-15(R)PGF<sub>2α</sub>; 2. 8-iso-PGF<sub>2α</sub>; 3. 11-PGF<sub>2α</sub>; 4. 15(R)-PGF<sub>2α</sub>, 5. 8-iso-PGF<sub>2α</sub>-d<sub>4</sub>).

**Fig. 5.** Stability of isoprostanes in EBC samples after six hours of storage at +20°C.



**Fig. 6.** Stability of isoprostanes in EBC samples after two weeks of storage at  $-80^{\circ}\text{C}$ ,  $+4^{\circ}\text{C}$  and  $+20^{\circ}\text{C}$ .

**Fig. 7.** LC-MS/MS chromatogram of a) student's EBC sample (concentration of isoprostanes  $\sim 15\text{pg/mL}$ ) showing chromatographic separation (1. 8-iso-15(R)-PGF<sub>2 $\alpha$</sub> ; 2. 8-iso-PGF<sub>2 $\alpha$</sub> ; 3. 11-PGF<sub>2 $\alpha$</sub> ; 4. 15(R)-PGF<sub>2 $\alpha$</sub> ), b) standards of four isoprostanes (100pg/ml) c) internal standard 8-iso-PGF<sub>2 $\alpha$</sub> -d<sub>4</sub>.

### Table Captions

**Table 1.** Calibration data of four isoprostanes obtained using column Agilent Extend C-18 (50mm $\times$ 2.1mm; 1,8 $\mu\text{m}$ ).

**Table 2.** Recoveries (%) and relative standard deviations RSD (%) obtained by LC-MS/MS analysis of EBC samples fortified with a standard solutions of four isoprostanes at three spiking levels (25, 100 and 250pg/mL).

**Table 3.** Intra-day repeatability and inter-day reproducibility of assay, analysed three times a day on three consecutive days.

**Table 4.** Stability of isoprostanes in water samples in different conditions of storage.

Analyte	Curve equation	$R^2$	LOD	LOQ
8,15-isoP	$y=0.0043x+0.109$	0.999	2	7
8-isoP	$y=0.00493x+0.0836$	0.999	1	4
11-isoP	$y=0.00337x+0.0364$	0.999	5	17
15-isoP	$y=0.00415x+0.000862$	0.999	3	10

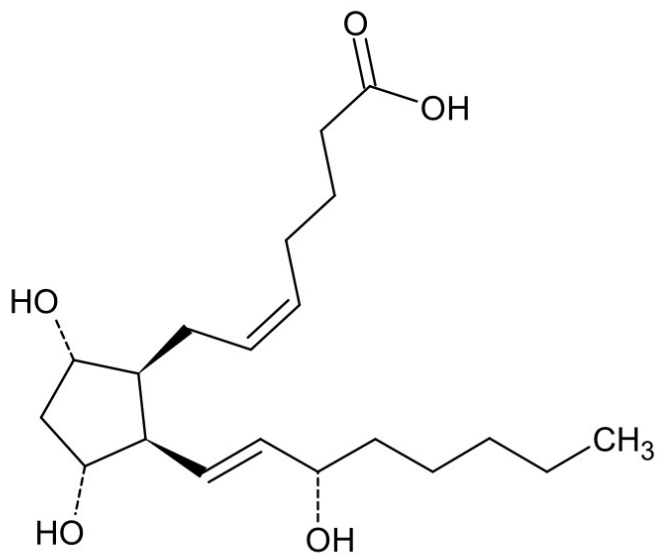


Compound	Recovery (%) of three concentration (RSD) (n=3)		
	25 pg/mL	100 pg/mL	250 pg/mL
8,15-isoP	97.0(4.7)	101.7(3.5)	98.8(2.1)
8-isoP	98.5(2.5)	98.9(3.6)	97.7(1.9)
11-isoP	100.6(6.6)	98.9(2.2)	98.4(2.5)
15-isoP	96.7(6.3)	99.6(2.8)	97.9(1.9)

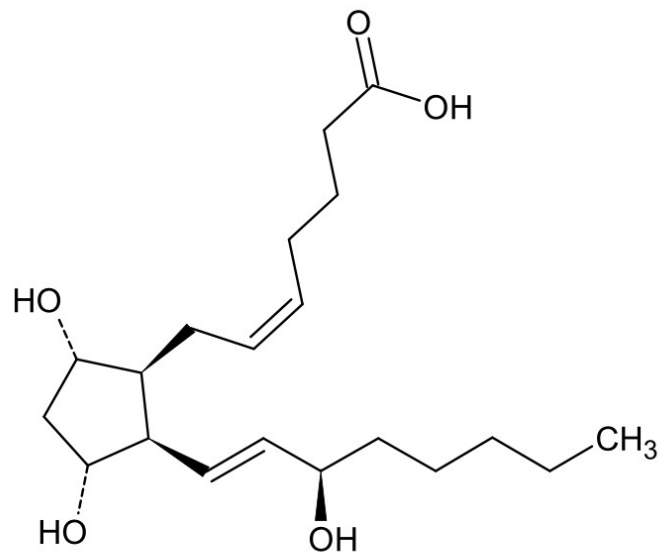
Analyte	Recovery (%) ( RSD) (n=3)			Inter-day
	Intra-day (n=3)			
	Day 1	Day 2	Day 3	
8,15-isoP	98.3(9.4)	101.7(8.3)	92.0(7.8)	97.3(4.9)
8-isoP	101.3(9.0)	104.8(8.0)	95.9(9.0)	100.6(4.5)
11-isoP	100.6(7.2)	104.1(7.5)	93.5(6.4)	99.4(5.4)
15-isoP	100.9(7.2)	103.8(6.7)	92.7(7.5)	99.1(5.8)

Time	Recovery (%) ± RSD (%) (n=4)			
Stability in water sample	8,15-isoP	8-isoP	11-isoP	15-isoP
Temperature -80°C				
0	100 ± 5.8	100 ± 7.8	100 ± 8.9	100 ± 2.5
7 days	98 ± 6.3	100 ± 3.2	96 ± 1.7	100 ± 3.8
14 days	98 ± 2.9	96 ± 5.2	96 ± 3.0	99 ± 7.1
21 days	93 ± 5.3	99 ± 6.9	94 ± 5.4	102 ± 1.0
28 days	88 ± 3.2	89 ± 6.6	88 ± 2.7	90 ± 4.1
35 days	92 ± 6.2	98 ± 6.8	93 ± 8.9	96 ± 2.2
42 days	95 ± 6.0	95 ± 4.6	97 ± 2.9	91 ± 5.3
49 days	97 ± 2.7	97 ± 2.6	92 ± 8.1	97 ± 2.3
56 days	94 ± 4.0	93 ± 2.6	89 ± 1.5	92 ± 4.6
Temperature -20°C				
0	100 ± 5.8	100 ± 7.8	100 ± 8.9	100 ± 2.5
7 days	99 ± 2.4	99 ± 3.2	99 ± 5.0	97 ± 0.3
14 days	93 ± 4.6	97 ± 1.6	91 ± 2.1	97 ± 5.2
21 days	95 ± 4.8	93 ± 1.9	95 ± 1.8	92 ± 3.8
28 days	90 ± 3.2	92 ± 1.8	92 ± 3.8	95 ± 6.0
35 days	92 ± 5.0	96 ± 7.4	100 ± 7.1	99 ± 10.6
42 days	96 ± 4.2	96 ± 4.2	96 ± 4.2	96 ± 4.2
49 days	94 ± 5.3	97 ± 4.4	95 ± 9.7	96 ± 2.8
56 days	91 ± 1.6	94 ± 4.8	90 ± 2.4	91 ± 6.2
Temperature +4°C				
0	100 ± 5.8	100 ± 7.8	100 ± 8.9	100 ± 3.4
7 days	93 ± 3.1	93 ± 2.7	94 ± 4.2	100 ± 2.9
14 days	92 ± 4.8	93 ± 2.4	91 ± 1.4	97 ± 5.8
21 days	83 ± 6.8	86 ± 3.9	87 ± 2.7	82 ± 5.4
28 days	88 ± 0.2	93 ± 3.0	92 ± 4.0	92 ± 8.8

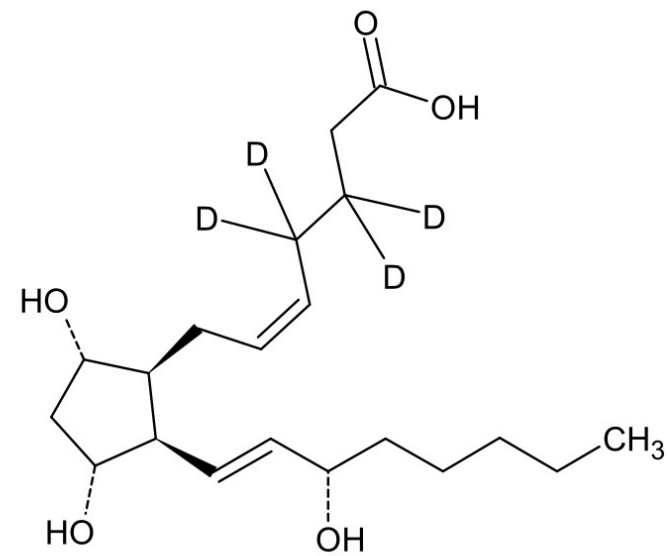
35 days	96 ± 1.5	101 ± 11.1	98 ± 8.5	98 ± 7.9
42 days	95 ± 1.7	95 ± 1.7	95 ± 1.7	95 ± 1.7
49 days	94 ± 1.1	94 ± 3.0	93 ± 4.3	100 ± 3.6
56 days	93 ± 0.3	96 ± 8.1	93 ± 4.2	95 ± 3.4
Temperature +20°C				
0	100 ± 5.8	100 ± 7.8	100 ± 8.9	100 ± 2.1
7 days	94 ± 3.1	94 ± 2.4	95 ± 4.6	99 ± 1.5
14 days	93 ± 5.5	91 ± 2.4	91 ± 4.9	94 ± 2.6
21 days	80 ± 6.9	83 ± 1.6	80 ± 1.5	80 ± 3.0
28 days	85 ± 0.4	96 ± 1.8	88 ± 0.4	90 ± 0.3
35 days	95 ± 1.5	97 ± 2.0	98 ± 1.1	100 ± 1.4
42 days	95 ± 2.3	95 ± 2.3	95 ± 2.3	95 ± 2.3
49 days	93 ± 0.9	95 ± 0.3	87 ± 0.7	93 ± 0.9
56 days	91 ± 0.7	92 ± 1.1	88 ± 0.3	95 ± 0.7



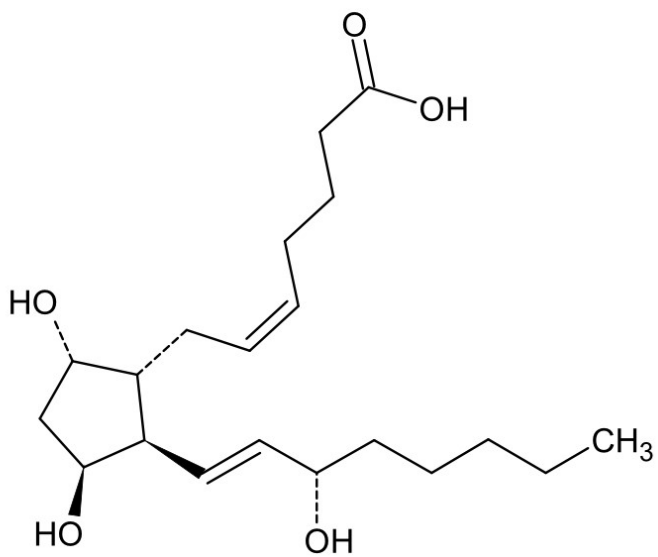
8-iso-PGF<sub>2</sub>α



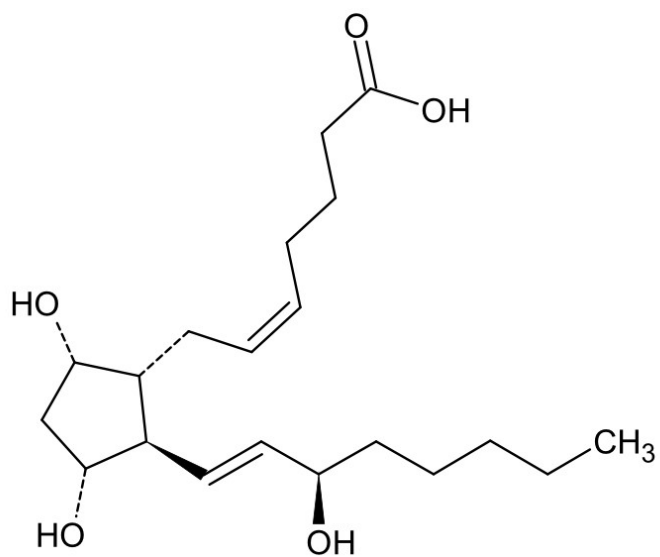
8-iso-15(R)-PGF<sub>2</sub>α



8-iso-PGF<sub>2</sub>α-d<sub>4</sub>



11 $\beta$ -PGF<sub>2</sub>α



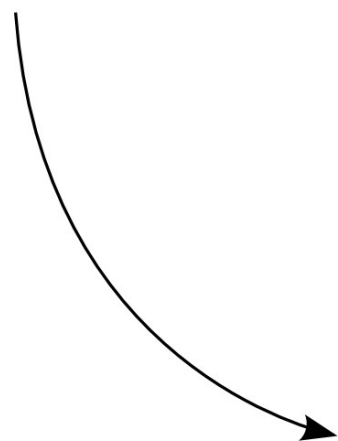
15(R)-PGF<sub>2</sub>α



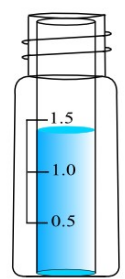
Collection of EBC



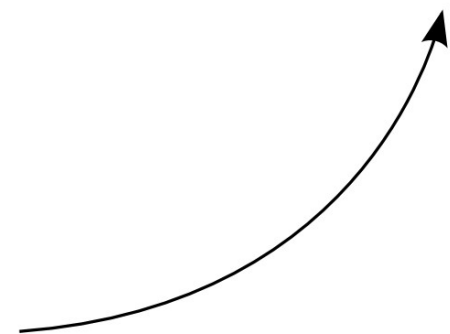
Analysis

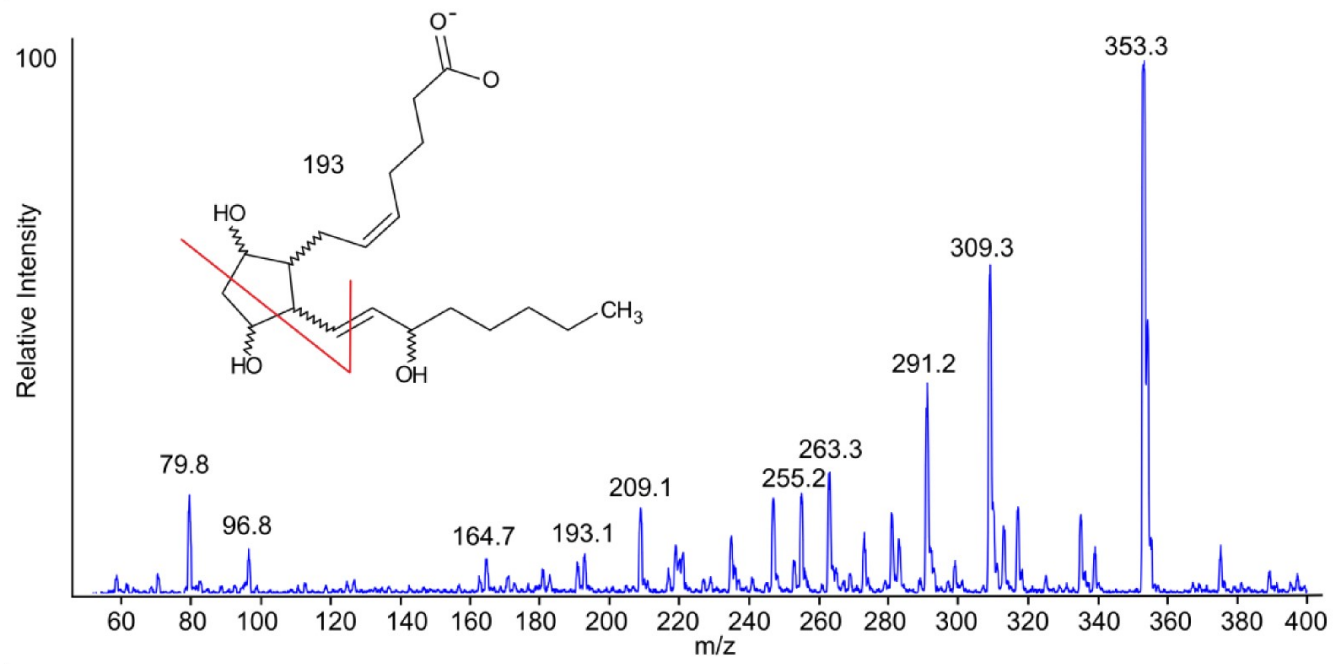
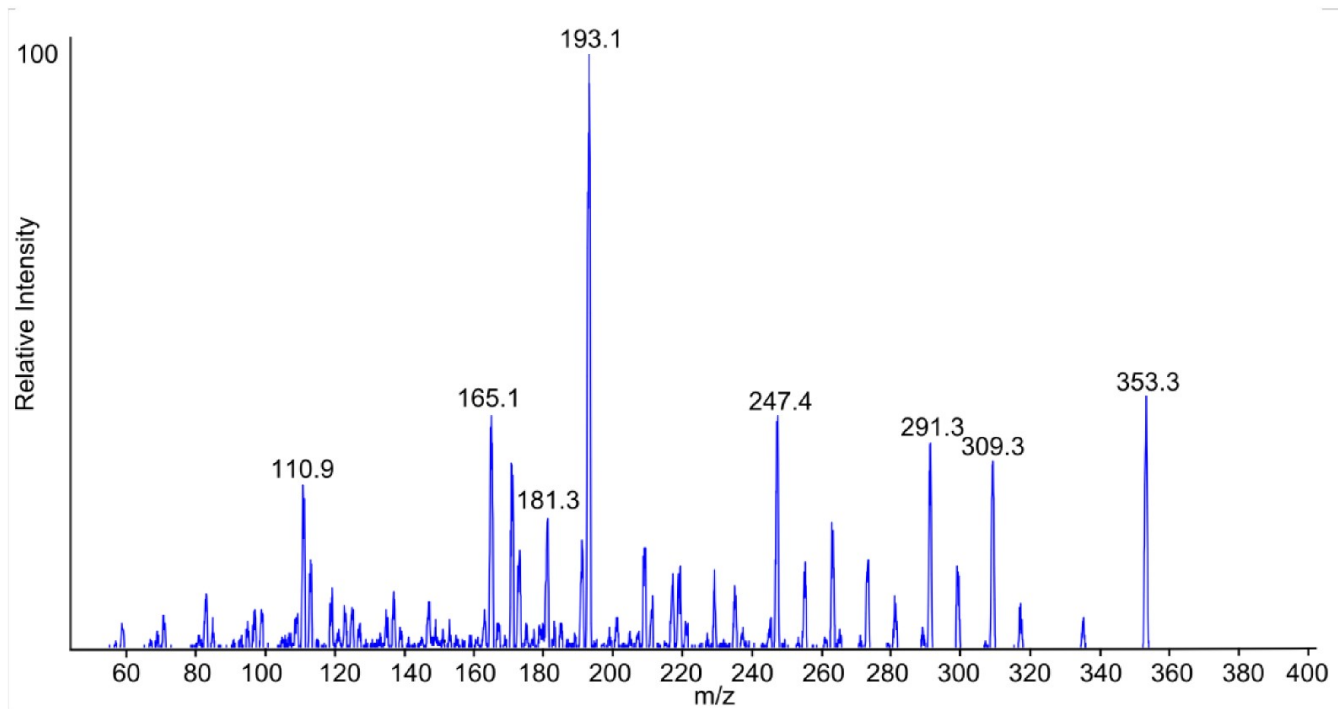


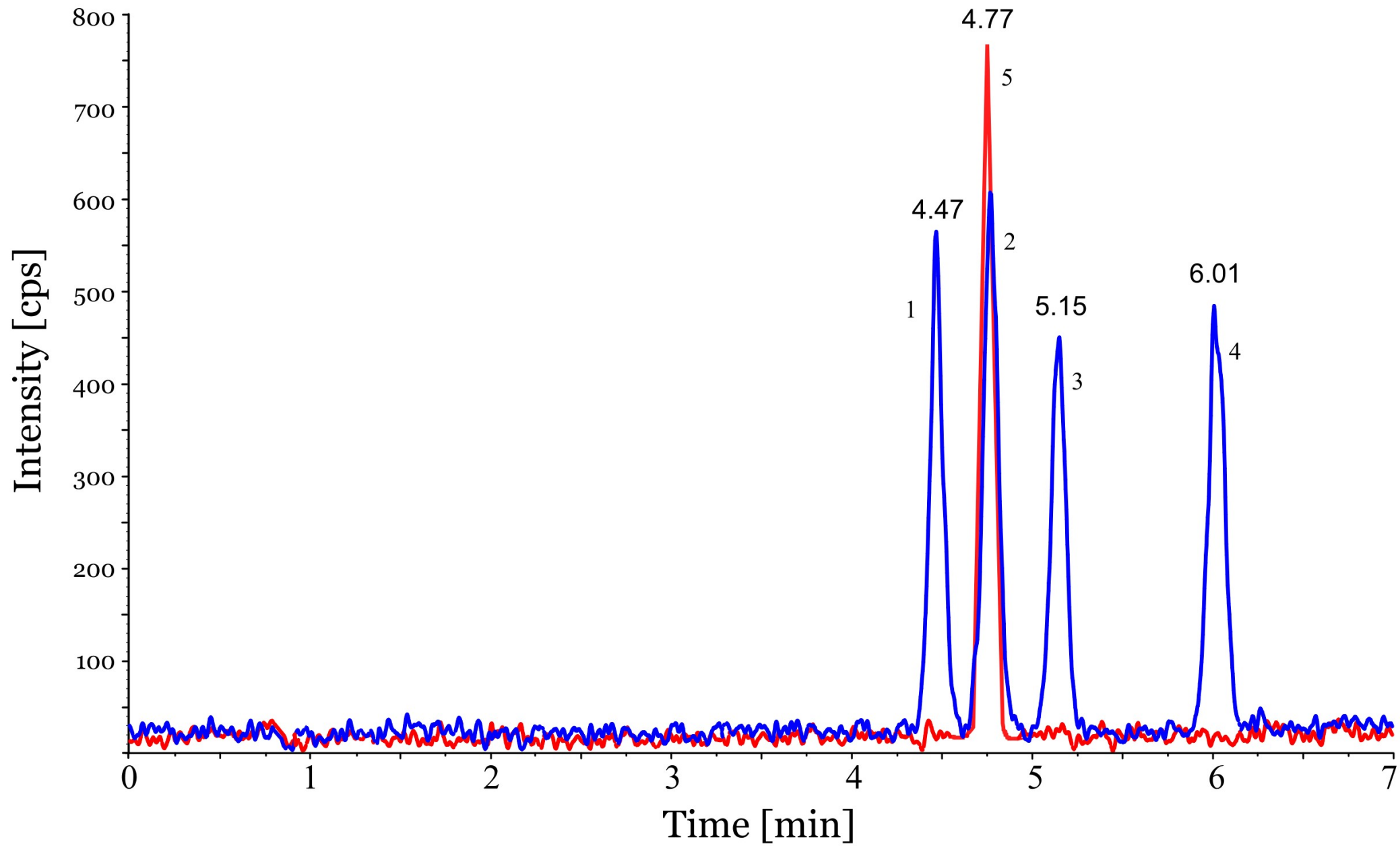
Addition of internal standard and lyophilization

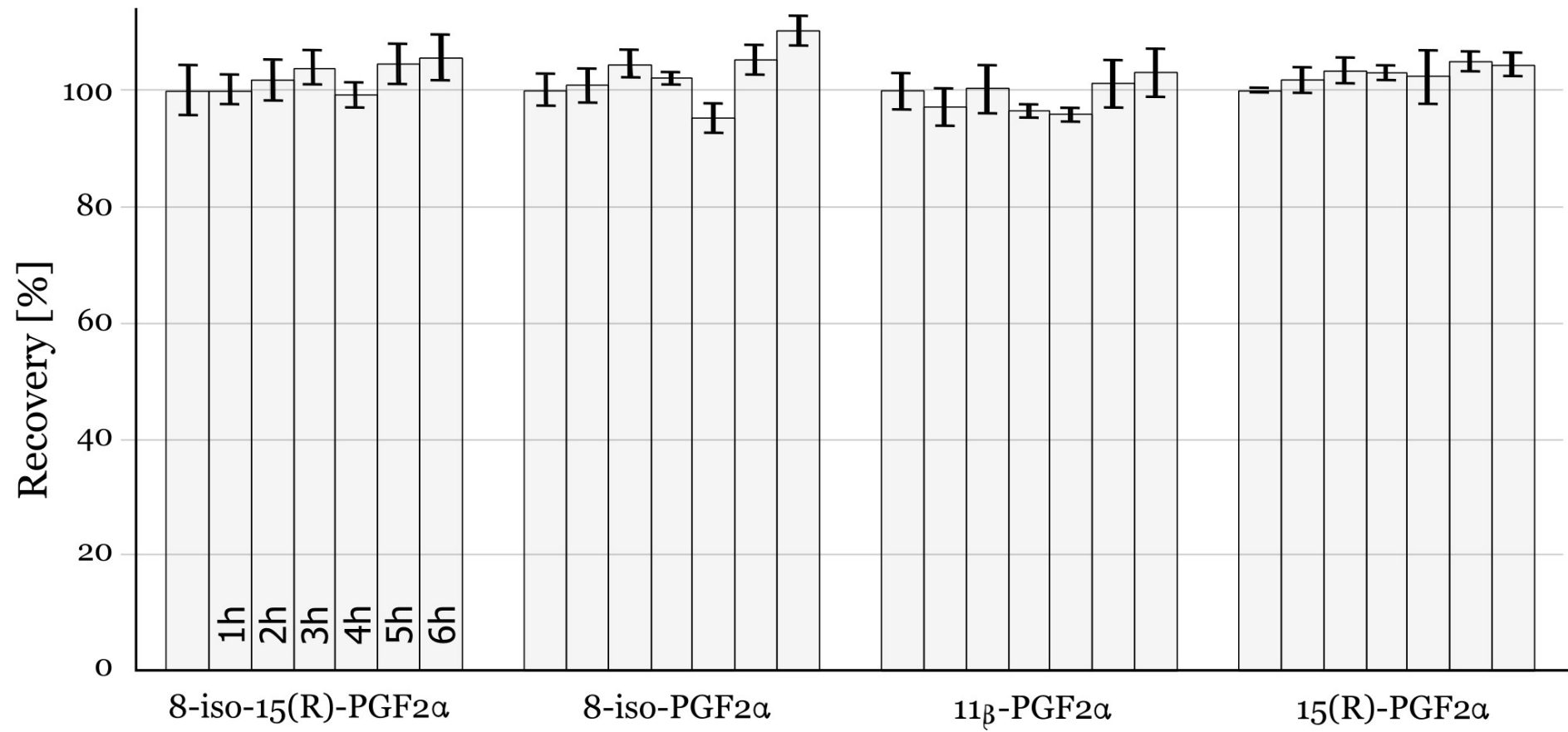


Redissolution in mobile phase

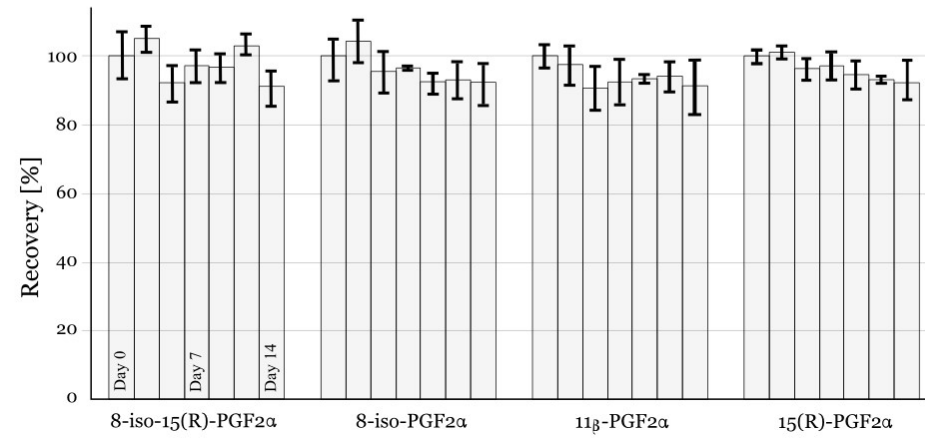


**A)****B)**

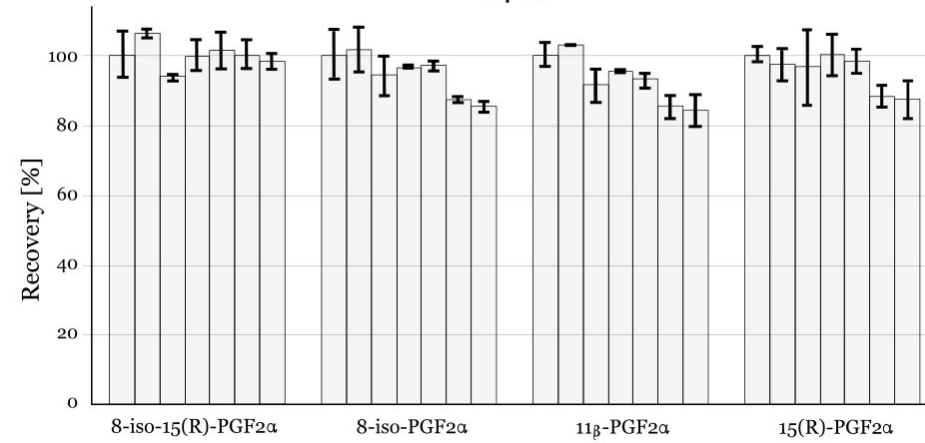




-80°C



+4°C



+20°C

