methods

Control of matrix effects in the analysis of urinary F_2-isoprostanes using novel multidimensional solid-phase extraction and LC-MS/MS

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Abstract **F_2-isoprostanes (F_2-iPs), established markers of oxidative stress, exist as four sets of regioisomers. Simultaneous and specific determination of F_2-iPs can be achieved by liquid chromatography-tandem mass spectrometry (LC-MS/MS). We developed novel methods for urine sample preparation and HPLC to control matrix-related ion suppression effects in the LC-MS/MS analysis of F_2-iPs. A selective solid-phase extraction (SPE) wash protocol was developed with an Oasis HLB (hydrophilic-lipophilic balance) SPE cartridge using an elution profile of [^{3}H]8-iso-prostaglandin (PG)$F_{2\alpha}$ ($iPF_{2\alpha}$-III) when the methanol concentration was increased under acidic, neutral, and base wash conditions. A multidimensional (MD)-SPE method that incorporated size exclusion, reverse-phase chromatography, and normal-phase chromatography was developed using an Oasis HLB SPE cartridge and an HLB μElution SPE plate. Average extraction recoveries of the deuterated internal standards of $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI were 62 ± 8% and 60 ± 10%. A buffer-free HPLC method for the separation of F_2-iP isomers was developed on base-deactivated C8 columns. Average matrix effects for $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI were 95 ± 6% and 103 ± 5%. The clean extraction of urine F_2-iPs using MD-SPE and the separation of F_2-iP isomers using a novel HPLC method did not cause apparent ion suppression in the analysis of $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI using LC-MS/MS. These findings should be useful for establishing a routine LC-MS/MS method for the analysis of F_2-iPs.—**Zhang, B., and K. Saku. **Control of matrix effects in the analysis of urinary F_2-isoprostanes using novel multidimensional solid-phase extraction and LC-MS/MS.** ***J. Lipid Res.*** **2007.** 48: **733–744.**

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Isoprostanes (iPs) are products of the free radical-initiated autoxidation of arachidonic acid (1). F_2-iPs are established markers for oxidative stress (2–4) and have been linked to cardiovascular diseases and risk factors (5). In addition, some F_2-iPs exert potent biological activity by acting as ligands for either plasma membrane-bound prostaglandin (PG) receptors or nuclear receptors (6–8). F_2-iPs are generated in situ esterified to phospholipids (9). Cleavage by phospholipase A_2 generates free F_2-iPs that are excreted in urine (10).

The measurement of F_2-iPs in biological samples presents several challenges. First, the methods used for measurement have to be specific, because F_2-iPs are isomers of F_2-PGs and exist as four sets of regioisomers (1, 11). In addition, the methods used for measurement have to be sensitive, because F_2-iPs exist at very low concentrations in biological samples (12–15).

F_2-iPs are usually measured noninvasively in urine. The 8-iso-$PGF_{2\alpha}$ [8-iso-15(S) $PGF_{2\alpha}$, also $iPF_{2\alpha}$-III] is the F_2-iP that has been studied most often. $iPF_{2\alpha}$-VI, a regioisomer of $iPF_{2\alpha}$-III, has been shown to be the more abundant F_2-iP (16). GC-MS methods are the "gold standard" for the measurement of F_2-iPs (13, 17, 18). GC-MS methods are highly sensitive but also laborious and are not sufficiently selective (13, 18). ELISA kits for measuring F_2-iPs are commercially available, but the simultaneous analysis of F_2-iP regioisomers is not possible. Selective and simultaneous measurements of different F_2-iPs could be important because they may have different biological activities and may be formed and metabolized differently under disease conditions that are linked to oxidative stress (19).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been developed for the measurement of F_2-iPs and their metabolites (1, 14, 15, 20). LC-MS/MS methods are specific, because HPLC can separate F_2-iP stereoisomers and electrospray ionization (ESI)-MS/MS can separate F_2-iP regioisomers. Also, LC-MS/MS methods are sensitive because the ESI-MS/MS technique permits the detection of very low levels of target analytes in the presence of a complex matrix background. However, LC-MS/MS is also associated with a major drawback when analyzing target analytes in biological fluids: the

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electrospray source is very susceptible to matrix-related ion suppression effects (21), and these matrix effects may vary from sample to sample (22).

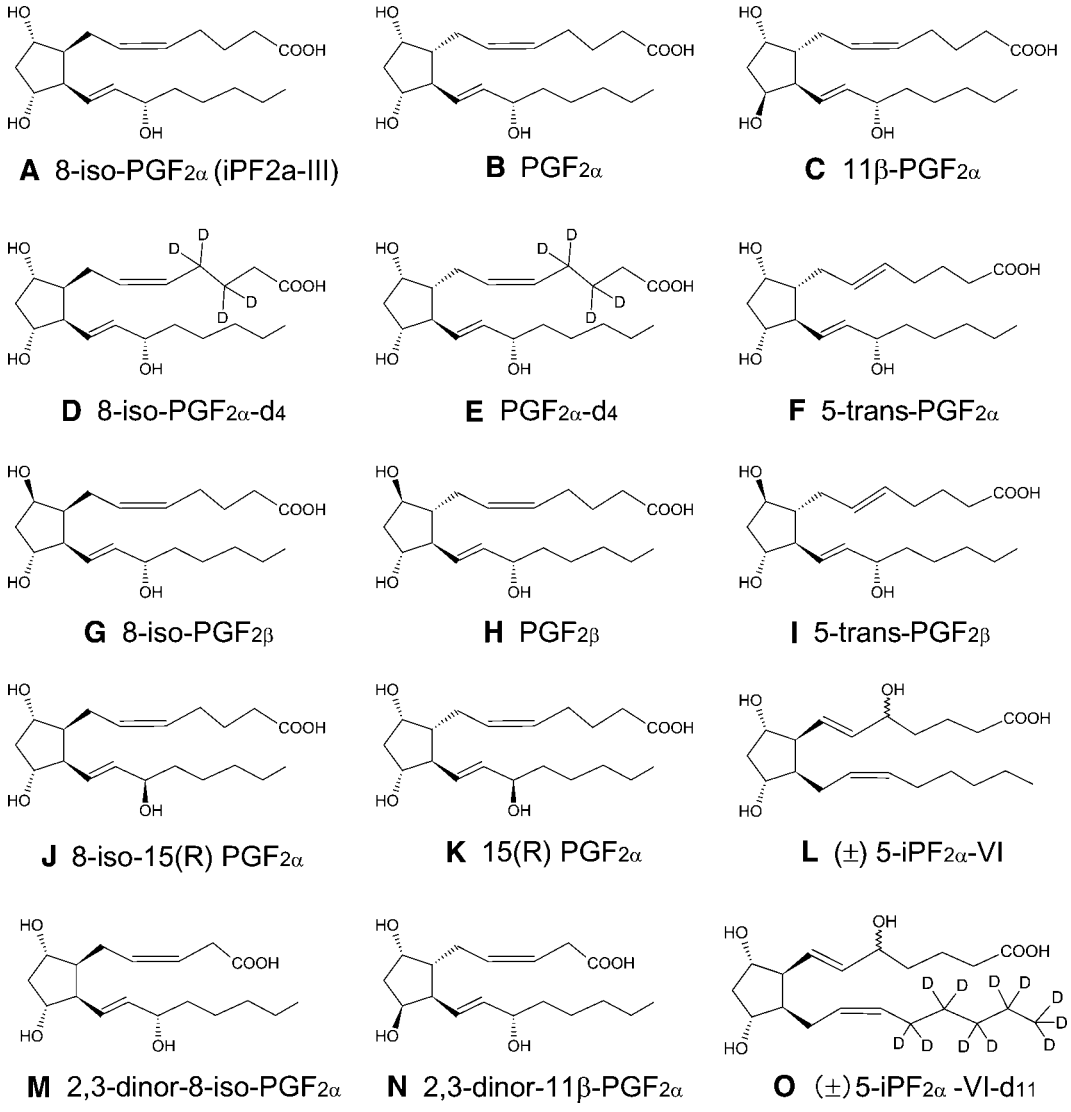
Stable isotope-labeled analogs, which are chemically and structurally the same as the target analytes but differ in molecular mass, have been used as internal standards (IS) to compensate for variations in injection, sample preparation, instrument parameter, and matrix effects. However, interference compounds that coelute with deuterated IS of 8-iso-$PGF_{2\alpha}$ have been shown to be contained in plasma samples prepared by solvent extraction (23). This gives inaccurate results, because target analytes and IS ratios are used to calculate analyte concentrations. This problem can be detected and circumvented by prolonged gradient HPLC separation (23). However, matrix-related compounds that coelute with the target analytes may also give inaccurate results, and these problems cannot be detected. Therefore, the clean extraction of biological samples is essential for the accurate and reproducible analysis of F_2-iPs using LC-MS/MS.

Matrix-related ion suppression effects have not been examined in previously reported LC-MS/MS methods for the analysis of F_2-iPs in biological samples (14, 15, 23, 24). Because the control of matrix effects is important for establishing LC-MS/MS as a routine technique for the measurement of F_2-iPs in biological samples, we developed a novel urine sample clean-up technique using multidimensional (MD)-solid-phase extraction (SPE) and a novel buffer-free HPLC separation method to minimize matrix- and buffer-additive-related ion suppression effects in the analysis of F_2-iPs using LC-MS/MS.

METHODS

Materials

All of the F_2-iPs and F_2-PG standards and deuterated IS of $iPF_{2\alpha}$-III, (±)5-$iPF_{2\alpha}$-VI, and $PGF_{2\alpha}$ shown in **Scheme 1** were obtained from Cayman Chemical (Ann Arbor, MI). Captiva Filter



Scheme 1. Structures of commercially available standards and deuterated internal standards (IS) of F_2-isoprostanes (iPFs) and F_2-prostaglandins (PGFs).

cartridges (0.2 μm) were obtained from Varian, Inc. (Palo Alto, CA). Oasis HLB (hydrophilic-lipophilic balance) SPE cartridges, Oasis MAX (mixed-mode anion exchange) SPE cartridges, Oasis MCX (mixed-mode cation exchange) SPE cartridges, and Oasis HLB μElution SPE plates were purchased from Waters Corp. (Milford, MA). Strata X SPE cartridges were obtained from Phenomenex (Torrance, CA). Methanol (MeOH), acetonitrile (ACN), and water were LC-MS grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan). All other reagents were HPLC grade.

Tandem mass spectrometry

F_2-iPs were detected with a Quattro Premier tandem mass spectrometer controlled with MassLynx version 4.1 software (Waters Corp.). Ionization was achieved using electrospray in the negative ESI mode. The position of the ESI probe and parameters of MS and MS/MS tuning were optimized for maximum sensitivity by infusing 200 ng/ml 8-iso-$PGF_{2\alpha}$ standard solution with a microsyringe pump (10 μl/min). The optimum tuning conditions for ESI were as follows: capillary voltage, 3 kV; source block temperature, 120°C; extractor, 3.0 V; radio frequency (RF) lens, 0.1 V; desolvation gas (nitrogen) heated to 400°C and delivered at a flow rate of 1,200 l/h; cone gas flow, 50 l/h; ion energy, 1.0; multiplier, 650 V; and low and high mass resolutions, 13. Entrance, collision, and exit for MS tuning were 50, 2, and 50 V, respectively, and entrance, exit, and collision gas (argon gas) flow for MS/MS tuning were −2 V, 2 V, and 0.35 ml/min, respectively.

A fully automated instrument mass calibration (static calibration, scanning calibration, and scan speed compensation) was performed using MassLynx version 4.1 software in the ES+ mode with MS tuning parameters (capillary voltage, 3 kV; cone voltage, 40 V; source block temperature, 80°C; extractor, 3.0 V; RF lens, 0.1 V; and desolvation gas heated to 150°C and delivered at a flow rate of 350 l/h) and the NAICS calibration reference file. Waters Atmospheric Pressure Ionization Calibration solution (NaCsI; catalog No. 700001593) was introduced using the instrument's syringe pump (250 μl) at a pump flow of 10 μl/min.

Nominal mass data acquisition parameters for 2,3-dinor-8-iso-$PGF_{2\alpha}$ (2,3-dinor-$iPF_{2\alpha}$-III), $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-III-d_4, $iPF_{2\alpha}$-VI, and $iPF_{2\alpha}$-VI-d_{11} were determined using flow injection (5 μl, 0.2 ml/min) (without the HPLC column) of standard solutions of the individual compounds (1,000 ng/ml). The mass-to-charge ratio (m/z) of the precursor ion of an individual compound was determined in the MS scan mode, and the sample cone voltage for maximum signal intensity of the deprotonated species $[M\text{-}H]^-$ was determined in the selected-ion reaction mode. Collision-induced dissociation of each deprotonated molecule was performed using MS/MS tuning. The most abundant daughter ion of F_2-iPs and deuterated IS was determined in the daughter scan mode, and the collision energy for maximum signal intensity of precursor-product ions was determined in the multiple reaction monitoring (MRM) mode. An interchannel delay time of 0.01 s, interscan delay time of 0.01 s, and dwell time of 0.1 s were used in data acquisition. The optimized cone voltage and collision energy for the individual compounds are given in **Fig. 1**.

Development of HPLC separation of isomers of F_2-iPs and F_2-PGs

Reverse-phase HPLC was performed using Waters Alliance 2796 and 2695 Separation Modules (Waters Corp.), which were interfaced directly with the mass spectrometer. Instrument control and data acquisition were carried out with MassLynx version 4.1 software. A two pump/four solvent system was used to make the gradient. Solvent A was water, solvent B was MeOH, and solvent C was ACN. ACE 3 μm C8 50 × 2.1 mm inner diameter (i.d.) columns (Advanced Chromatography Technologies, Aberdeen, Scotland) and Hypersil BDS 3 μm C8 50 × 2.1 mm i.d. columns (Thermo Electron Corp., Waltham, MA) were used for HPLC method development and the analysis of urine samples. The HPLC separation method was developed using the computer simulation software DryLab 2000 Plus (LC Resources, Walnut Creek, CA) (25, 26). The column was held at 24°C, and the mobile phase flow rate was 0.2 ml/min. The gradient program was as follows: 0–6 min, linear gradient from 21% to 40.5% B/C (B:C = 2:1, curve 1); 6–17 min, linear gradient from 40.5% to 43.5% B/C (B:C = 2:1, curve 6); 17–22 min, linear gradient from 43.5% to 100% B/C (B:C = 2:1, curve 1); and 22–27 min, linear gradient from 100% to 21% B/C (B:C = 2:1, curve 1).

Development of a selective SPE procedure

For the efficient development of a selective SPE procedure for the extraction of F_2-iPs in urine samples, [^{3}H]8-iso-$PGF_{2\alpha}$ (~10,000 cpm/ml; Cayman Chemical, Ann Arbor, MI) was added to acidified urine samples and extracted together with 8-iso-$PGF_{2\alpha}$ (3 ng/ml). Oasis SPE cartridges (3 cc/60 mg) were conditioned with 2 ml of MeOH and 2% formic acid, and SPE extraction was performed under vacuum using a Vac Elut SPS 24 Manifold (Varian, Inc.). The retention capacities of different Oasis SPE sorbents (i.e., HLB, MAX, and MCX) were examined by applying urine samples (1 ml each up to 10 ml) to the respective SPE cartridges. Two hundred microliters of wash waste and eluate were collected and counted for radioactivity in 3 ml of liquid scintillation solution (Clear-sol I; Nacalai Tesque, Inc., Kyoto, Japan) on a liquid scintillation counter (Tri-carb 2500; Packard Instrument Co., Meriden, CT).

A selective SPE wash procedure was developed by taking advantage of the differences in the elution profiles of F_2-iPs and sample matrix interference as a function of both the pH of the wash and the concentration of the elution solvents (27). The percentage of MeOH in acidic, neutral, and base washes was determined by washing the cartridges with 2% formic acid, water, and 2% NH_4OH solutions containing varying concentrations (10–100%) of MeOH, respectively, and collecting the waste for radioactivity counting. Elution volume was determined by eluting F_2-iPs with six 0.5 ml portions of diethyl ether-acetic acid (100:2) solvent and counting the radioactivity in the eluate.

Development of a MD-SPE method

An Oasis HLB cartridge (1 cc/30 mg) and an Oasis HLB μElution plate (750 μl/2 mg) were used to develop the MD-SPE method.

Two milliliters of centrifuged urine samples was mixed with one-fifth volume of MeOH and mixtures of IS solutions containing 8-iso-$PGF_{2\alpha}$-d_4, $PGF_{2\alpha}$-d_4, and (±)5-$iPF_{2\alpha}$-VI-d_{11} and put on ice for 30 min. The samples were adjusted to pH ≈ 3 with 1–2 ml of 1% formic acid before extraction. The HLB SPE cartridges were conditioned with 1 ml of MeOH and 1 ml of 5% MeOH/2% formic acid solution before use. The acidified and diluted urine samples were applied to a 0.2 μm Captiva Filter cartridge that was attached to the top of an Oasis HLB SPE cartridge using a Bond Elute adaptor (Varian, Inc.) with a 20 Port Vacuum Manifold (Agilent Technologies, Palo Alto, CA) at a flow rate of <3 ml/min. After sample application, filter cartridges were discarded and the SPE cartridge was washed with 1 ml of 5% MeOH. Subsequent wash and elution steps were continued using a Cerex System 48 Positive-Pressure Manifold (SPEware Corp., San Pedro, CA). Nitrogen gas was used for positive-pressure SPE.

Eluate from Oasis HLB SPE cartridges was applied to an Oasis μElution plate. SPE extraction with an Oasis μElution plate was

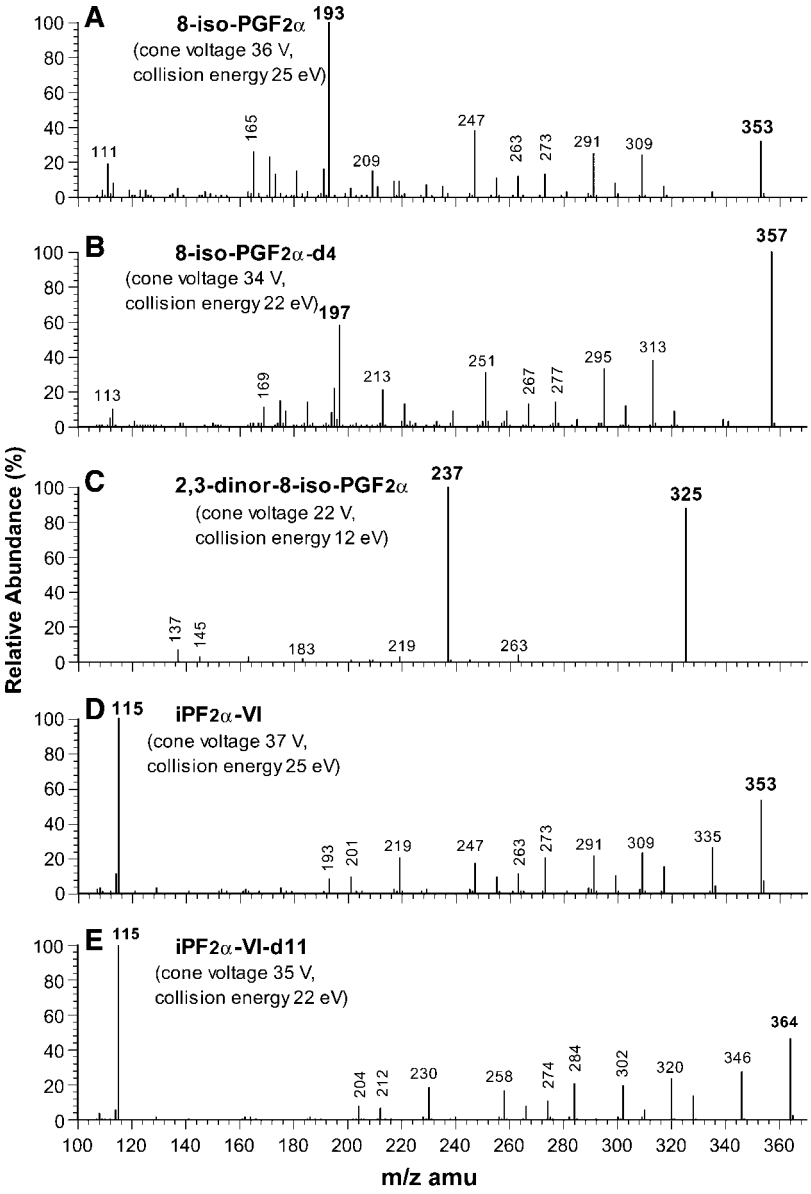


Fig. 1. Product ion spectra of 8-iso-$PGF_{2\alpha}$ (A), 8-iso-$PGF_{2\alpha}$-d_4 (B), 2,3-dinor-8-iso-$PGF_{2\alpha}$ (C), (±)5-$iPF_{2\alpha}$-VI (D), and (±)5-$iPF_{2\alpha}$-VI-d_{11} (E).

performed using a Plate SPE Extraction Manifold (Waters Corp.). Eluate from the Oasis μElution plate was diluted with water and analyzed directly using LC-MS/MS.

Assessment of extraction recovery and matrix-related ion suppression effects

Recovery in urine sample extraction was examined by adding the IS mixtures [8-iso-$PGF_{2\alpha}$-d_4 and (±)5-$iPF_{2\alpha}$-VI-d_{11}] to urine samples before and after extraction. Recovery (%) was calculated as peak area of IS spiked into urine samples before extraction/peak area of IS spiked into urine samples after extraction × 100 (28).

Ion suppression effects of sample matrices were examined by adding mixtures of standard solutions [2,3-dinor-8-iso-$PGF_{2\alpha}$, 15(R)-8-iso-$PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, and (±)5-$iPF_{2\alpha}$-VI] and deuterated IS solutions [8-iso-$PGF_{2\alpha}$-d_4 and (±)5-$iPF_{2\alpha}$-VI-d_{11}] to water and sample matrix, respectively. The matrix effect (%) was calculated as peak area of the standard or IS added into urine samples/peak area of the standard or IS added into water × 100 (28).

Data analysis

Peak-to-peak signal-to-noise ratios for $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI peaks on mass chromatograms were determined using MassLynx version 4.1 software after smoothing using the Moving Mean method [window size (scans) set at ±3, number of smoothings set at 2]. Significant differences in the signal-to-noise ratios of $iPF_{2\alpha}$-III or $iPF_{2\alpha}$-VI peaks determined from 1 and 2 ml urine samples were determined by the Wilcoxon sign-rank test using the SAS (Statistical Analysis System) software package (version 9.1; SAS Institute, Inc., Cary, NC) at Fukuoka University. QuanLynx software (Waters Corp.) was used for automated peak identification and the integration of peak area on the MRM chromatogram. The integrated area data for the analyzed samples were exported as text files and combined using a self-made

Excel macro. Recovery and matrix effects were calculated using the SAS software package.

RESULTS

MS/MS detection of F_2-iPs

Four groups of regioisomers of F_2-iPs [groups III (15-series), IV (8-series), V (12-series), and VI (5-series)] are formed from the oxidation of arachidonic acid (11). Groups III and VI F_2-iPs have been shown to be abundant F_2-iPs (16, 17). Scheme 1 shows the structures of commercially available standards of group III F_2-iPs [A, 8-iso-$PGF_{2\alpha}$; G, 8-iso-$PGF_{2\beta}$; J, 8-iso-15(R) $PGF_{2\alpha}$], group VI F_2-iPs [L, (±)5-$iPF_{2\alpha}$-VI], F_2-PG [B, $PGF_{2\alpha}$; C, 11β-$PGF_{2\alpha}$; F, 5-*trans*-$PGF_{2\alpha}$; H, $PGF_{2\beta}$; I, 5-*trans*-$PGF_{2\beta}$; K, 15(R) $PGF_{2\alpha}$], and metabolites of 8-iso-$PGF_{2\alpha}$ (M, 2,3-dinor-8-iso-$PGF_{2\alpha}$) and $PGF_{2\alpha}$ (N, 2,3-dinor-$PGF_{2\alpha}$). Scheme 1 also shows the structures of deuterated IS of $iPF_{2\alpha}$-III (D, 8-iso-$PGF_{2\alpha}$-d_4), $PGF_{2\alpha}$ (E, $PGF_{2\alpha}$-d_4), and $iPF_{2\alpha}$-VI [O, (±)5-$iPF_{2\alpha}$-VI-d_{11}].

Figure 1 shows the product ion spectra of F_2-iPs. Abundant molecular ions generated under the ES-mode were *m/z* 353 for 8-iso-$PGF_{2\alpha}$ (Fig. 1A) and (±)5-$iPF_{2\alpha}$-VI (Fig. 1D), *m/z* 357 for 8-iso-$PGF_{2\alpha}$-d_4 (Fig. 1B), *m/z* 325 for 2,3-dinor-8-iso-$PGF_{2\alpha}$ (Fig. 1C), and *m/z* 364 for (±)5-$iPF_{2\alpha}$-VI-d_{11} (Fig. 1E). Product ions at *m/z* 193 (Fig. 1A) and *m/z* 115 (Fig. 1D) were the most abundant product ions and were specific for 8-iso-$PGF_{2\alpha}$ and $iPF_{2\alpha}$-VI, respectively. The most abundant product ions were *m/z* 197 for 8-iso-$PGF_{2\alpha}$-d_4 (Fig. 1B), *m/z* 237 for 2,3-dinor-8-iso-$PGF_{2\alpha}$ (Fig. 1C), and *m/z* 115 for $iPF_{2\alpha}$-VI-d_{11} (Fig. 1E). Therefore, precursor/product ion pairs for the detection of F_2-iPs under MRM mode were determined to be as follows: 8-iso-$PGF_{2\alpha}$ *m/z* 353/193 for 8-iso-$PGF_{2\alpha}$, *m/z* 357/197 for 8-iso-$PGF_{2\alpha}$-d_4, *m/z* 325/237 for 2,3-dinor-8-iso-$PGF_{2\alpha}$, *m/z* 353/115 for $iPF_{2\alpha}$-VI, and *m/z* 364/115 for $iPF_{2\alpha}$-VI-d_{11}.

8-Iso-$PGF_{2\alpha}$ and $PGF_{2\alpha}$ showed similar fragmentation patterns in the product ion spectra (data not shown). Therefore, the separation of 8-iso-$PGF_{2\alpha}$ from $PGF_{2\alpha}$ isomers is necessary for the specific determination of 8-iso-$PGF_{2\alpha}$.

Development of a reverse-phase HPLC method for the separation of F_2-iP isomers

As a first step in the development of a novel HPLC method, experiments were performed to determine the composition of the mobile phase using an ACE 3 μm C8 50 × 2.1 mm i.d. column. Because we found that the ionization efficiency of 8-iso-$PGF_{2\alpha}$ was higher in MeOH than in ACN (data not shown), the separation of isomers of F_2-iPs began with MeOH, and the proportion of ACN was increased to increase the selectivity. The resolution of four isomers, 15(R) $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-III, 15(R) $PGF_{2\alpha}$, and $PGF_{2\alpha}$, was increased when the proportion of ACN to MeOH was increased, and enough separation was obtained when the proportion of ACN to MeOH was increased to 1:2 (data not shown).

Because ammonium acetate is the common buffer additive used in the analysis of 8-iso-$PGF_{2\alpha}$ by LC-MS/MS (1, 14, 15), we examined the effects of ammonium acetate on the ionization efficiency of 15(R) $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-III, 15(R) $PGF_{2\alpha}$, and $PGF_{2\alpha}$ by adding ammonium acetate solutions (pH 6.8) to the standard solutions. Surprisingly, the ion intensities of all four isomers were greatly reduced when even low concentrations (0.4 and 2 mM) of ammonium acetate were present in the standard solutions (data not shown). Ammonium acetate decreased the retention of all four isomers but did not affect the separation (data not shown).

The effects of pH on the separation of isomers were examined by analyzing mixtures of four standard solutions prepared with water and 0.001, 0.01, and 0.1% acetic acid, respectively. Retention of the four isomers increased with an increase in the concentration of acetic acid, but separation was not affected. Therefore, the following HPLC method was developed under neutral pH without buffer additives.

The optimum temperature and gradient conditions for separation of the four isomers were determined using the computer simulation software DryLab 2000 Plus. Four experiments were performed with simultaneous changes in temperature (20°C and 40°C) and gradient steepness [gradient time, 6 and 18 min; gradient, 40–90% MeOH/ACN (2:1)]. The computer simulation indicated that better separation can be achieved under a shallow gradient condition and at lower temperature.

Finally, to optimize peak shape and separation under neutral conditions, C8 columns from different makers were screened for the separation of standard solutions of six $PGF_{2\alpha}$ isomers: 8-iso-15(R) $PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, 11β-$PGF_{2\alpha}$, 15(R) $PGF_{2\alpha}$, 5-*trans*-$PGF_{2\alpha}$, and $PGF_{2\alpha}$. A Hypersil BDS 3 μm C8 50 × 2.1 mm i.d. column was chosen as the analysis column based on the symmetric peak, good separation, and slightly stronger retention of F_2-iP isomers than with the ACE C8 column (data not shown).

Figure 2 shows LC-MS/MS results with the standard solutions of F_2-iPs and F_2-PGs. As shown in Fig. 2A, $iPF_{2\alpha}$-III (8-iso-$PGF_{2\alpha}$) was baseline-separated from 15(R) $iPF_{2\alpha}$-III [8-iso-15(R) $PGF_{2\alpha}$], 11β-$PGF_{2\alpha}$, 15(R) $PGF_{2\alpha}$, 5-*trans*-$PGF_{2\alpha}$, and $PGF_{2\alpha}$. As shown in Fig. 2B, 8-iso-$PGF_{2\beta}$ and $PGF_{2\beta}$ were coeluted with $iPF_{2\alpha}$-III and 5-*trans*-$PGF_{2\beta}$ was coeluted with 11β-$PGF_{2\alpha}$. Other stereoisomer pairs (Fig. 2C–F), $iPF_{2\alpha}$-III-d_4 (8-iso-$PGF_{2\alpha}$-d_4) and $PGF_{2\alpha}$-d_4, 2,3-dinor-8-iso-$PGF_{2\alpha}$ and 2,3-dinor-$PGF_{2\alpha}$, $iPF_{2\alpha}$-VI and 5-epi-$iPF_{2\alpha}$-VI, and $iPF_{2\alpha}$-VI-d_{11} and 5-epi-$iPF_{2\alpha}$-VI-d_{11}, were all baseline-separated from each other. As also shown in Fig. 2, not only baseline separation but also sharp and symmetric peaks were achieved using the Hypersil BDS 3 μm C8 column.

Development of a selective SPE wash procedure

Because F_2-iPs are weakly acidic, weakly polar, hydrophobic compounds, three packings of polymer-based sorbents, Oasis HLB, MAX, and MCX, were screened for their ability to retain the analytes in urine samples. **Figure 3A** shows the breakthrough curve of [^{3}H]8-iso-$PGF_{2\alpha}$ added to a urine sample for Oasis HLB, MAX, and MCX SPE cartridges (3 cc/60 mg). As shown, [^{3}H]8-iso-$PGF_{2\alpha}$ in up to 10 ml urine sample was consistently retained by Oasis HLB cartridges but not by Oasis MAX or MCX cartridges. This result indicates that Oasis HLB had the greatest

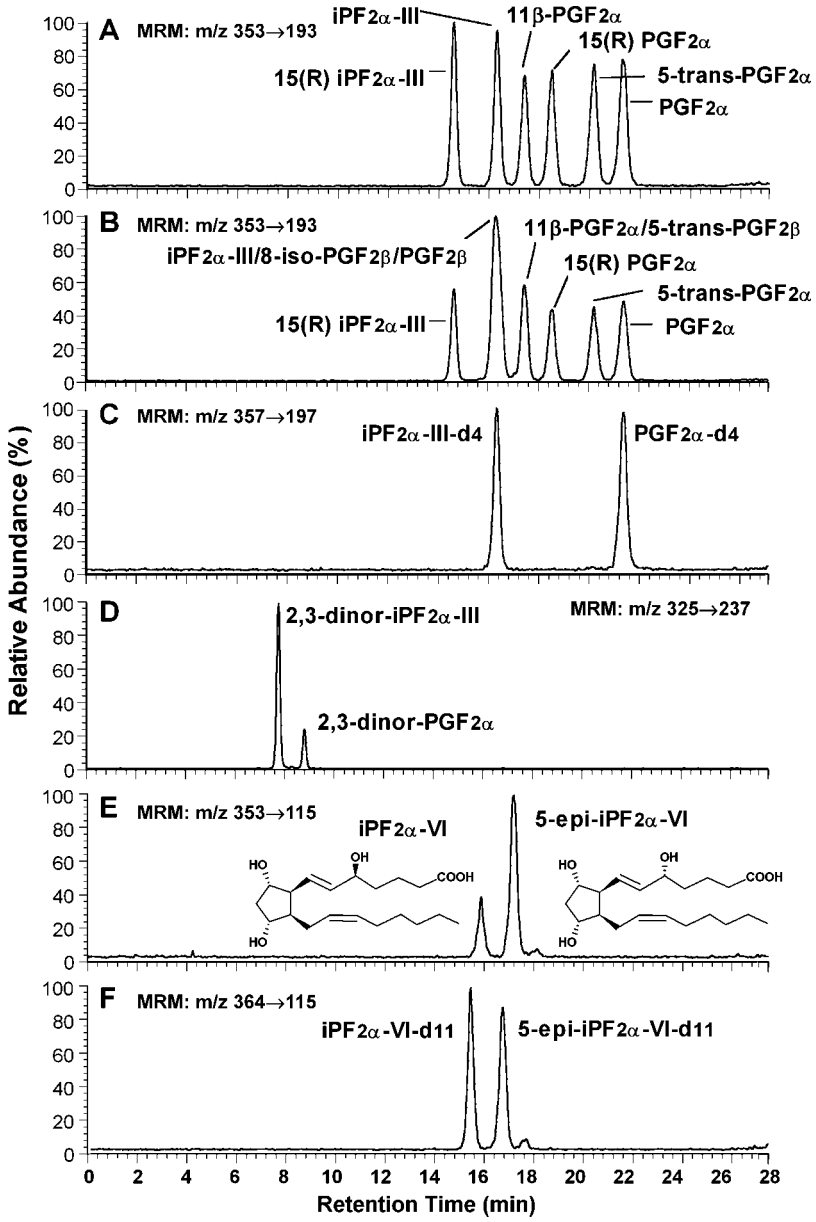


Fig. 2. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) results of the standard solutions containing $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI isomers and deuterated IS detected under multiple reaction monitoring (MRM) mode. A, B: Ion channel of *m/z* 353 → 193. Peaks are 8-iso-15(R) $PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$ ($iPF_{2\alpha}$-III), 11β-$PGF_{2\alpha}$, 15(R) $PGF_{2\alpha}$, 5-*trans*-$PGF_{2\alpha}$, $PGF_{2\alpha}$, 8-iso-$PGF_{2\beta}$ (9β-8-iso-$PGF_{2\alpha}$), $PGF_{2\beta}$ (9β-$PGF_{2\alpha}$), and 5-*trans*-$PGF_{2\beta}$ (9β-5-*trans*-$PGF_{2\alpha}$). C: Ion channel of *m/z* 357 → 197. Peaks are 8-iso-$PGF_{2\alpha}$-d_4 ($iPF_{2\alpha}$-III-d_4) and $PGF_{2\alpha}$-d_4. D: Ion channel of *m/z* 325 → 237. Peaks are 2,3-dinor-$iPF_{2\alpha}$-III and 2,3-dinor-$PGF_{2\alpha}$. E: Ion channel of *m/z* 353 → 115. Peaks are $iPF_{2\alpha}$-VI and 5-epi-$iPF_{2\alpha}$-VI. F: Ion channel of *m/z* 364 → 115. Peaks are $iPF_{2\alpha}$-VI-d_{11} and 5-epi-$iPF_{2\alpha}$-VI-d_{11}.

retention capacity; therefore, it was used in the following SPE method development.

To develop a selective SPE procedure, we examined the retention behavior of [^{3}H]8-iso-$PGF_{2\alpha}$ when the percentage of MeOH was increased from 0% in 10% increments at acidic pH (with 2% formic acid), neutral pH (with water), and alkaline pH (with 2% NH_4OH) washes. As shown in Fig. 3B, [^{3}H]8-iso-$PGF_{2\alpha}$ was retained until the content of MeOH was increased to 50% under acidic and neutral wash conditions, but it was only retained when the content of MeOH was <20% under basic wash conditions. This result indicates that the elution profile of [^{3}H]8-iso-$PGF_{2\alpha}$ as a function of MeOH concentration was different at low and high pH. According to these results, a selective SPE procedure was developed and incorporated into MD-SPE.

MD-SPE of urine samples for LC-MS/MS analysis

Figure 3C shows the MD-SPE method developed for the extraction of F_2-iPs in urine. First, an acidified (pH ≈ 3) urine sample containing 10% MeOH was applied to an

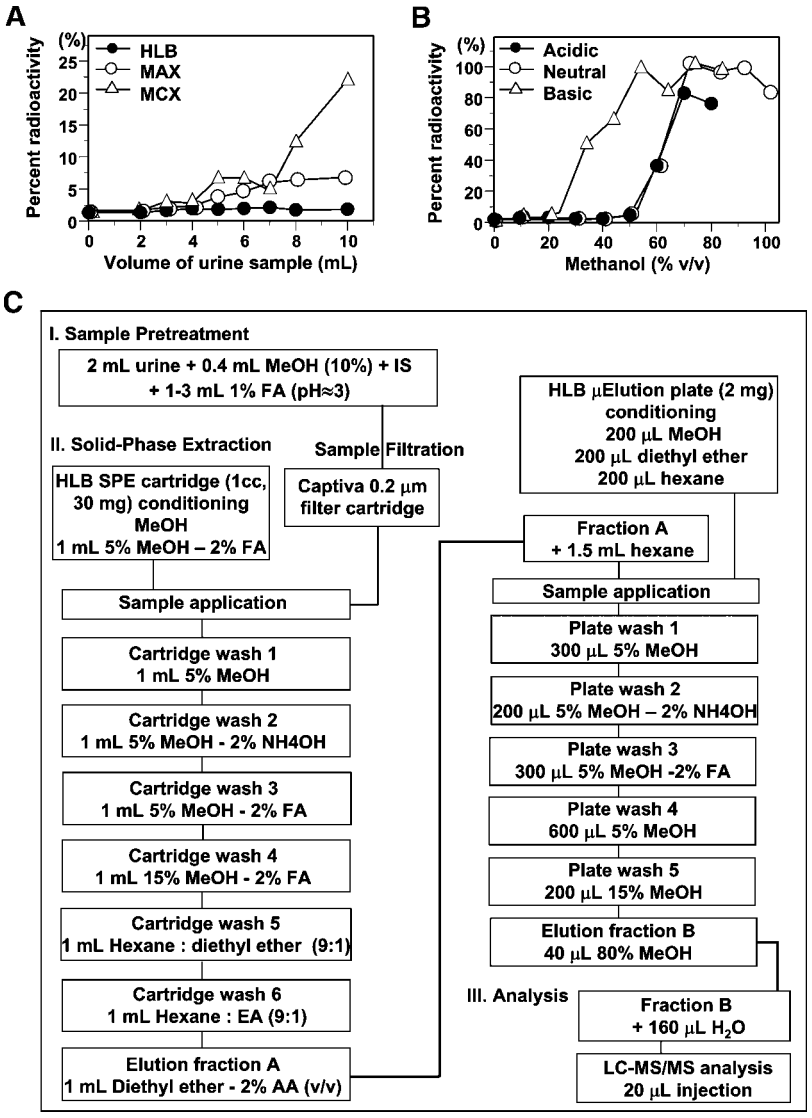


Fig. 3. A: Breakthrough curves of [^{3}H]8-iso-PGF$_{2\alpha}$ in urine samples for Oasis HLB (hydrophilic-lipophilic balance), MAX (mixed-mode anion exchange), and MCX (mixed-mode cation exchange) solid-phase extraction (SPE) cartridges (3 cc/60 mg). B: Wash-eluate profile of [^{3}H]8-iso-PGF$_{2\alpha}$ in urine samples when the concentration of methanol (MeOH) increased under acidic [2% formic acid (FA)], neutral (water), and base (2% NH_4OH) wash conditions for Oasis HLB SPE cartridges. C: Selective SPE protocol and multi-dimensional (MD)-SPE method. AA, arachidonic acid. EA, ethyl acetate.

HLB cartridge and washed with 5% MeOH. In this sample application and cartridge wash step 1 (Fig. 3C), high-molecular-weight sample components (such as proteins) were size-excluded because of the small pore diameter (8 nm) of the sorbent, and salts and other polar interference such as carbohydrates were not retained by reverse-phase chromatography. In cartridge wash step 2 (base wash), acidic, moderately polar, hydrophobic interference was washed off using 5% MeOH with 2% NH_4OH (Fig. 3C). The elution of yellow interference from urine samples was observed in this step. In cartridge wash step 3, pH was switched to acidic by washing with 5% MeOH and 2% formic acid (Fig. 3C). In cartridge wash step 4, basic, moderately polar, hydrophobic interference was washed off using 15% MeOH with 2% formic acid. In cartridge wash steps 5 and 6, neutral interference was washed off with hexane-diethyl ether (9:1) and hexane-ethyl acetate (9:1), respectively (Fig. 3C). In elution fraction step A, the fatty acid fraction was selectively eluted with diethyl ether with 2% acetic acid (Fig. 3C). Visible yellow interference remained on the SPE cartridges.

Eluted fraction A from the HLB SPE cartridge was applied to the HLB µElution plate after dilution with hexane (Fig. 3C). In this step, F_2-iPs were retained on the SPE sorbent by normal-phase chromatography. In plate wash step 1, acetic acid was washed off with 5% MeOH (Fig. 3C). In plate wash step 2 (base wash), yellow interference was washed off with 5% MeOH and 2% NH_4OH. In plate wash steps 3 and 4, the pH was switched to acidic by washing with 5% MeOH and 2% formic acid, and formic acid was

subsequently washed off with 5% MeOH (Fig. 3C). In plate wash step 5, moderately polar, hydrophobic interference was washed off with 15% MeOH. Finally, in elution fraction step B, F_2-iP fractions were eluted with 80% MeOH. The final eluted fraction was clear and used directly for LC-MS/MS analysis after dilution with water.

Analysis of urine extracts using LC-MS/MS

Figure 4 shows MD-SPE LC-MS/MS results for a urine sample detected under MRM mode. As shown in Fig. 4A, 8-iso-15(R) $PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ were baseline-separated from $PGF_{2\alpha}$ and other unknown isomers. 8-Iso-15(R) $PGF_{2\alpha}$, 8-iso-$PGF_{2\alpha}$, 15(R) $PGF_{2\alpha}$, and $PGF_{2\alpha}$ were identified by adding mixtures of the standard solutions to the extracted sample (Fig. 4B). Figure 4C shows deuterium-labeled 8-iso-$PGF_{2\alpha}$ (8-iso-$PGF_{2\alpha}$-d_4) and $PGF_{2\alpha}$ ($PGF_{2\alpha}$-d_4) extracted from the urine sample. Extraction of blank sample (not spiked with deuterated IS) indicated that no interference was coeluted with 8-iso-$PGF_{2\alpha}$-d_4 and $PGF_{2\alpha}$-d_4 (Fig. 4D). Figure 4E shows that 2,3-dinor-$iPF_{2\alpha}$-III was baseline-

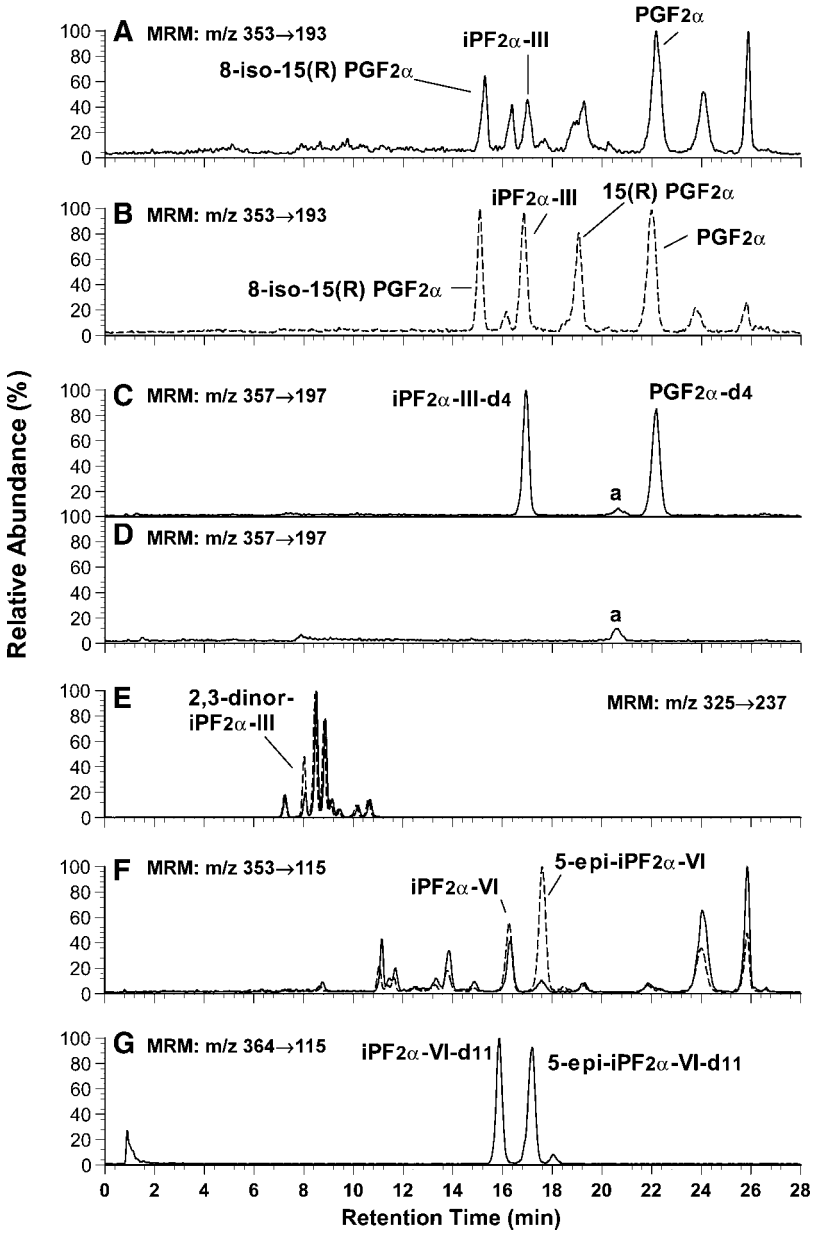


Fig. 4. MRM chromatograms of F_2-isoprostanes (F_2-iPs) extracted from a urine sample using MD-SPE. A: $iPF_{2\alpha}$-III isomers in a urine sample. B: $iPF_{2\alpha}$-III isomers (dotted line) in a urine sample spiked with standard solutions of 8-iso-15(R) $PGF_{2\alpha}$, $iPF_{2\alpha}$-III, 15(R) $PGF_{2\alpha}$, and $PGF_{2\alpha}$ for peak identification. C: Ion channel of *m/z* 357 → 197 for spiked IS $iPF_{2\alpha}$-III-d_4 and $PGF_{2\alpha}$-d_4 in a urine sample. D: Ion channel of *m/z* 357 → 197 in a blank sample of urine. E: 2,3-Dinor-$iPF_{2\alpha}$-III isomers in a urine sample and a urine sample spiked with the standard solution of 2,3-dinor-$iPF_{2\alpha}$-III (dotted line). F: $iPF_{2\alpha}$-VI isomers in a urine sample and a urine sample spiked with the standard solution of (±)5-$iPF_{2\alpha}$-VI (dotted line). G: Ion channel of *m/z* 364 → 115 for spiked IS (±)5-$iPF_{2\alpha}$-VI-d_{11} in a urine sample.

separated from other unknown isomers. Increasing the gradient time and/or the use of a longer column baseline-separated other unknown isomers (data not shown). As shown in Fig. 4F, $iPF_{2\alpha}$-VI and 5-epi-$iPF_{2\alpha}$-VI were baseline-separated from other unknown isomers. Figure 4G shows that $iPF_{2\alpha}$-VI-d_{11} and 5-epi-$iPF_{2\alpha}$-VI-d_{11} extracted from the urine sample were baseline-separated from each other. No interference was coeluted with $iPF_{2\alpha}$-VI-d_{11} and 5-epi-$iPF_{2\alpha}$-VI-d_{11}, as demonstrated by extraction of a blank sample (data not shown).

To examine whether or not $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI were quantitatively recovered from different volumes of urine samples, 1 and 2 ml urine samples from eight volunteer subjects were spiked with the same amount of $iPF_{2\alpha}$-III-d_4 and $iPF_{2\alpha}$-VI-d_{11} (2 ng) and extracted using the MD-SPE method. **Figure 5** shows that the relative areas of $iPF_{2\alpha}$-III to $iPF_{2\alpha}$-III-d_4 (Fig. 5A) and $iPF_{2\alpha}$-VI to $iPF_{2\alpha}$-VI-d_{11} (Fig. 5B) in 2 ml urine samples were well correlated ($r = 0.992$ and 0.997, respectively) with those in 1 ml urine samples. The signal-to-noise ratios for $iPF_{2\alpha}$-III (Fig. 5C) and $iPF_{2\alpha}$-VI (Fig. 5D) ranged from 16 to 118 and from 143 to 355, respectively. As shown in Fig. 5, the signal-to-noise ratios for $iPF_{2\alpha}$-III (Fig. 5C) and $iPF_{2\alpha}$-VI (Fig. 5D) extracted from 2 ml urine samples were greater than those extracted from 1 ml urine samples. These results indicate that peak-to-peak signal-to-noise ratios of >20 were achieved for $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI analysis by extracting 2 ml urine samples from eight randomly selected volunteers.

SPE recovery and matrix-related ion suppression effects

IS mixtures of 8-iso-$PGF_{2\alpha}$-d_4 and (±)5-$iPF_{2\alpha}$-VI-d_{11} (2 ng) were added to 1 or 2 ml urine samples before and after extraction to examine sample extraction recovery (**Table 1**). As shown in Table 1 (extraction 1), ~50–70% of the IS was recovered from 1 and 2 ml urine samples from four volunteer subjects. Four repetitive extractions (extractions 1–4) of 2 ml urine samples from four subjects showed consistent recoveries, and the average recoveries for $iPF_{2\alpha}$-III-d_4, $iPF_{2\alpha}$-VI-d_{11}, and 5-epi-$iPF_{2\alpha}$-VI-d_{11} were 62 ± 8%, 60 ± 10%, and 61 ± 8%, respectively (Table 1).

The effects of urine matrix on the ionization efficiency of F_2-iPs were examined by comparing the ion intensities of standard solutions in water and urine sample matrices. As shown in **Table 2**, 12–23% of the ion suppression (matrix effects, 77–88%) was consistently observed for 2,3-dinor-$iPF_{2\alpha}$-III, which was extracted from 1 and 2 ml

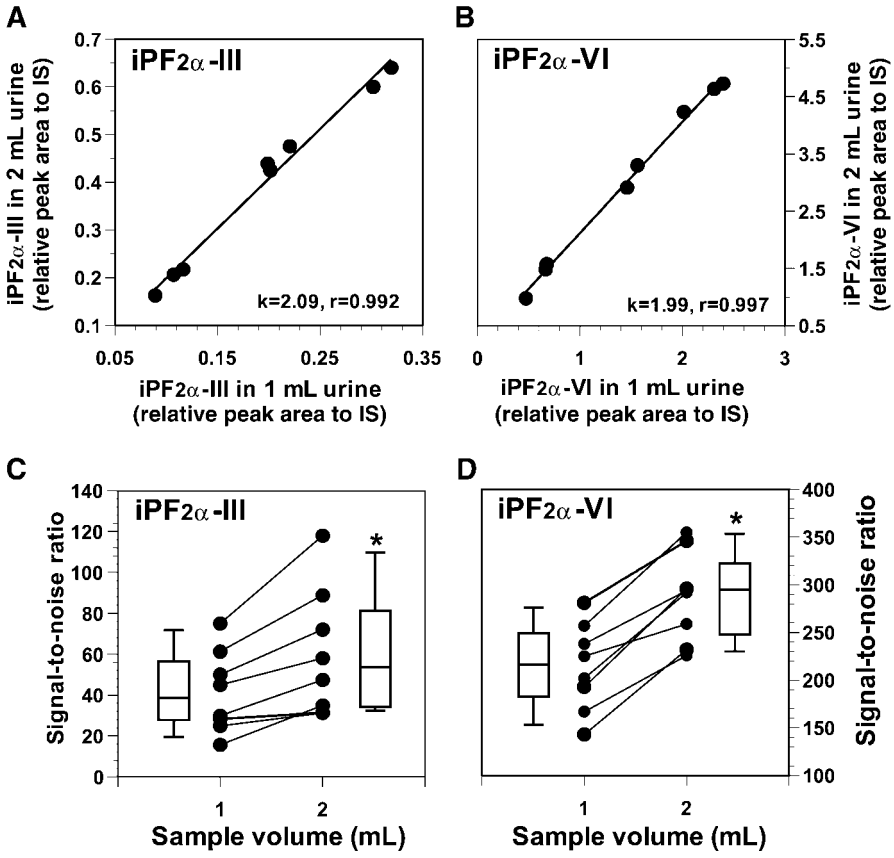


Fig. 5. Correlation of relative peak areas of $iPF_{2\alpha}$-III to $iPF_{2\alpha}$-III-d_4 (A) and $iPF_{2\alpha}$-VI to $iPF_{2\alpha}$-VI-d_4 (B) and signal-to-noise ratios of $iPF_{2\alpha}$-III (C) and $iPF_{2\alpha}$-VI (D) in 1 and 2 ml urine samples from eight volunteer subjects using MD-SPE. The signal-to-noise ratios of $iPF_{2\alpha}$-III (C) and $iPF_{2\alpha}$-VI (D) are also presented in whisker-box plots in which the upper and lower boundaries of the box and the line within the box indicate the 75th and 25th percentiles and the median, respectively. The error bars above and below the box indicate the 90th and 10th percentiles. The same amounts (2 ng) of $iPF_{2\alpha}$-III-d_4 and $iPF_{2\alpha}$-VI-d_{11} were added to 1 and 2 ml urine samples.

TABLE 1. Recovery in solid-phase extraction of urine samples from four volunteers

Isoprostane	Extraction 1		Extraction 2	Extraction 3	Extraction 4		
	1 ml of Urine (n = 4)	2 ml of Urine (n = 4)	2 ml of Urine (n = 4)	2 ml of Urine (n = 4)	2 ml of Urine (n = 4)	Average Recovery	Coefficient of Variation
$iPF_{2\alpha}$-III-d_4	63 ± 10	72 ± 13	57 ± 12	68 ± 7	51 ± 16	62 ± 8	13
$iPF_{2\alpha}$-VI-d_{11}	54 ± 4	62 ± 8	60 ± 12	68 ± 3	56 ± 15	60 ± 6	10
5-Epi-$iPF_{2\alpha}$-VI-d_{11}	53 ± 8	58 ± 9	62 ± 8	74 ± 5	57 ± 9	61 ± 8	13

iP, isoprostane. All values shown are percentages.

urine samples (extraction 1) or at different times (extractions 1–4). The matrix effects for 1 and 2 ml urine samples and different extractions on other F_2-iPs ranged from 88% to 127% (Table 2), and the average matrix effects for 15(R) $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-III, $iPF_{2\alpha}$-III-d_4, $iPF_{2\alpha}$-VI, 5-epi-$iPF_{2\alpha}$-VI, $iPF_{2\alpha}$-VI-d_{11}, and 5-epi-$iPF_{2\alpha}$-VI-d_{11} were 95 ± 6%, 100 ± 6%, 103 ± 5%, 96 ± 3%, 100 ± 3%, 109 ± 15%, and 104 ± 16%, respectively. These results indicate that the ion suppression effects of urine matrices did not significantly affect the ionization efficiency of these F_2-iPs.

DISCUSSION

Oxidative stress is related to many diseases, including cardiovascular disease (2, 3, 16, 17). Methods for the routine measurement of markers of oxidative stress are urgently needed. F_2-iPs are products of the reactive oxygen species-mediated peroxidation of arachidonic acid and have been shown to be reliable markers of oxidative stress (1, 4, 5). LC-MS/MS methods have recently been developed for the specific determination of F_2-iP regioisomers in biological samples (14, 15, 23, 24). However, matrix effects are not controlled in these methods, which are known to affect the accuracy, reproducibility, and sensitivity of ESI-MS (21, 28, 29).

In this work, we developed a novel MD-SPE and buffer-free HPLC method to control matrix- and buffer-additive-related ion suppression in the LC-MS/MS analysis of F_2-iPs in urine samples.

We developed the MD-SPE method based on two-step SPE, applying orthogonal retention mechanisms, and on a selective SPE wash and elution protocol for F_2-iPs. In the first-step SPE on Oasis HLB SPE cartridges, F_2-iPs were retained by a reverse-phase chromatography mechanism, and in the second-step SPE on an Oasis μElution SPE plate, F_2-iPs were retained by a normal-phase chromatography mechanism. Because Oasis HLB is a hydrophilic-lipophilic balanced polymer sorbent (27), both reverse-phase chromatography and normal-phase chromatography mechanisms can be used for the retention of F_2-iPs. The solvents were exchanged for LC-MS/MS analysis in the second-step SPE, which eliminated the need to evaporate organic solvents that is normally part of the SPE of urine samples (14, 15, 23, 24). Also, the samples were concentrated using small-bed SPE sorbent (2 mg) in the second-step SPE (Fig. 3C). Concentration is normally achieved by the evaporation of organic solvent, and both target analytes and matrix interference are concentrated. However, in the second-step SPE on the Oasis μElution plate, F_2-iPs were concentrated but matrix interference was removed, which further cleaned up the samples. Because of the small elution volume (40 μl), a 10-fold concentration of 2 ml urine samples was easily achieved by the MD-SPE method. In addition, using MD-SPE, the time required to prepare samples was greatly shortened, because organic solvents did not have to be evaporated.

In MD-SPE, the clean extraction of urine samples was achieved by a novel selective SPE wash and elution procedure. Oasis HLB is a polymer sorbent and can be used from pH 1 to 14 (27). Therefore, a selective SPE wash procedure was developed by taking advantage of the different elution profiles of F_2-iPs and urine matrix interference as a function of both the concentration of MeOH and pH. With HLB sorbent, acid and base compounds show opposite retention behaviors under low and high pH. Under low pH, acid compounds have strong retention and base compounds have weak retention, whereas under high pH, acid compounds have weak retention and base compounds have strong retention. Therefore, when acidified urine samples containing 10% MeOH were applied to an

TABLE 2. Matrix-related ion suppression effects

Isoprostane	Extraction 1		Extraction 2	Extraction 3	Extraction 4		
	1 ml of Urine (n = 4)	2 ml of Urine (n = 4)	2 ml of Urine (n = 4)	2 ml of Urine (n = 4)	2 ml of Urine (n = 4)	Average Matrix Effects	Coefficient of Variation
2, 3-Dinor-$iPF_{2\alpha}$-III	85 ± 11	77 ± 4	80 ± 8	82 ± 10	88 ± 13	82 ± 4	5
15(R) $iPF_{2\alpha}$-III	100 ± 12	96 ± 15	87 ± 3	91 ± 3	101 ± 5	95 ± 6	7
$iPF_{2\alpha}$-III	106 ± 15	101 ± 18	91 ± 6	96 ± 10	106 ± 6	100 ± 6	6
$iPF_{2\alpha}$-III-d_4	101 ± 10	97 ± 10	102 ± 2	104 ± 8	110 ± 4	103 ± 5	5
$iPF_{2\alpha}$-VI	98 ± 5	91 ± 7	97 ± 6	100 ± 11	98 ± 5	96 ± 3	4
5-Epi-$iPF_{2\alpha}$-VI	102 ± 4	97 ± 6	101 ± 6	104 ± 9	98 ± 3	100 ± 3	3
$iPF_{2\alpha}$-VI-d_{11}	108 ± 18	127 ± 14	103 ± 34	119 ± 24	88 ± 25	109 ± 15	14
5-Epi-$iPF_{2\alpha}$-VI-d_{11}	91 ± 9	87 ± 7	112 ± 9	126 ± 5	103 ± 2	104 ± 16	15

All values shown are percentages.

HLB SPE cartridge, base interference was not retained. Acidic wash with 15% MeOH/2% formic acid (Fig. 3C, cartridge wash step 4) was also used to eliminate base interference. Base wash with 5% MeOH/2% NH_4OH was used to remove acid interference. Elution of yellow interference was observed during the base wash.

Neutral interference in the urine samples was washed off with hexane. Because hexane is immiscible with MeOH and water, one part of diethyl ether was added to the wash solvent (Fig. 3C, cartridge wash step 5). A wash step with hexane-ethyl acetate (9:1) (Fig. 3C, cartridge wash step 6) was further included to remove residual water on the HLB SPE cartridge because ethyl acetate is miscible with water. Diethyl ether/2% acetic acid was used for the elution of F_2-iPs in the first-step SPE because diethyl ether is less polar and more selective than ethyl acetate. Therefore, selective wash and selective elution gave a selective SPE procedure for the clean extraction of F_2-iPs.

The cleanliness of the extracted urine samples as examined by MS was not markedly affected by slight variations in extraction parameters, including the concentration of organic solvent and pH (data not shown), indicating that the selective SPE procedure was robust. Also, the use of Strata X SPE cartridges, another polymer sorbent, instead of Oasis HLB SPE cartridges did not affect the cleanliness of extraction or recovery (data not shown), indicating that the MD-SPE method is robust.

Because F_2-iPs are isomers of F_2-PGs and consist of many diastereomers (1, 11), separation of these isomers is important for the specific analysis of individual F_2-iPs. Li et al. (15) showed that 15(R) 8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ were baseline-separated using Hypersil BDS 3 μm C18 150 mm × 2.1 mm i.d. columns. We achieved a good resolution of 15(R) 8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ in standard solutions and urine samples using Hypersil BDS 3 μm C18 50 mm × 2.1 mm i.d. columns (data not shown), which confirms the finding of Li et al. (15). However, when urine samples were analyzed using Hypersil BDS 3 μm C8 columns, an additional peak was detected between 15(R) 8-iso-$PGF_{2\alpha}$ and 8-iso-$PGF_{2\alpha}$ and next to 8-iso-$PGF_{2\alpha}$ (Fig. 4A). Our new finding is that an unknown F_2-iP isomer was coeluted with 8-iso-$PGF_{2\alpha}$ on C18 columns but was separated from 8-iso-$PGF_{2\alpha}$ on C8 columns. Therefore, C8 columns were used in the novel HPLC method to give better specificity than C18 columns.

Because we found that buffer additives caused significant ion suppression of F_2-iPs and the separation of F_2-iP isomers was not affected by including acetic acid in the extracted urine samples (data not shown), an HPLC method was developed without the use of buffers to control pH. Alternatively, we used LC-MS-grade water for LC-MS/MS analysis. Buffer-free HPLC methods minimize the maintenance of the LC-MS/MS machine and save time required for the daily preparation of buffers. Only water, MeOH, and ACN were supplemented. The novel HPLC method is robust because slight variations in temperature, mobile phase composition, gradient steepness, pH, sample volume, and replacement of HPLC columns did not significantly affect the separation of the commercially available F_2-iPs standards (Scheme 1) (data not shown). To demonstrate the robustness of the novel HPLC method, we also analyzed standard solutions and extracted urine samples in different HPLC machines with different system volumes (Waters Alliance 2796 and 2695 Separation Modules). Similar separation of F_2-iPs was achieved by changing the prevolume parameter (300 and 600 μl for Waters Alliance 2796 and 2695 Separation Modules, respectively) and slightly adjusting the starting gradient organic content while keeping the gradient steepness constant (data not shown). This is the first report that a robust HPLC method for the separation of F_2-iPs was efficiently developed using computer simulation software. This agrees with the finding of Snyder and Dolan (26) that the separation of stereoisomers as a function of temperature and gradient was predictable by a computer simulation using DryLab.

We found that the maintenance of HPLC columns and MS detectors could be minimized using the novel MD-SPE and HPLC methods. More than 400 injections of the extracted urine samples did not cause a significant increase in HPLC column backpressure (data not shown). Therefore, guard columns and precolumn filters were not used in the HPLC analysis. More than 40 injections of the extracted urine samples did not cause visible contamination of the sample cone in the MS detector. This not only saves time for MS maintenance but also reduces the variation in detection sensitivity. Also, our findings indicate that these novel MD-SPE and HPLC methods made possible the consistent recovery of IS of $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI from urine samples and the nonsignificant matrix-related ion suppression for $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI (Table 1).

One great advantage of the novel MD-SPE method is that the urine sample-processing time for LC-MS/MS analysis is short. We normally extract eight urine samples in 3 h. Because of the robustness of the MD-SPE method, it should not be difficult to increase sample throughput through automation. Also, the MD-SPE method can be used to process 10 ml of culture medium from cells, including endothelial cells and smooth muscle cells, or 1 or 2 ml urine samples from animals, including rabbits and rats, with only very slight modification [i.e., by replacing a 0.2 μm, 3 ml Captiva Filter cartridge with a 10 μm, 10 ml Captiva Filter cartridge (Varian, Inc.)] (data not shown).

However, the MD-SPE method will require major modifications if plasma samples are to be prepared for the analysis of F_2-iPs using LC-MS/MS. Because F_2-iPs are generated in situ esterified to phospholipids (1), alkaline hydrolysis of plasma samples is needed to cleave F_2-iPs into free F_2-iPs to measure total F_2-iPs. Also, it may be worthwhile to add a free radical scavenger such as butylhydroxytoluene or an inhibitor of cyclooxygenase such as indomethacin (30) to EDTA plasma samples to prevent autoxidation during sample separation, storage, and processing. Iuliano et al. (31) added [$^2H_8$]arachidonic acid to plasma samples after collection to detect any artifactual formation of 8-iso-$PGF_{2\alpha}$-III. We found that retention of [^{3}H]8-iso-$PGF_{2\alpha}$ by Oasis HLB SPE cartridges was much lower in plasma samples that had been treated with 15% KOH in ethanol than in plasma samples that were not treated (data not shown). One

milliliter of plasma sample overloaded the 30 mg/1 cc Oasis HLB cartridge. Therefore, Oasis HLB or Strata X SPE cartridges with a higher retention capacity (i.e., a greater sorbent amount such as 60 or 200 mg) are needed for plasma sample preparation. Therefore, the urine MD-SPE method has limitations in the analysis of F_2-iPs in plasma samples.

In conclusion, we developed a novel sample clean-up method using MD-SPE and a novel HPLC method for the specific analysis of F_2-iPs using LC-MS/MS. With this novel MD-SPE LC-MS/MS method, it should no longer be difficult to perform the routine analysis of F_2-iPs in urine samples. Validation of the novel MD-SPE LC-MS/MS method for the routine analysis of $iPF_{2\alpha}$-III and $iPF_{2\alpha}$-VI in urine is now in progress in our laboratory.

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ERRATA

In the article "Control of matrix effects in the analysis of urinary F_2-isoprostanes using novel multidimensional solid-phase extraction and LC-MS/MS" by Bo Zhang and Keijiro Saku, published in the March 2007 issue of the *Journal of Lipid Research* (Volume 48, pages 733–744), the authors would like to note the following changes:

Page 733, Abstract section, fourth sentence: size exclusion, should read: size exclusion chromatography.

Page 734, Scheme 1, part A: iPF2a-III, should read: $iPF_{2\alpha}$-III.

Page 737, Results section, second paragraph, second sentence: under the ES-mode, should read: under the ES- mode.

Page 737, Results section, second paragraph, last sentence: as follows: 8-iso-$PGF_{2\alpha}$ *m/z* 353/193 for 8-iso-$PGF_{2\alpha}$, should read: as follows: *m/z* 353/193 for 8-iso-$PGF_{2\alpha}$.

Page 739, Results section, first paragraph, fourth sentence and second paragraph, last sentence: 5% MeOH and 2% formic acid should read: 5% MeOH with 2% formic acid.

Page 741, Figure 5 legend, first sentence: (A) and $iPF_{2\alpha}$-VI to $iPF_{2\alpha}$-VI-d4, should read: (A) and $iPF_{2\alpha}$-VI to $iPF_{2\alpha}$-$VI\text{-}d_{11}$.