IDENTIFICATION OF NOVEL CYTOTOXIC PROSTAGLANDIN METABOLITES PRODUCED IN ARACHIDONOYL ETHANOLAMIDE-TREATED TUMOREGENIC KERATINOCYTES

By

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Arachidonoyl ethanolamide (AEA) induces apoptosis in mouse tumorigenic keratinocytes (JWF-2 cells). Our previous data show that AEA is metabolized by COX-2 to pro-apoptotic J-series prostaglandins. COX-2 is an enzyme that is abundant in tumor cells but not in the normal epithelial cells surrounding the tumor. Thus, the pro-apoptotic J-series prostaglandins should be selectively synthesized in AEA-exposed tumor cells with elevated COX-2 expression. As such, AEA could be developed as a topical agent to treat non-melanoma skin cancer. *The main goal of this project is to identify the specific J-series prostaglandins that are produced as a result of the metabolism of AEA by COX-2 using mass spectrometry.* (ELISA analysis can detect J-series family prostaglandins but cannot distinguish between the individual J-series isoforms).

Exogenous J-series prostaglandins were added to fresh cell culture medium, and the prostaglandins were extracted using solid phase extraction. Concentrated samples were then subjected to Liquid Chromatography/Electrospray Ionization /Mass Spectrometry (LC-ESI-MS) in negative mode for identification of J-series prostaglandin isoforms. Our data show good recovery of extracted species and acceptable resolution of these chemically similar standards.

The culture medium from AEA- and ethanol-treated was extracted using the validated extraction protocol and analyzed using the method developed with LC-ESI-MS. The mass spectrum of the culture medium obtained from AEA-treated, extracted and concentrated cell culture media clearly shows peaks at m/z ratio identified as the parent ion peak $(M-H)^-$ of ethanolamide conjugates of the J-series prostaglandins. The identification of the ethanolamide conjugates was confirmed by performing tandem mass spectrometry. It was also observed that with increased AEA concentration, the mass spectral intensity of the ethanolamide conjugates of J-series prostaglandins increased. The effect of a COX-2 inhibitor and the antioxidant N-acetyl cysteine(NAC), on the production of ethanolamide conjugates of J-series was also studied. The identification of the cytotoxic ethanolamide conjugates of J-series prostaglandins as the metabolites of AEA synthesized in tumor cells help us to determine the mechanism by which AEA induces apoptosis.

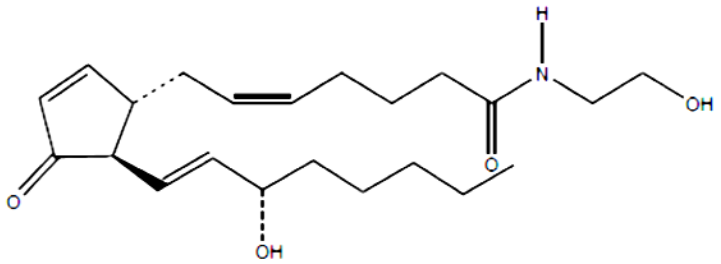

Figure 1 Structure of PG-J_2 ethanolamide (Exact mass 377.2)

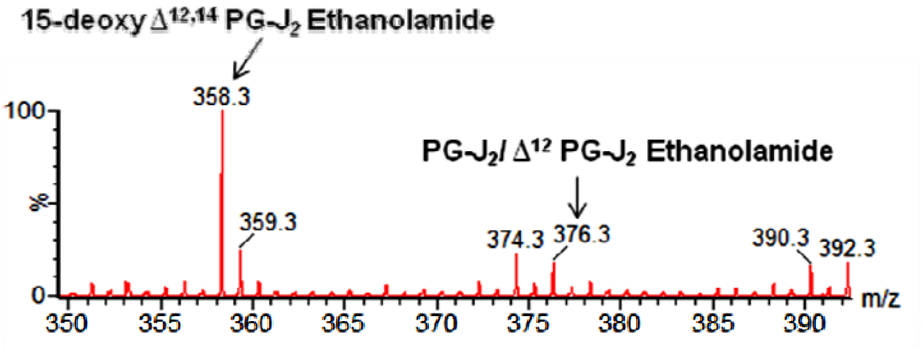

Figure 2 The LC-ESI-MS spectrum of AEA treated sample in negative ion mode

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Chapter 1: Introduction

Cancer is uncontrollable growth of abnormal cells in the body[1]. Besides growing uncontrollably these cells also invade the surrounding tissues which actually make them cancerous[2]. The cells become cancerous due to DNA damage which can be caused by several factors such as environmental carcinogens, DNA replication errors by normal cells or by inheritance[2]. Any organ or tissue can develop cancer such as skin, lung, breast or nervous tissue[1].

1.1 Skin Cancer

Skin cancer is the most common cancer in United States[3]. The incidence of skin cancer is reported to be higher than the incidence of all other cancers combined including lung, breast, colon and prostate[3]. More than two million cases of skin cancer are diagnosed every year[3].

1.1.1 Types of Skin Cancer

Skin cancers are classified based on the type of skin cells they affect. The cancer occurring in melanocytes is referred to as melanoma skin cancer and the cancers occurring in basal keratinocytes and squamous keratinocytes is referred to as non-melanoma skin cancers.

1.1.2 Causes, Signs and Symptoms of Skin Cancer

There are several factors which are found to have the potential to induce skin cancer[4]:

a) Excessive exposure to UV-radiation while outdoors

b) Excessive exposure to artificial UV-radiation in indoor tanning industries

c) Declining stratospheric ozone layer protection

d) Aging

e) Chemicals such as arsenic

f) Viruses such as human papilloma virus

Skin cancers occur mostly in the sun exposed areas such as upper arms legs, face, nose etc. Like many other cancers, skin cancers also start as precancerous lesions which have potential to develop into cancers over time[5]. The appearance of the tumor varies depending upon the type of skin cancer. A basal cell carcinoma appears as waxy or pearly bump with visible blood vessels whereas a squamous cell carcinoma appears as a scaly or crusty reddish nodule[5].

1.1.3 Non Melanoma Skin Cancer (NMSC)

NMSC is not only the most common form of skin cancer but also it is also the most common human malignancy[4]. In the United States alone, every year more than 3.5 million new tumors are identified in more than 2 million people[3] every year. It was reported that about 90% of non-melanoma skin cancers are caused due to exposure to UV-radiation from the sun[3]. Between 1992 and 2006, treatment for NMSC has increased 77 percent[3].

1.2 Cannabinoids as Chemotherapeutic agents

Cannabinoids are the molecules which interact with the cannabinoid receptors and produce "tetrad effects" such as hypomotility, hypothermia, analgesia and catalepsy[6]. They possess certain therapeutic properties that are used to mitigate symptoms such as nausea and vomiting in cancer patients and neuropathic pain in individuals with multiple sclerosis[7]. They are potential anticancer agents found to suppress tumor growth in various cultured cells and animal models[7]. They can be broadly classified into phytocannabinoids, endocannabinoids and synthetic cannabinoids [6,7].

1.2.1 Phytocannabinoids

Phytocannabinoids are the chemically active components derived from the plant *Cannabis sativa* (marijuana)[7]. Several physiological and pathological effects of cannabinoids such as the reduction of pain or inflammation were identified for the phytocannabinoid

tetrahydrocannabinol (Δ^9 THC)[6]. Cannabidiol (CBD) and cannabinol (CBN) are examples of other phytocannabinoids.

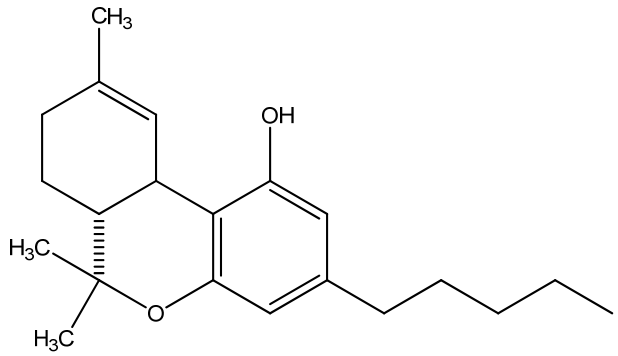


Figure 1.2.1 Structure ofΔ^9 Tetrahydrocannabinol(Δ^9 THC).

1.2.2 Synthetic cannabinoids

Synthetic cannabinoids are the chemically synthesized compounds which mimic the effects of the phytocannabinoids[6] . The examples of synthetic cannabinoids are WIN-55,222-2, AM-94 and JWH-200.

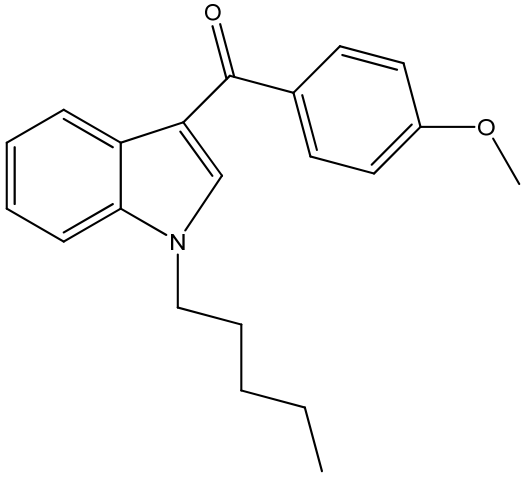


Figure 1.2.2 Structure of JWH-018.

1.2.3 Endocannabinoids

Endocannabinoids are produced by mammalian cells and also found to mimic the effects of phytocannabinoids. Arachidonoyl ethanolamide, also called anandamide, was the first

endocannabinoid discovered[7]. Endocannabinoids play a major role in the central nervous system and are also involved in regulation of immunity, cell growth and inflammation[8].Other examples are 2-arachidonoyl glycerol (2-AG) and oleoyl ethanolamide(OEA).

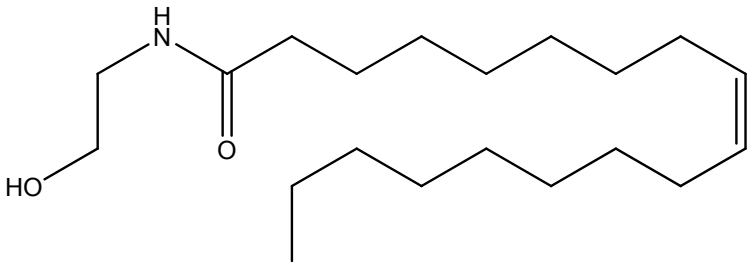


Fig. 1.2.3 Structure of oleoyl ethanolamide(OEA).

1.3 Arachidonoyl ethanolamide

Arachidonoyl ethanolamide (AEA) is an N-acyl ethanolamide of arachidonic acid (Fig.1.3.1). AEA controls various cellular responses such as cell proliferation, growth arrest, cell death and inflammation[8]. This endocannabinoid is also found to exert cytotoxic effects in many types of tumor cell lines including breast, thyroid and skin[9, 10].

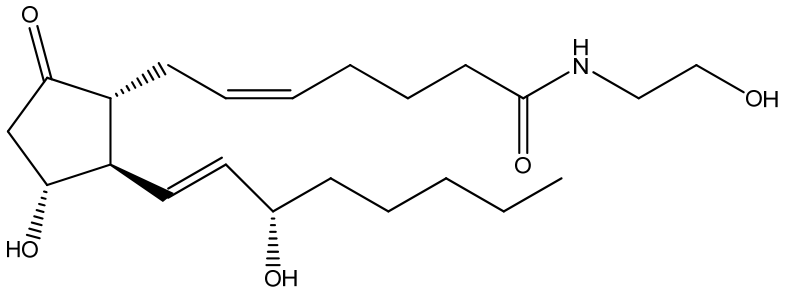


Figure 1.3 Structure of Arachidonoyl ethanolamide(AEA).

1.3.1 Selectivity and Cytotoxicity of AEA

In the previous studies conducted by Van Dross, et al, AEA was found to inhibit the growth of skin tumor cells by inducing apoptosis[11]. It was also reported that AEA was selectively

metabolized to cytotoxic J-series prostaglandins in skin tumor but not in normal keratinocytes[11]. The mechanism of its cytotoxicity is not very well understood. On the other hand, the selectivity of AEA towards the tumor cells may be explained by the fact that the COX-2, which plays a key role in the metabolism of AEA, is more abundant in tumor cells than the surrounding normal skin cells.

1.3.2 Metabolism of AEA to J-series Prostaglandins

Van Dross, et al, have reported that AEA-induced apoptosis in the skin tumor cells is mediated by the production of J-series prostaglandins[6]. We hypothesize that AEA is metabolized to J-series prostaglandins through one of two possible pathways (Figure 1.3.2). Pathway A is a well-established pathway in normal cells in which AEA is metabolized to arachidonic acid (AA) and ethanolamide (EA) in the presence of the enzyme fatty acid amide hydrolase (FAAH). Arachidonic acid in turn is metabolized in a series of reactions to J-series prostaglandins.

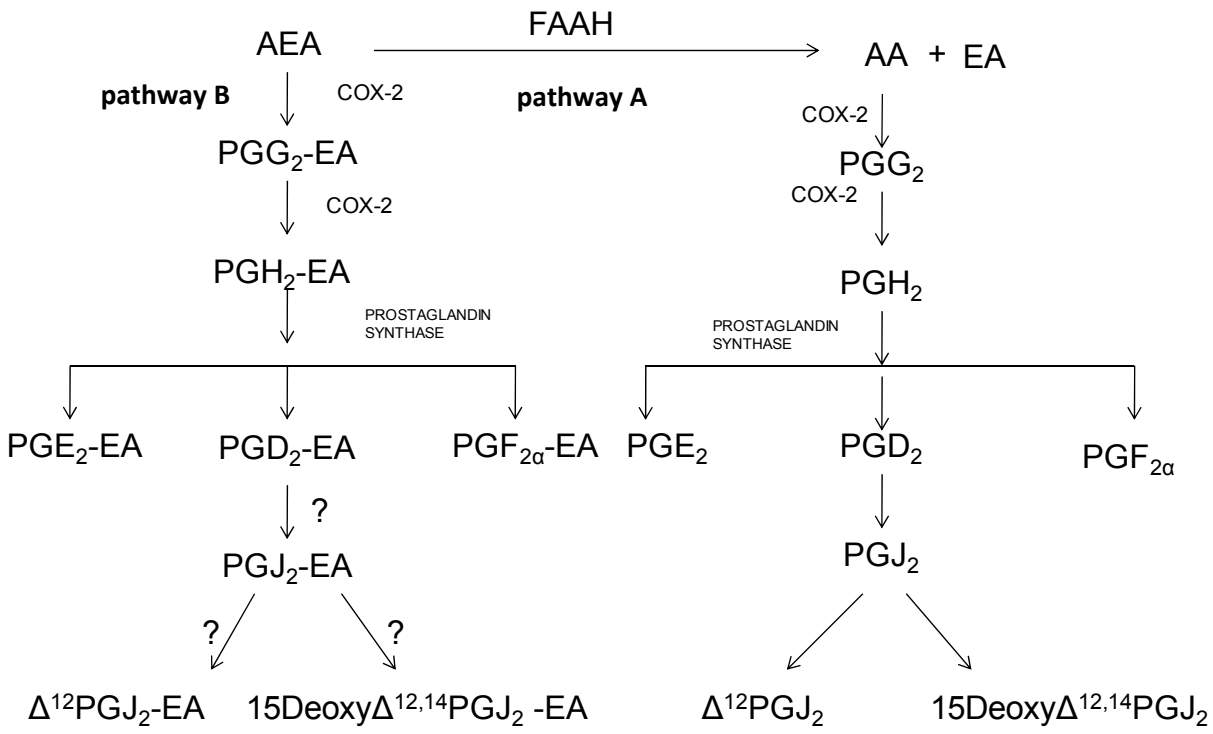


Figure 1.3.2 Metabolic pathway of the conversion of AEA to J-series prostaglandins (pathway A) or J-series ethanolamide conjugates (pathway B). Question marks represent data that is not published in the literature.

Various studies have reported that as shown in pathway B, AEA is directly metabolized by COX-2 to ethanolamide prostaglandins of E-, F- and D-series[13, 14.] The subsequent metabolism of D-series ethanolamide prostaglandins to J-series ethanolamide prostaglandins has not been reported in the literature to date. *Our main goal is to identify the chemical nature and the type of J-series prostaglandin/s produced by the skin tumor cells when treated with AEA* using LC-ESI-MS. Although ELISA was used to quantify the J-series prostaglandins by Van Dross, et al, identification of the specific classes of molecules was not possible with ELISA.

The type of tumor cells being studied is the squamous cell carcinoma cell line which is the non-melanoma type of skin cancer cell line. Research indicates that AEA has the potential to

be developed as topical chemotherapeutic agent to treat non-melanoma skin cancer cells because of its selectivity and cytotoxicity to the skin tumor cells[6]. Our research is focused towards the better understanding the metabolism of AEA which is critical for further research and development of AEA as a chemotherapeutic drug.

Chapter 2: Techniques and Instrumentation

2.1 Techniques

Besides LC-ESI-MS, the other important bioanalytical techniques used either for the validation of the protocols or for monitoring the cellular response to the drug treatment were ELISA and Western blot. ELISA was used for the validation of the extraction protocol discussed in detail in chapter 4.4.2. The AEA-treated cells were subjected to Western blot analysis to monitor cell death.

2.2.1 ELISA

ELISA is used for the detection of specific antibodies, soluble antigens or cell surface antigens[15]. The different types of ELISA protocols used are indirect, direct competitive, antibody sandwich, double antibody sandwich, direct cellular and indirect cellular[15]. Although the most common applications of all the ELISA protocols listed are either antigen or antibody screening, some of them are also used to measure cellular antigen expression.

We have used a 15-deoxy $\Delta^{12,14}$ PG-J_2 Enzyme Immunoassay Kit (Assay Designs, Ann Arbor, MI) to quantify the amount of J-series prostaglandins in the solvent washes and the eluate collected at different steps in the extraction process. This kit is a competitive immunoassay used for quantitative determination of 15-deoxy $\Delta^{12, 14}$ PG-J_2 in the biological sample. As discussed earlier in Chapter 1, this kit is not specific for just the 15-deoxy $\Delta^{12, 14}$ PG-J_2 because it is shown to have some cross-reactivity with the other types of J-series prostaglandins. Also, this kit does not differentiate between the 15-deoxy $\Delta^{12, 14}$ PG-J_2 and the ethanolamide conjugates of the same. However, irrespective of the lack of selectivity, this kit provided good correlation between the increasing dose of AEA and the production of J-series prostaglandins quantified by Van Dross, et al[6].

The principle involved in the quantification is the competitive binding of the 15-deoxy-$\Delta^{12,14}$PG-J_2 antibody to 15-deoxy $\Delta^{12,14}$ PG-J_2 in the sample or the 15-deoxy-$\Delta^{12,14}$PG-J_2 attached to an alkaline phosphatase buffer. The ELISA plate coated with the goat antibody is incubated either with a 15-deoxy-$\Delta^{12,14}$ PG-J_2 standard or the sample containing 15-deoxy-$\Delta^{12,14}$ PG-J_2 along with the15-deoxy-$\Delta^{12,14}$PG-J_2 attached to an alkaline phosphatase and the 15-deoxy $\Delta^{12,14}$PG-J_2 antibody for 2hours on plate shaker at ~500 rpm. During this time, the analyte and the analyte-enzyme complex compete for binding with the primary antibody which in turn bind to the goat antibody. The unbound analyte and the analyte-enzyme complex are washed away using a wash buffer and the substrate p-nitrophenyl phosphate in buffer is added and incubated for 3 hours. After incubation the enzyme reaction is stopped by using a stop buffer and the yellow colour developed due to the metabolite that is formed due to reaction between the phosphatase enzyme and the p-nitrophenyl phosphate substrate is read on a microplate reader(UV-vis spectrophotometer) at 405 nm. Figure 2.2.1 describes a representative ELISA measurement of 15-deoxy $\Delta^{12,14}$ PG-J_2 using 15-deoxy $\Delta^{12,14}$ PG-J_2 phosphatase conjugate.

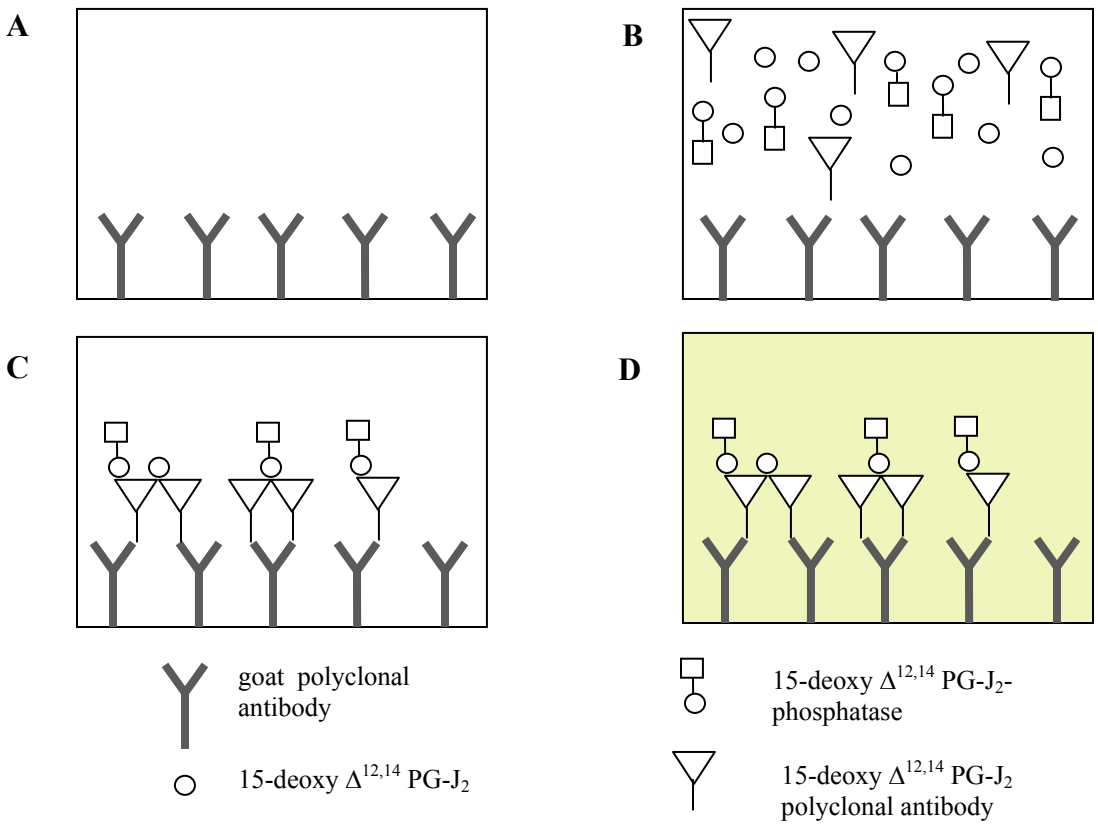


Figure 2.1.1 A representative ELISA measurement of 15-deoxy $\Delta^{12,14}$ PG-J_2 using 15-deoxy $\Delta^{12,14}$ PG-J_2 -phosphatase conjugate. (Adapted from *Current Protocols in Immunology*(1999) 7.33.2)[15].

2.1.2 Western Blot Analysis

Western blot is proteomic technique used for the detection of proteins in cells, tissue lysates or extracts. It can also be used for the determining the size of the protein, its relative abundance and also phosphorylation status. The proteins are extracted from the cell, quantified and then loaded on to the gel for purification. The proteins are separated on the gel based on their size by electrophoresis. After separation, the proteins from the gel are transferred to a membrane followed by blotting with the specific antibody and signal detection as illustrated in the

Figure2.1.2. The brief protocol used to analyze the form of the protein poly (ADP-ribose), abbreviated as PARP, in AEA-treated JWF-2 cells is discussed in chapter 3.

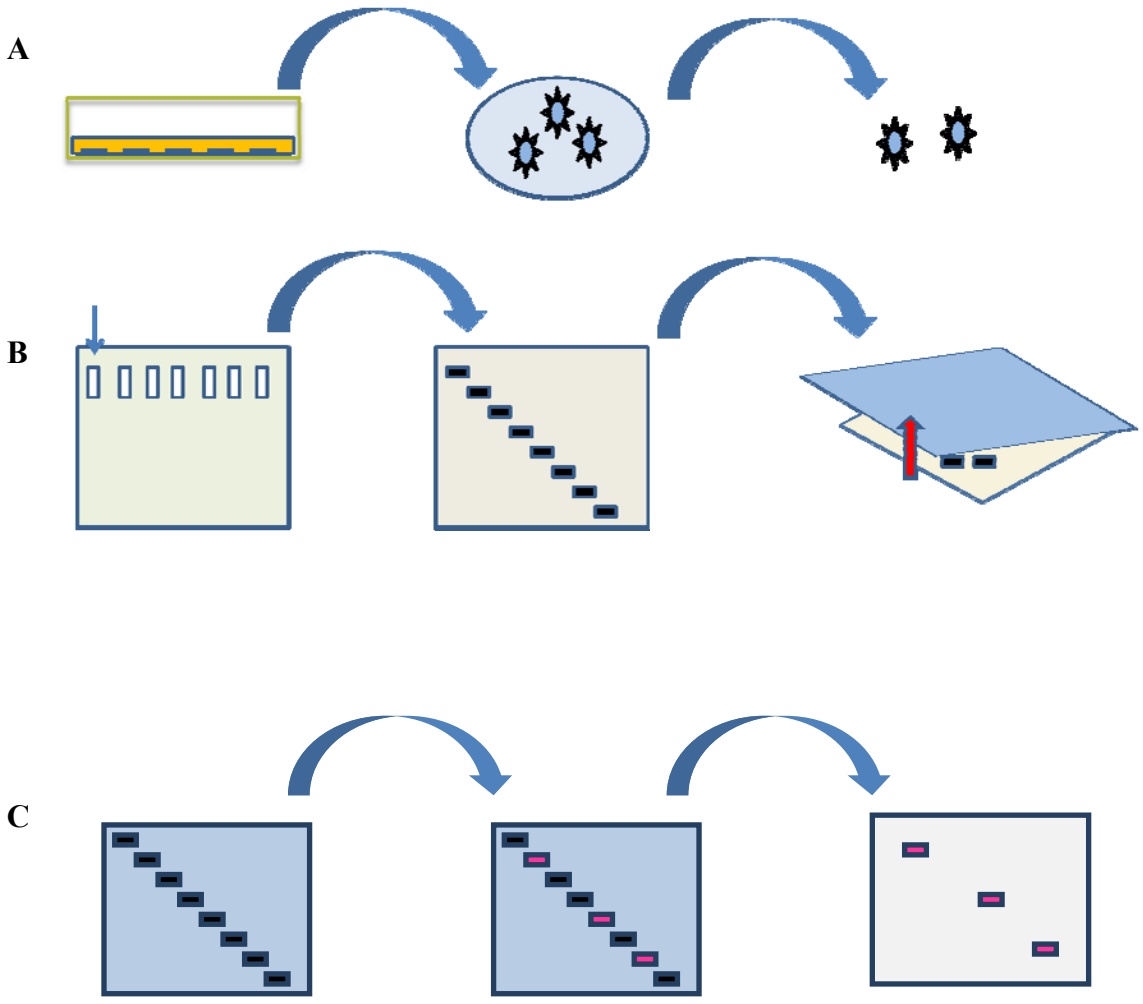


Figure 2.1.2 An illustration of Western blot analysis. A) Extraction of proteins from the cells attached to the plate. B) Gel electrophoresis followed by membrane transfer. C) Membrane blotting and signal detection. (adapted from *Molecular station, Western blot*)

2.2 Instrumentation

LC-ESI-MS was performed on a Waters Acquity UPLC system coupled with a Micromass QToF mass spectrometer. A schematic of the instrument is illustrated in Figure 2.2. The prostaglandins are chromatographically resolved on an Acquity UPLC using a BEH (Ethylene Bridged Hybrid) column with C_8 particles (particle diameter 1.7µM). The column has a 2.1 mm inner diameter and 100mm length (Waters, Milford, MA). The mobile phase consisted of 90/10 water/acetonitrile (solvent A) and 100% acetonitrile (solvent B). UV grade Acetonitrile was filtered through a 0.1 micron filter by the manufacturer (Honeywell B&J, Muskegon, MI) and is 99.9% pure. Solvent A was prepared by mixing ultrapure deionized water (18MΩ-cm) and acetonitrile. The gradient started with solvent A decreasing from 65% to 50% A at 5%/minute, and then from 50% to 35% A at 7.5%/minute. A constant flow rate of 0.4 mL/minute was used and the injection volume was 20 µL.

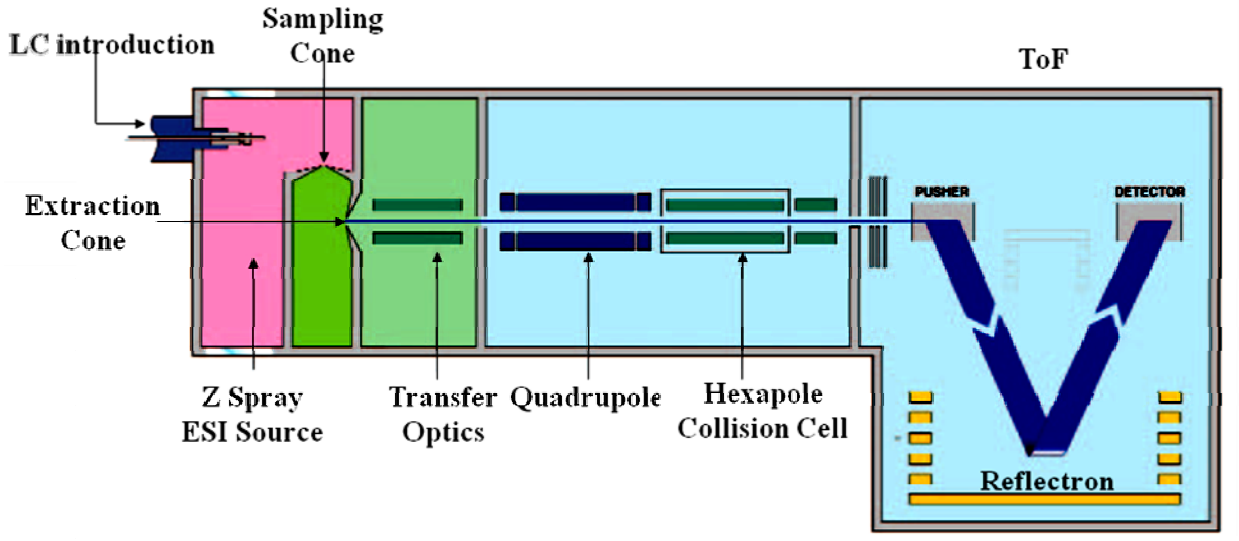


Figure 2.2 A schematic representation of the LC-ESI-MS instrument

2.2.1 Electrospray Ionization source

The mass spectrometer, equipped with an electrospray ionization source, was used for ESI-MS and ESI-MS-MS. The instrument was operated in negative ion mode with the capillary voltage set at 3.2kV, which creates a strong electric field in which ions are formed. Nitrogen gas promotes the formation of an aerosol with the charged particles. The sampling cone and the extraction cone shown in the schematic of the instrument (Figure 2.2) act to steer the ions to the mass spectrometer and aid in "skimming" the remaining solvent from the electrospray plume. The potential difference at the skimmers controls the extent of fragmentation, referred to as in-source dissociation. The essential parameters optimized for an LC-ESI-MS analysis are listed in Table 2.2. The desolvation and source temperatures for LC-ESI-MS analysis are much higher than for direct ESI-MS analysis because the flow rate is higher. Also the flow rate of cone gas is maintained at 10 L/hour during an LC-MS analysis to reduce the comparatively higher volume of sample entering into the mass spectrometer. The eluate from the UPLC is directed into the electrospray ionization source. When the eluate enters the steel nebulizer of the ESI source, a Taylor cone is formed due to high potential difference between the ESI needle and its counter electrode .The ions are formed at the tip of the Taylor cone[16] and enter as gas phase ions into the Q-ToF.

Table 2.2 Settings for LC-ESI-MS analysis

Parameter	LC-MS	ESI-MS
Desolvation Temperature (ºC)	120	80
Source Temperature (ºC)	275	120
Cone gas (L/hr)	10	0
Desolvation gas (L/hr)	500	500

2.2.2. Quadrupole-Time of Flight mass analyzer

The ions in the gas phase are directed towards the Q-ToF mass analyzer. During MS analysis the quadrupole focuses all the ions towards the ToF analyzer. In MS/MS analysis, the quadrupole is operated in a mass filter mode to transmit only the parent ion of interest[17]. The selected parent ion is accelerated to energy of 20-40 eV before it enters the collision cell and undergoes collision-induced dissociation by colliding with the neutral gas molecules (argon).

Chapter 3: Experimental

3.1 Experimental Design

The entire experimental analysis can be summarized under two categories: extracellular and intracellular analysis. After treating the JWF-2 cells with AEA, the media is extracted, concentrated and analyzed using LC-ESI-MS. The treated cells containing the protein markers for apoptosis are analyzed using Western blot technique.

3.2 Cell Culture

The type of cells cultured is the murine squamous cell carcinoma cell lines called the JWF2 cells (Dr.Susan Fischer, University of Texas; MD Anderson Cancer Center, Smithfield TX). The cells are cultured in Eagle's Minimal Essential Media (SMEM) (US Biological, Marblehead, MA). The cells are split when they are close to being 100% confluent and have no more space to grow. If they are not confluent enough to split, the cells are fed with media until they are ready to split. Cells need to be regularly split or fed on every alternate day or within a maximum gap of two days, again depending on the confluency of cells. The following are the detailed protocols for cell splitting, cell feeding and preparation of the media for the cell culture.

Cell splitting protocol

The SMEM media containing heat inactivated fetal bovine serum (FBS), penicillin (100mg/mL), streptomycin (100mg/mL), non essential amino acids and glutamine and trypsin are warmed to 37ºC prior to the experiment. Media is removed from a 75cm^2 flask, washed twice with 10 mL of phosphate buffer each and incubated with 2 mL of warmed trypsin in a CO_2 incubator maintained at 37ºC. Trypsin is removed from the flask after a minute followed by incubation for another 4 minutes. The residual trypsin acts on the cells and helps them detach from the surface. After incubation, the bottom of the flask is tapped to dislodge the cells from the

flask. Approximately 10 mL of the warmed SMEM media is added to each flask containing cells, transferred into a sterile centrifuge tube (15/50mL) tube and centrifuged for 5 minutes at 1200 rpm. Media is removed and the appropriate amount of fresh SMEM media is added depending on the pellet size (2-7 mL). The resulting cell suspension is mixed well to get a uniform cell suspension. The cell suspension is distributed into new set of $75cm^2$ flasks followed by incubation. For each experiment, cells are plated in 100mm dishes at a density of 75,000 cells/mL. To count the cells, 10µL of the cell suspension is added to 10µL of trypan blue dye, and cells are counted using a hemocytometer and microscope.

3.3 Cell treatment

The cells are plated 48 hours prior to the day of the experiment to achieve 85-90% confluency on cells. The cells are treated using SMEM media containing penicillin (100mg/mL), streptomycin (100mg/mL), non essential amino acids, glutamine and calcium chloride. As this media does not contain FBS, it is also called serum free SMEM media (SF-SMEM). The cell treatment slightly varies depending upon the experiment.

3.3.1 Cell treatment with AEA and ethanol as control

The concentration of AEA used to treat the cells was 10µM, 20µM or 30µM and was prepared by adding the appropriate amount of AEA stock to SF-SMEM media (warmed to 37ºC prior to the experiment). The control treatment is prepared by adding the same volume of ethanol (AEA solvent) as that of AEA stock added to the SF-SMEM media. Media, which was present on the cells for 48 hours (spent media), is removed from the culture dishes and 10 mL of either AEA or ethanol containing SF-SMEM is added to each plate. The plates are then labeled and incubated for approximately 5 hours in the CO_2 incubator. After the treatment, the media is

collected into clean 15 mL centrifuge tubes and stored at -80ºC until extraction. The plates which contain attached cells are washed twice with 10 mL of ice cold PBS and also stored at -80ºC.

3.3.2 Cell pretreatment with N-acetyl cysteine followed by treatment with AEA

A stock solution of 1M N-acetyl cysteine (NAC) was prepared and filter sterilized. A final concentration of 75 mM NAC is prepared by adding 3 mL of the 1M stock solution to 37 mL of SF-SMEM media (warmed to 37ºC prior to the experiment). Adding NAC to the media decreases the pH of the media which is restored to pH 7 with 1N NaOH solution. Spent media is removed from the cells and then cells are pre-treated for 2 hours with 75 mM NAC or an equal volume of water (solvent for NAC) prepared in SF-SMEM media. After 2 hours, 20 µM AEA or an equivalent volume of ethanol is added to the NAC-containing media used for the pretreatment. The media is then added back to the cells, and incubated for 5 hours in the CO_2 incubator. The treatments are summarized as follows:

a) Water (pretreatment) and Ethanol (treatment)
b) NAC (pretreatment) and Ethanol (treatment)
c) Water (pretreatment) and AEA (treatment)
d) NAC(pretreatment) and AEA (treatment)

After the treatment the media is collected into fresh 15 mL centrifuge tubes and stored at -80ºC until extraction. The plates containing the treated and attached cells are washed twice with 10 mL ice cold PBS and also stored at -80ºC.

3.3.3 Cell pretreatment with COX-2 inhibitor (NS-398) followed by treatment with AEA

Spent media is removed from the cells and the cells are pretreated with either 25 µM or 50 µM of NS-398 or with DMSO (NS-398 solvent) as control for 45 minutes prepared similarly as discussed in earlier sections (3.3.2 and 3.3.3). After 45 minutes, 20µM AEA or an equivalent

volume of ethanol is added to the same media used for the pretreatment. The media is then added back to the cells, and cells are incubated for 5 hours in the CO_2 incubator. The treatments are summarized as follows:

a. DMSO pretreatment and Ethanol treatment
b. NS-398 (25uM) pretreatment and Ethanol treatment
c. NS-398 (50uM) pretreatment and Ethanol treatment
d. DMSO pretreatment and AEA treatment
e. NS-398 (25uM) pretreatment and AEA treatment
f. NS-398 (50uM) pretreatment and AEA treatment

After the treatment the media is collected into fresh 15 mL centrifuge tubes and stored at -80ºC until extraction. The plates containing the treated and attached cells are washed twice with 10 mL of ice cold PBS and also stored at -80ºC.

3.4 Extracellular analysis

After the cell treatment, the media is extracted and then concentrated before LC-ESI-MS analysis. Solid phase extraction is used for isolating the prostaglandin molecules from the media. The solid phase C_{18} columns are fixed on to the extraction apparatus and are operated under vacuum. Prostaglandins tend to stick to plastic surfaces, so only glass pipettes are used throughout the extraction procedure, and the final eluates are collected in glass vials.

3.4.1.1 Extraction of prostaglandin standards from SF-SMEM media

Prostaglandins (PG-J_2, 15-deoxy $\Delta^{12,14}$ PG-J_2, and Δ^{12} PG-J_2) were dissolved in ethyl acetate and purchased from Cayman Chemical (Boston, MA). Samples were evaporated to dryness with nitrogen gas and reconstituted in ethanol immediately before use. SF-SMEM media is warmed to 37˚C prior to the experiment and PG-J_2, 15-deoxy $\Delta^{12,14}$ PG-J_2 and Δ^{12} PG-J_2 are added to the 1 mL of the either fresh or spent SF-SMEM media in a 1mL centrifuge tube to a final concentration of 3, 6 and 12 μM respectively. The same volume of ethanol as that of the total volume of all the prostaglandins is added to the media and also extracted similarly as a control. The media spiked with prostaglandins or ethanol is acidified by adding 2M HCl to adjust the pH to approximately 3.5. The media is then placed on ice for 15 minutes followed by centrifugation at a maximum speed for 2 minutes to remove any precipitates. The media is then transferred into a fresh tube for extraction. Solid phase C_{18} columns (GRACE, Grace Davidson Discovery Science, Deerfield, IL) of 1.5 mL capacity are fixed on to the extraction apparatus. The columns are equilibrated with 1 mL of 100% acetonitrile to condition the column and flush out the previously adsorbed species. The columns are then washed with 1mL of ultrapure deionized water and the supernatant is applied to the columns. The columns are washed with 1 mL of ultrapure deionized water followed by solvent wash with 1 mL each of 15% acetonitrile and hexane. The prostaglandins are finally eluted in 1 mL of 100% acetonitrile, transferred into a glass vial and stored at -80˚C before evaporation and reconstitution.

3.4.1.2 Protocol for extraction of prostaglandins from the media after the cell treatment

Thawed media is acidified with 2M HCl to adjust the pH to approximately 3.5, placed on ice for 15 minutes, and centrifuged at a maximum speed for 2 minutes to remove any

precipitates. The supernatant is then transferred into a fresh centrifuge tube for extraction, following the same extraction steps detailed in Section 3.4.1.1.

3.4.2 Evaporation and Reconstitution

The extracted media is evaporated to dryness under a stream of nitrogen gas followed by reconstitution in 0.010 mL acetonitrile. More dilute analyte solutions (prepared with larger volumes of acetonitrile) were not detectable at high signal-to-noise ratios with mass spectrometry.

3.5 Intracellular analysis

In their previous studies, Van Dross et al have demonstrated by Western blot analysis that AEA induces cell death by apoptosis in skin tumor cells. The following are the steps involved in the Western blot analysis of the AEA treated JWF-2 cells.

1. BCA assay(bicinchoninic acid assay)
2. Western Blot-gel electrophoresis
3. Transfer of proteins to polyvinylidene diflouride(PVDF) membrane
4. Membrane blotting
5. Signal detection

3.5.1 BCA assay

Protease inhibitors solution is prepared by adding1μL each of Aprotin, Pepstatin, and DDT; 2μL each of Sodium Vanadate and Okadaic acid; 5μL of Leupeptin; 8 μL sodium fluoride; and 10 μL Phenylmethylsulfonyl fluoride (PMSF) in 1ml of Tissue lysis buffer solution (TLB). This inhibitors prevent the lysis of the proteins present in the cell by proteases and phosphatases. The cell plates stored at -80˚C are left to thaw on an ice bed. Approximately 100 μL of TLB buffer containing the protease inhibitors is added to the plates and the entire surface of the plate

is scrapped with a cell scraper. The mixture is collected from one end of the plate and transferred to a labeled microfuge tube on ice. The cell scraper is cleaned in 70% ethanol between each sample collection. The samples are vortexed for 10 seconds each after each plate has been scrapped. Alternatively, cell lysate can be taken up and down with a needle and syringe (18 gauge) 8 times each. The samples are centrifuged at 12,000 rpm for 10 minutes at 4ºC and the supernatant is transferred into a clean labeled microfuge tube. An aliquot of each sample is transferred into a microfuge tube for protein concentration determination. For this cell line, 6 uL of the cell lysate is added into tubes containing 18 µL of water. The protein concentration in each sample is measured using a BCA reagent kit.

A 96 well plate is loaded with 10 µL of BSA standards in duplicates with concentrations of 0, 125, 250, 500, 1000, and 2000 (µg/mL). Similarly, 10 µL of the samples are loaded in duplicates. Exactly 200 µL of the BCA solution, prepared by adding reagents B and A (1:50 ratio), is added to each of the wells loaded with the BCA standards or the samples. The plate is incubated for 30 minutes at 37˚C and is read using a microplate reader (Biotek, Winooski, VT).

3.5.2 Western Blot-Gel electrophoresis

Samples are prepared in centrifuge tubes based on the BCA assay. Each sample is prepared in order to have the same amount of protein (~30 µg) loaded onto each well of an SDS-PAGE gel(sodium dodecyl sulfate polyacrylamide gel electrophoresis). A fixed amount of dye and water are also added to each sample to make up all the samples to the same final volume. The samples are heated for 5 minutes and then immediately loaded on the gel.The gel is run and at 56 mA until the blue dye front disappears from the gel.

3.5.3 Transfer of protein to PVDF membrane

The PVDF membrane is washed once in methanol and twice in water. The membrane and the gel are incubated in the transfer buffer for 15 minutes on a shaking rocker. The apparatus for the transfer is set-up and transfer can be conducted at 100V for 100 minutes using a 2 hour buffer (72.6g Tris Base, 75g Glycine, 4g SDS, 800 ml methanol- QS to 4L with water) or 20V for 16 hours at 4˚C using an overnight buffer (9.7g Tris Base, 44.7g Glycine- QS to 4L with water).

3.5.4 Membrane Blotting

The membrane is blocked for one hour in TBST (Tris-buffered saline with tween 20) containing 5% (w/v) non-fat dry milk (NFDM) on the rocker. Primary antibody (PARP and GAPDH) is added to fresh 5% non-fat dairy milk at a ratio 1:1000 dilution, and membrane is incubated in this solution for 3 hours at room temperature or overnight at 4˚C on the rocker. The membrane is then washed twice in TBST on the rocker for 5 min each. Secondary antibody is added to the appropriate blocking buffer (5% NFDM) in a 1:10,000 dilution membrane and is incubated in this solution for 1 hour at room temperature on the rocker. The membrane is then washed three times in TBST for 5 minutes each followed by one time in TBS (without tween) for 5 minutes on the rocker

3.5.5 Signal Detection

Enhanced chemiluminescence (ECL) Reagent is prepared by mixing 75 µL of reagent B(peroxide solution) and 3 mL of reagent A(luminal and enhancer). After the final wash excess TBS is blotted off the membrane and then ECL solution is added and incubated for a minute. The excess ECL solution is blotted off the membrane and the membrane is placed in the cassette. In the dark room, the signal is captured on the X-ray film and then developed.

Chapter 4: Analysis of Prostaglandin Standards and Validation of Protocols

The largest focus of this study was the development and validation of an LC-ESI-MS method for prostaglandins. The possible prostaglandin metabolites were either the J-series Prostaglandins which are Δ^{12} PG-J_2, PG-J_2 or 15-deoxy$\Delta^{12,14}$ PG-J_2 or their ethanolamide conjugates. Since the ethanolamide conjugated J-series prostaglandins are not available commercially, method development and validation were done using the unmodified J-series prostaglandins.

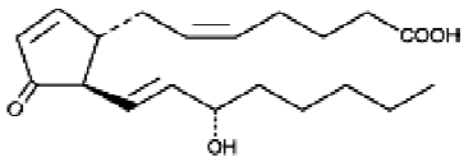
4.1 ESI-MS analysis of the unmodified J-series prostaglandins

Reference mass spectra of the three J-series prostaglandins (Δ^{12} PG-J_2, PG-J_2 and 15-deoxy-$\Delta^{12,14}$ PG-J_2) were obtained in negative ion mode ESI. Table 4.1 lists the *m/z* ratios of the deprotonated molecules and the common product ions of J-series

Table 4.1 Possible mass spectral fingerprints of the J-series prostaglandin ethanolamides

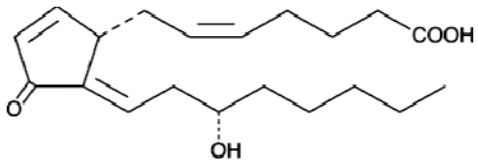
Molecule	*m/z* $(M-H)^-$	*m/z* Fragments of $(M-H)^-$
PG-J_2	333.2	315.2,271.2,189.2
15-deoxy-$\Delta^{12,14}$-PG-J_2	315.2	271.2, 189.2
Δ^{12}-PG-J_2	333.2	315.2, 271.2, 189.2

prostaglandins calculated using the monoisotopic masses up to one decimal place. Δ^{12} PG-J_2 and PG-J_2 are positional isomers, so they have the same *m/z* ratios.



$PG\text{-}J_2$

Exact mass 334.2



$\Delta^{12}\text{-}PG\text{-}J_2$

Exact mass 334.2

Figure 4.1.1 Structures of the two isomeric J-series Prostaglandins and their exact molar masses.

Prostaglandin ions fragment easily during transfer in the mass spectrometer, giving good signal intensity of the deprotonated molecule and the product ions. A mass spectrum showing significant product ions can be more informative than a spectrum showing just an intact ion. As seen in Figure 4.1.2, ESI-MS of Δ^{12} PG-J_2 and PG-J_2 yield mass spectra with the same deprotonated molecule and product ion peaks formed from in-source dissociation. Additionally, a significant number of peaks are observed in the mass spectra that are not related to the prostaglandin ions. They are a polymeric distribution of ions (separated by 14 m/z units) that are likely introduced from the solvents used.

4.2 ESI-MS-MS analysis of the unmodified J-series prostaglandins

ESI-MS-MS allows us to confirm the relationship among product and parent ions and remove the interfering signal from the solvent contamination. In an MS-MS experiment, a parent ion of interest, here the deprotonated molecule, is isolated and dissociated, resulting in the spectra shown in Figure 4.2.1. A peak at *m/z* 271 was observed in all MS/MS spectra. In Figure 4.2.1 (A) and (B), product ion peaks at m/z 189, 233, and 315 also were observed. Product ion structures are shown in Figure 4.2.2.[18] These *m/z* values can be used as markers for prostaglandins in our LC-ESI-MS spectra.

The isomeric prostaglandins were subjected to further ESI-MS-MS analysis at higher collision energy, expecting that these would dissociate differently resulting in the production of

unique product ions. However, the isomers were found to have identical product ions even at higher collision energies and no unique product ions were identified.

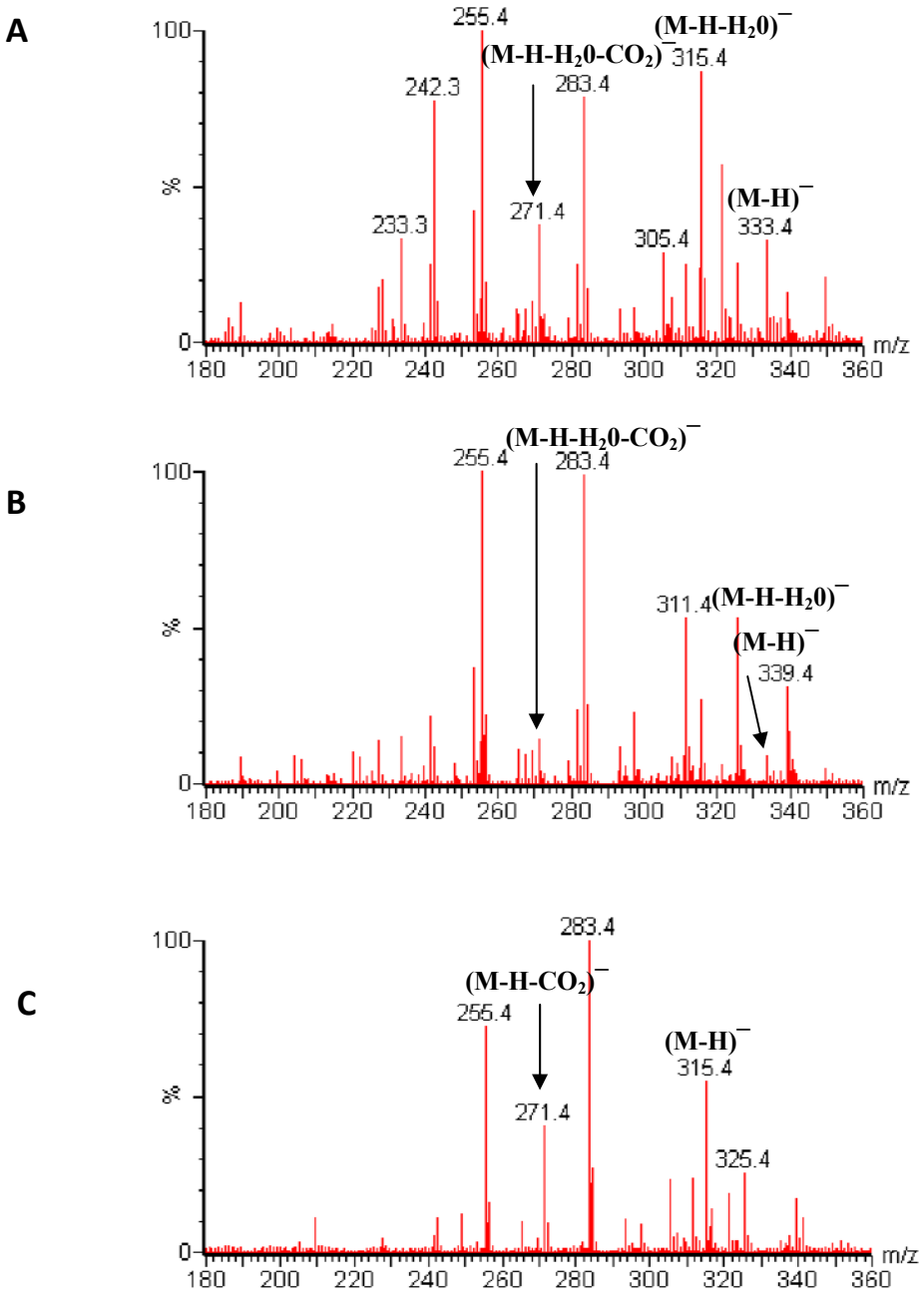


Figure 4.1.2 Negative-mode ESI-MS analysis of A) PG-J_2 B) Δ^{12} PG-J_2 and C) 15-deoxy$\Delta^{12,14}$ PG-

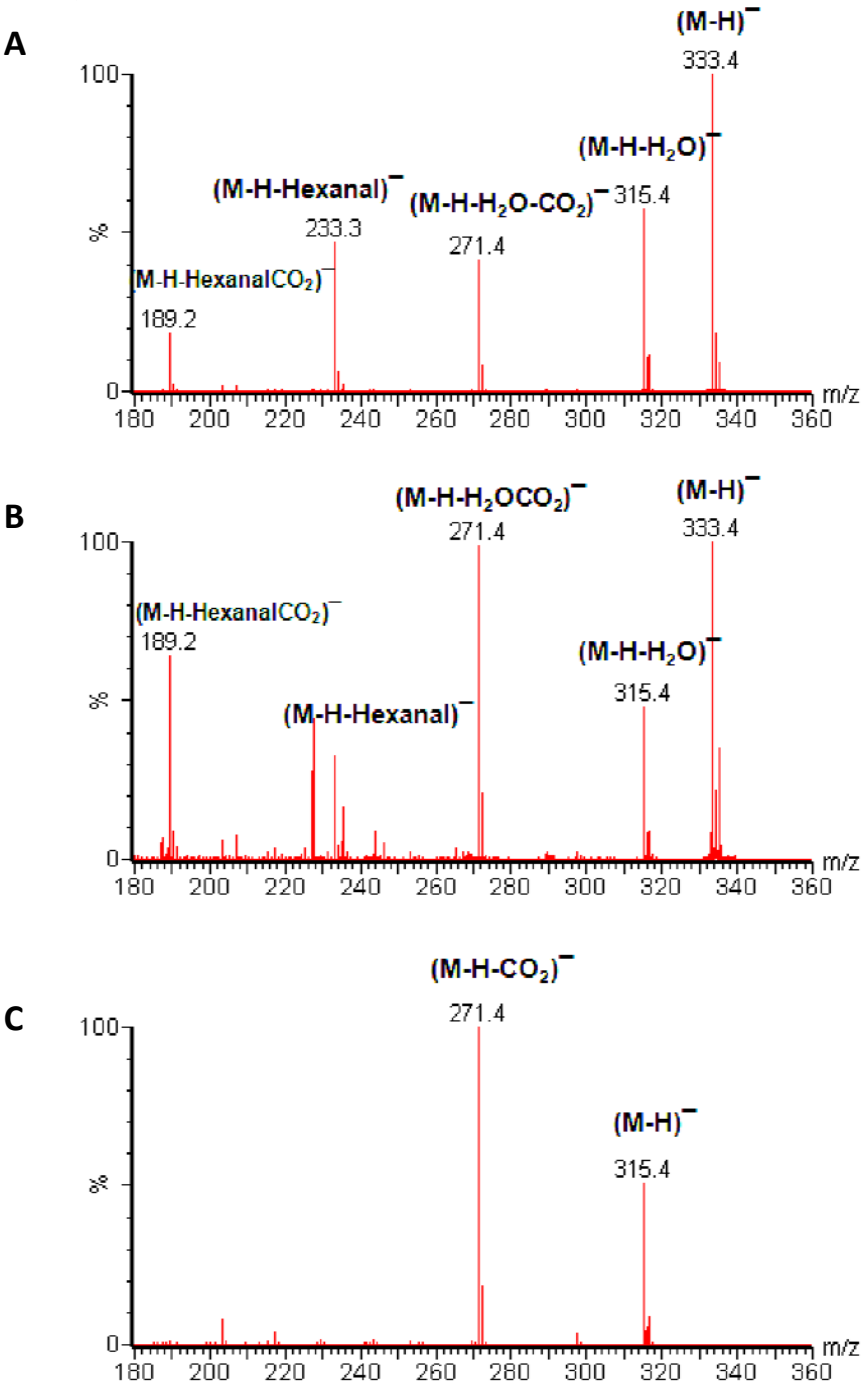


Figure 4.2.1 Negative mode ESI-MS-MS analysis of A) PG-J_2 B) Δ^{12} PG-J_2 and

C) 15-deoxy-$\Delta^{12,14}$ PG-J_2

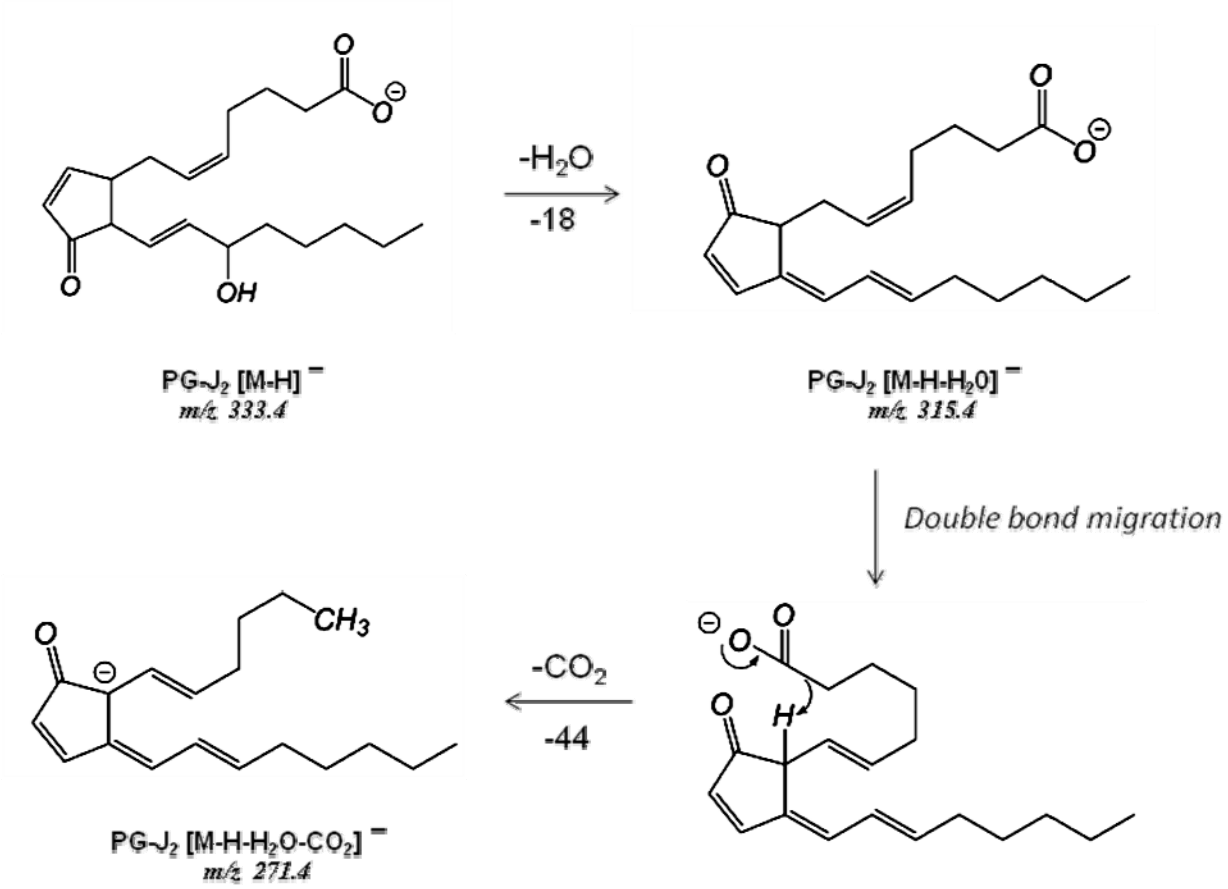


Figure 4.2.2 Structures of product ions formed from PG-J_2[18]

4.3 Method development with LC-ESI-MS for analysis of the prostaglandins

LC-ESI-MS can be used to first separate the prostaglandin molecules and then detect them using the mass spectrometer. LC removes unwanted biological material from entering the mass spectrometer, as well.

A sample containing a mixture of all three prostaglandin standards was analyzed. Figure 4.3.1 shows the most effective chromatographic separation of the isomers identified based on the *m/z* values detected and the ratios of concentrations of standards used to prepare the mixture.

However, resolution of the two isomers PG-J_2 and Δ^{12} PG-J_2 has been achieved only in this experiment; in most other experiments attempted, the isomers tend to co-elute because of their very close structural similarity compared to 15-deoxy-$\Delta^{12,14}$ PG-J_2.

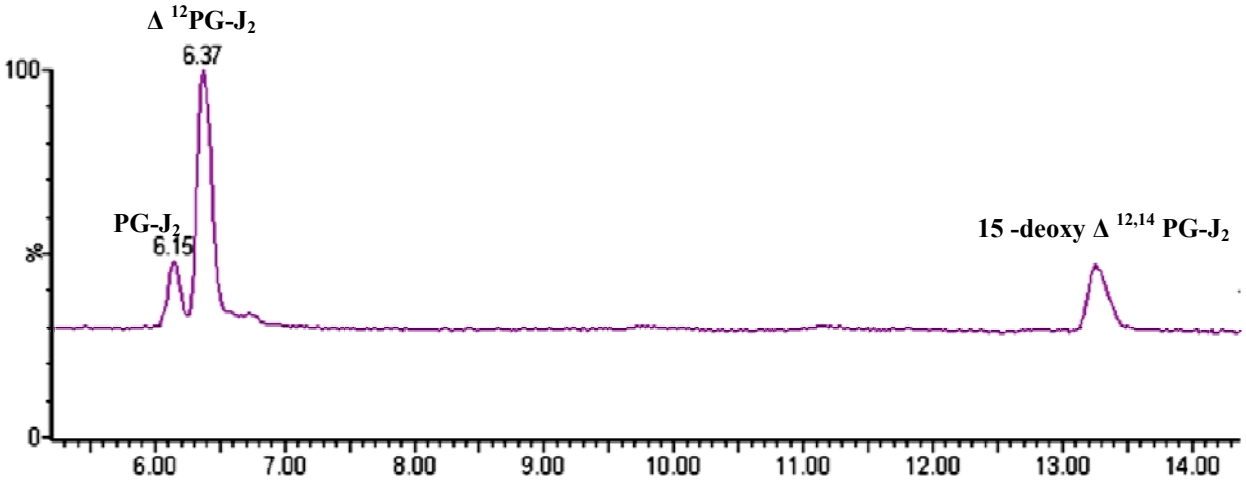


Figure 4.3.1 Demonstration of an LC separation of a mixture of J-series prostaglandins

A variety of reversed-phase gradient elution programs were developed in an attempt to efficiently separate the prostaglandins before introduction into the mass spectrometer. No separation of the two isomers was achieved; thus, the gradient elution that produced the sharpest peaks was chosen, where the mobile phase solvent was decreased from 65% to 50% A at 5%/minute, and then from 50% to 35% A at 7.5%/minute.

The LC-ESI-MS chromatogram of the mixture of the J-series prostaglandins shown in Figure 4.3.2A demonstrates separation of J-series prostaglandins typically achieved with the optimized gradient. It was observed that PG-J_2 and Δ^{12} PG-J_2 co-eluted first followed by the elution of 15-deoxy $\Delta^{12,14}$ PG-J_2. The mass spectra scanned across the peak eluting around 0.5-0.8 minutes did not have any prostaglandin related peaks. This chromatographic signal may be

attributed to either the sample matrix or the carryover from the column. The latter peaks were identified as the prostaglandin related based on the mass spectra scanned across the peaks. Mass spectra summed across the two chromatographic peaks are shown in Figure 4.3.2 (B) and (C). This gradient method was used for the validating the extraction technique and for the LC-ESI-MS analysis of the real-world samples.

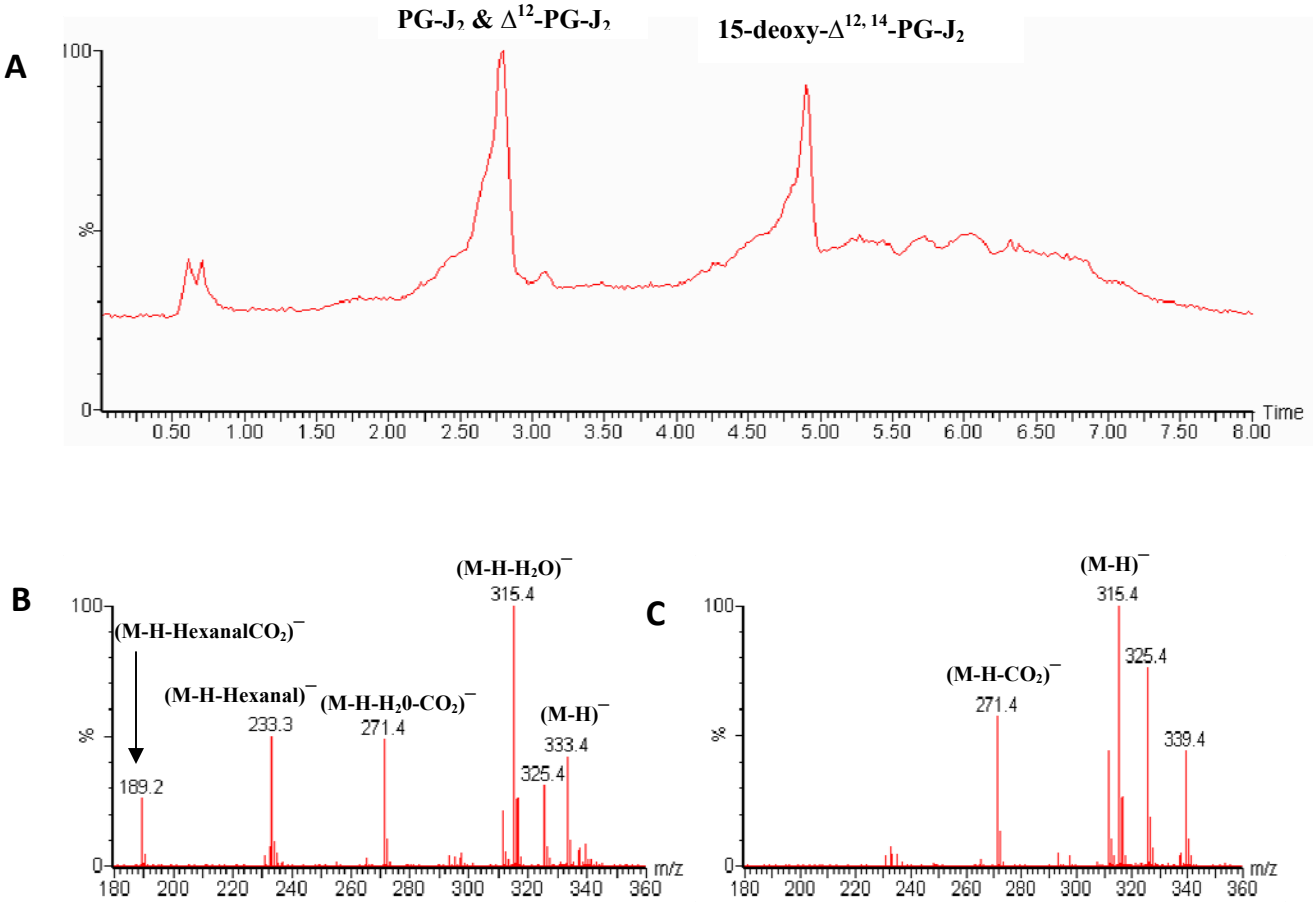


Figure 4.3.2 A) LC-ESI-MS chromatogram of the mixture of the J-series prostaglandins. LC-ESI-MS mass spectra across the peak identified as PG-J_2 & Δ^{12}-PG-J_2 (B), 15-deoxy-$\Delta^{12, 14}$-PG-J_2 (C)

4.4 Validation of the extraction technique

The extraction technique developed was validated first by extracting the spiked media and analyzing those using LC-ESI-MS. Later the real samples were also used to validate the extraction protocol using ELISA.

4.4.1 Validation of extraction protocol using LC-ESI-MS.

The extraction protocol was validated using both the spiked fresh media and spiked spent media. The fresh media is the media that has not been exposed to the cells, whereas the spent media is exposed to cells for 48 hours. By extracting the spiked fresh media, we initially wanted to check the efficiency of our extraction protocol. Later, the same protocol was used for validation with the spiked spent media to check if cells release anything into the media which ionize to give the same *m/z* ratio as that of the prostaglandin molecules. The fresh or the spent media was spiked with the known concentration of the unmodified J-series prostaglandins and extracted using the protocol discussed in chapter 3.4.1. After the extraction, the samples were subjected to LC-ESI-MS.

The LC-ESI-MS chromatograms shown in Figure 4.4.1 A and B demonstrate that the prostaglandins added to the media before extraction were recovered.. The mass spectral intensities of the prostaglandins were in reasonable agreement with the intensities obtained during an LC-ESI-MS analysis of the same mixture in acetonitrile, indicating the extraction protocol developed was efficient. Similar extraction efficiency was also obtained with the spiked spent media. Hence the extraction protocol was validated with the unmodified J-series prostaglandins using LC-ESI-MS.

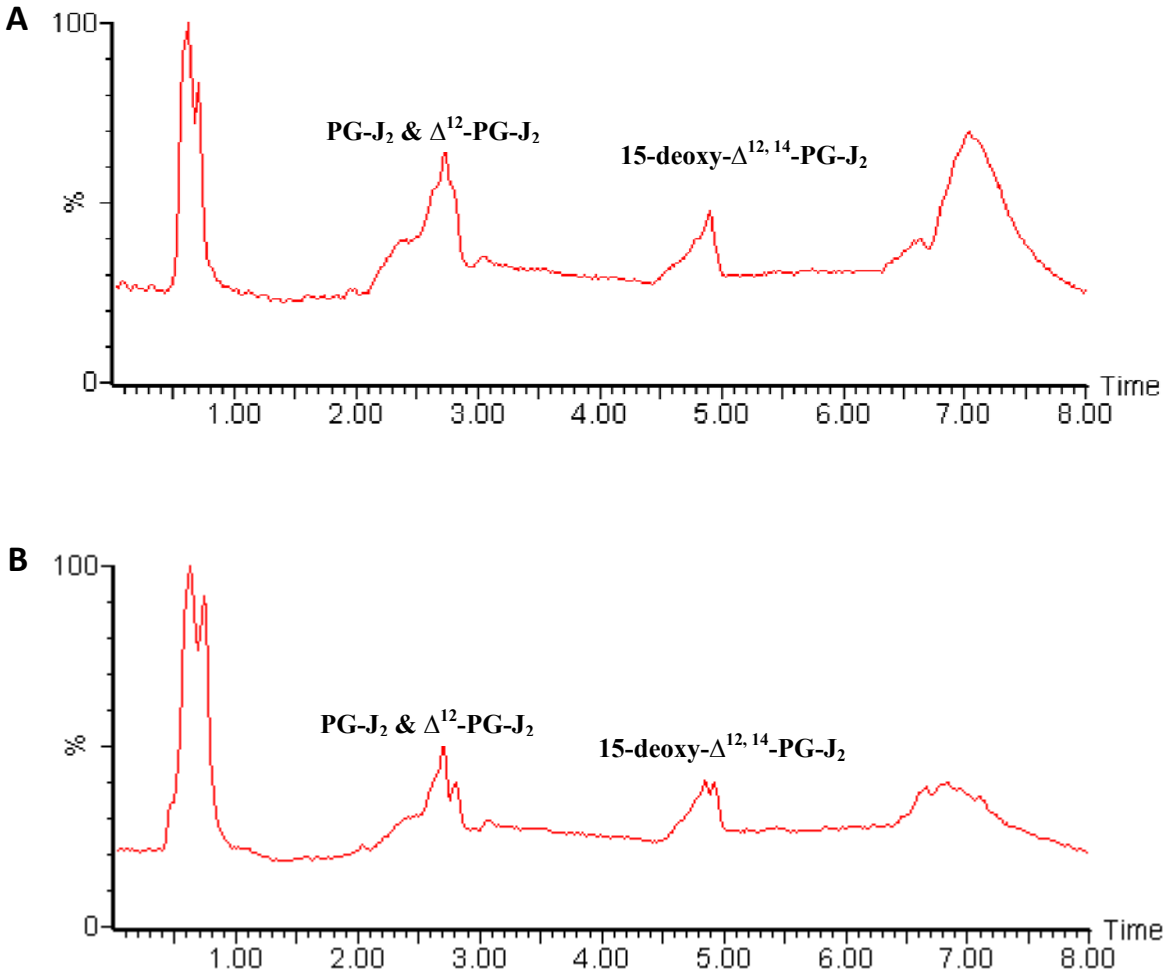


Figure 4.4.1 LC-ESI-MS chromatogram of the A) spiked fresh media after extraction B) spiked spent media after extraction

4.4.2 Validation of extraction protocol using ELISA.

If AEA metabolizes to ethanolamide conjugates of the J-series prostaglandins, the same extraction protocol might still need to be validated as the ethanolamides are structurally different from the unmodified ones. But, irrespective of the type of prostaglandins produced, Van Dross, et al[6], were able to quantify the J-series prostaglandins using ELISA. They have used the media to quantify the prostaglandins which was used to treat the cells with AEA. Similarly, we monitored all fractions during SPE with ELISA. The results from ELISA indicated that the final

eluent had a significant amount of prostaglandins and the other washes including the flow through from the column did not have any prostaglandins. Thus, prostaglandins were not being lost during washings or other extraction steps, and they were being eluted during the final elution step of SPE. The extraction protocol was validated for the prostaglandin metabolites which could be either the ethanolamide or the unmodified J-series prostaglandins.

Chapter 5: Identification of Prostaglandin-Ethanolamides

5.1 Analysis of AEA-treated cell media by LC-ESI-MS

After successful validation of the extraction technique using ELISA, the culture medium from the AEA-treated cells was extracted using the validated extraction protocol. Extraction was followed by evaporation and reconstitution in acetonitrile. The samples were then analyzed using LC-ESI-MS and LC-ESI-MS/MS. As already stated, because the J-series Prostaglandin-ethanolamides are not commercially available, the method development with LC-ESI-MS was done using unmodified J-series prostaglandin standards.

The table below summarizes the possible *m/z* ratios of the molecular ion $(M+EA-H)^-$ of the ethanolamide conjugates of the J-series prostaglandins. 'M' represents the exact molecular mass of the J-series prostaglandin and 'EA' stands for ethanolamide group.

Table 5.1 Possible Mass spectral fingerprints of the ethanolamide J-series prostaglandins

Molecule	*m/z*$(M+EA-H)^-$
PG-J_2	376.2
15-deoxy-$\Delta^{12,14}$-PG-J_2	358.2
Δ^{12}-PG-J_2	376.2

The following are the chromatograms acquired for the control and AEA-treated samples. The control here refers to the ethanol-treated cells instead of AEA. Ethanol is the solvent for AEA. The purpose of having a control is to check if ethanol, media or anything released from the cells into the media in the absence of the AEA ionize to give the *m/z* ratio similar to our analyte.

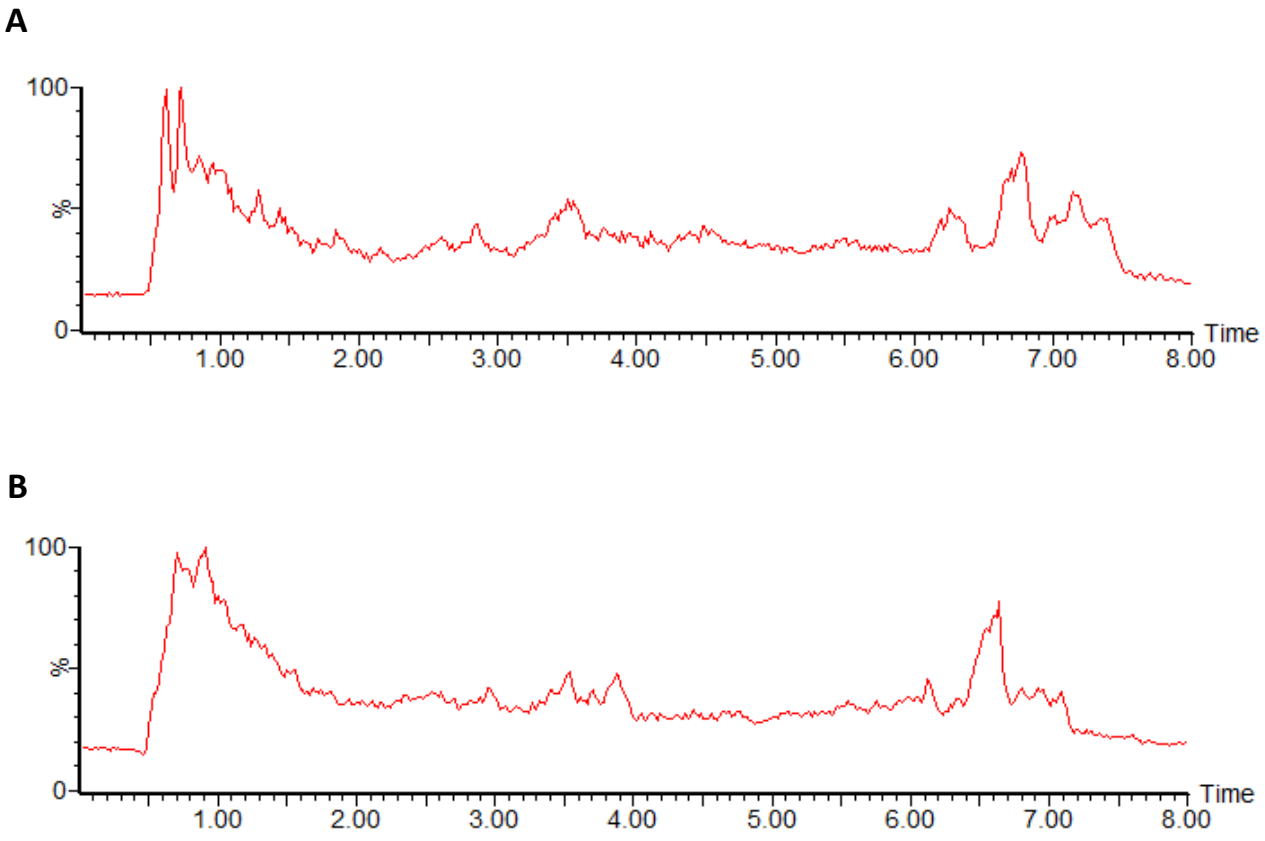


Figure 5.1.1 Total ion chromatograms of an LC-ESI-MS analysis of the media of cells treated with A) ethanol (control) and B) AEA.

The chromatograms were then extracted using a data processing tool to obtain an extracted ion chromatogram for the *m/z* ratio of one of the possible ions of the analyte. This process simplifies spectral interpretation by showing the intensity of only one selected *m/z* ion as a function of time. Extracted ion chromatograms were generated for the *m/z* values listed in Table 5.1, and signal consistent with ethanolamide conjugates of the J-series Prostaglandins was obtained at retention times of 0.5-3.0 minutes. Figure 5.1.2 (A) and (B) illustrate this process for the ethanol treated and AEA treated media. These are the extracted ion chromatograms, in which only the intensity of *m/z* 358 is plotted as a function of time.

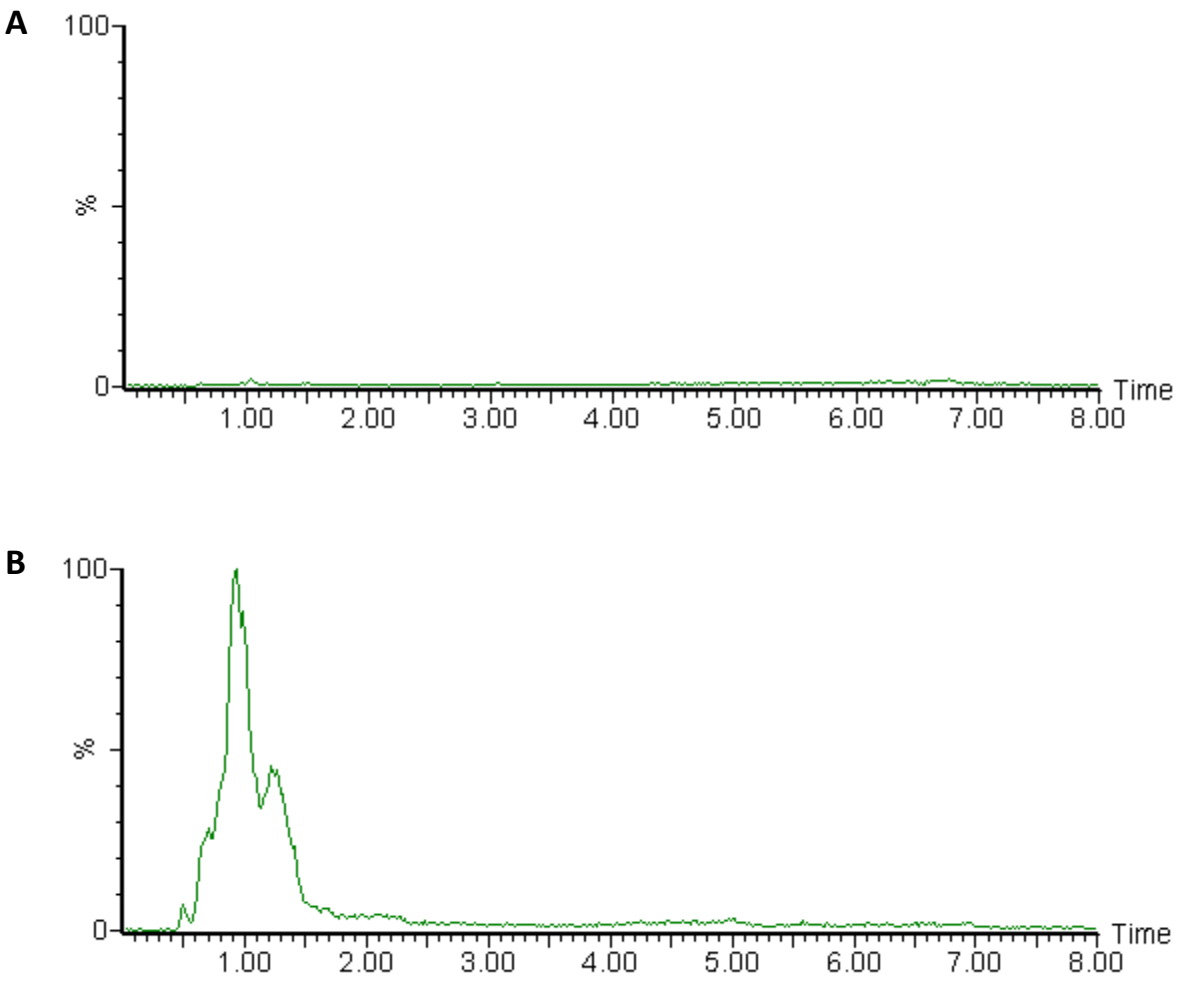


Figure 5.1.2 Extracted ion chromatogram of an LC-ESI-MS analysis of the media of cells treated with A) ethanol (control) and B) AEA.

The extracted ion chromatogram of the ethanol-treated media was normalized against the signal intensity of the *m/z* 358 of the AEA treated media. Figure 5.1.2(A) illustrates that all the signal intensity of *m/z* 358 shown in the Figure 5.1.2(B) is associated with the AEA treatment and none from the reagents or the cells. Also, the extracted ion chromatogram quickly illustrates to the user where to look for more mass spectral fingerprints. Mass spectra were summed over the time range indicated by *m/z* 358 signal in the extracted ion chromatogram in Figure 5.1.2(A) and (B). In Figure 5.1.3(B), in addition to signal arising from *m/z* 358, the ethanolamide

conjugate of 15-deoxy$\Delta^{12,14}$ PG-J_2, m/z 376 is also observed which corresponds to the ethanolamide conjugate of either prostaglandin isomer, Δ^{12} PG-J_2/PG-J_2.

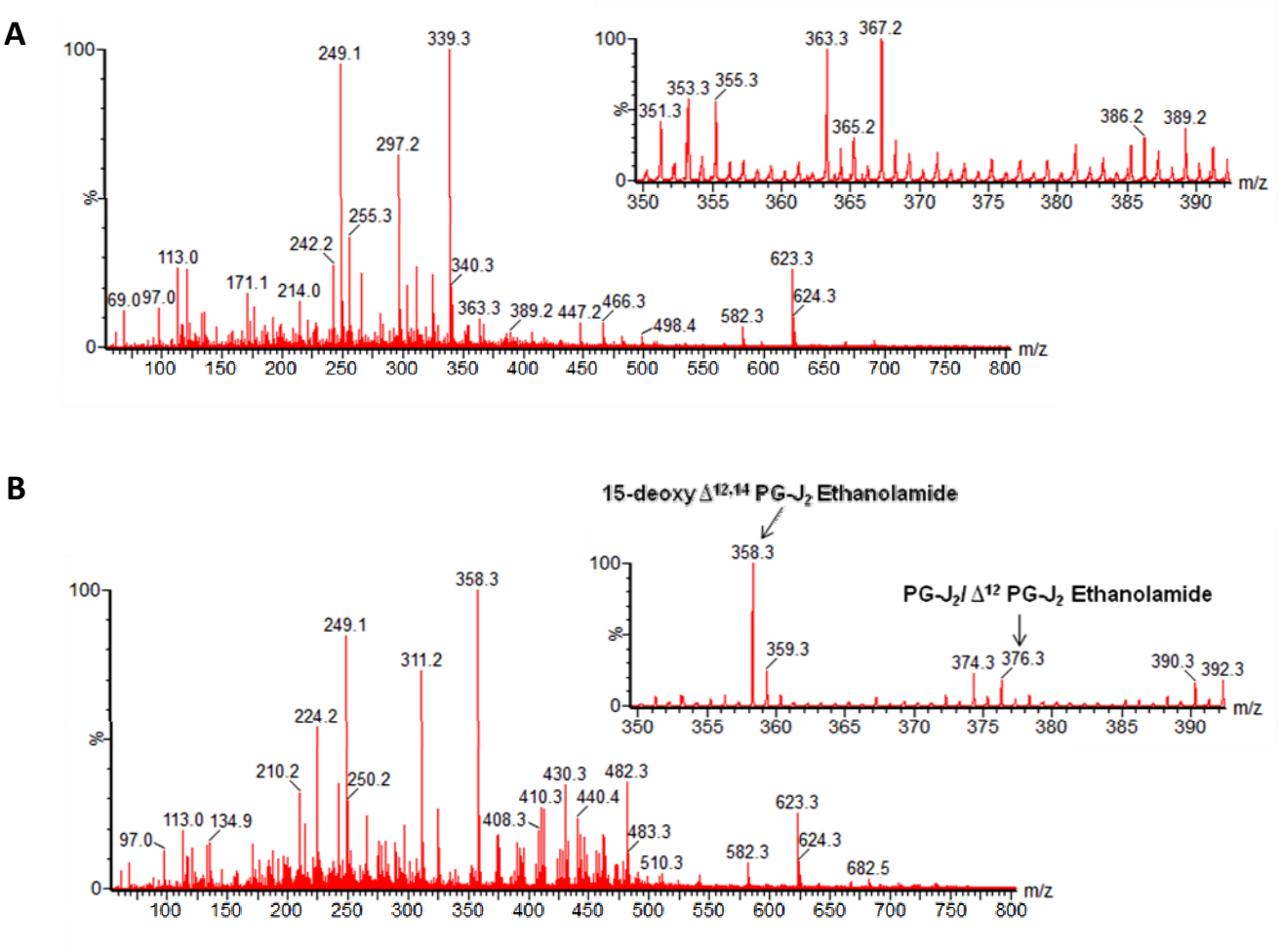


Figure 5.1.3 LC-ESI-MS analysis of the media of cells treated with A) ethanol (control) and B) AEA.

The *m/z* 358 and 376 peaks, corresponding to $(M+EA-H)^-$ ion of 15-deoxy$\Delta^{12,14}$ PG-J_2 and Δ^{12} PG-J_2/PG-J_2 respectively, were identified only in the mass spectrum of culture medium of AEA-treated cells as shown in the Figure 5.1.3(B). Further confirmation was essential to confirm the identity of the peaks as ethanolamide conjugates of the J-series prostaglandins. This was achieved by subjecting the identified peaks to LC-ESI-MS-MS analysis. As discussed earlier

in Chapter 2.2, the MS-MS was performed with collision induced dissociation. The parameter that is critical for a MS-MS analysis is the collision energy. Optimizing the collision energy to favor reasonable signal intensity of the parent and product ions is very crucial. As very high collision energy would cause scattering of the parent ion making its passage unlikely through the hexapole collision cell. On other hand very low collision energy would result in little to no dissocaition. This depends on the nature of the parent ion and collision gas used. In a QToF, collision energies ranging from 10-40eV were tested, and the collision energy for analyzing the ethanolamide conjugates was optimized to 15eV. The parent ions (*m/z* 376 and 358) were then subjected to CID at 15eV and the spectra were scanned across the peak eluting from the LC at 0.5-3.0 minutes.

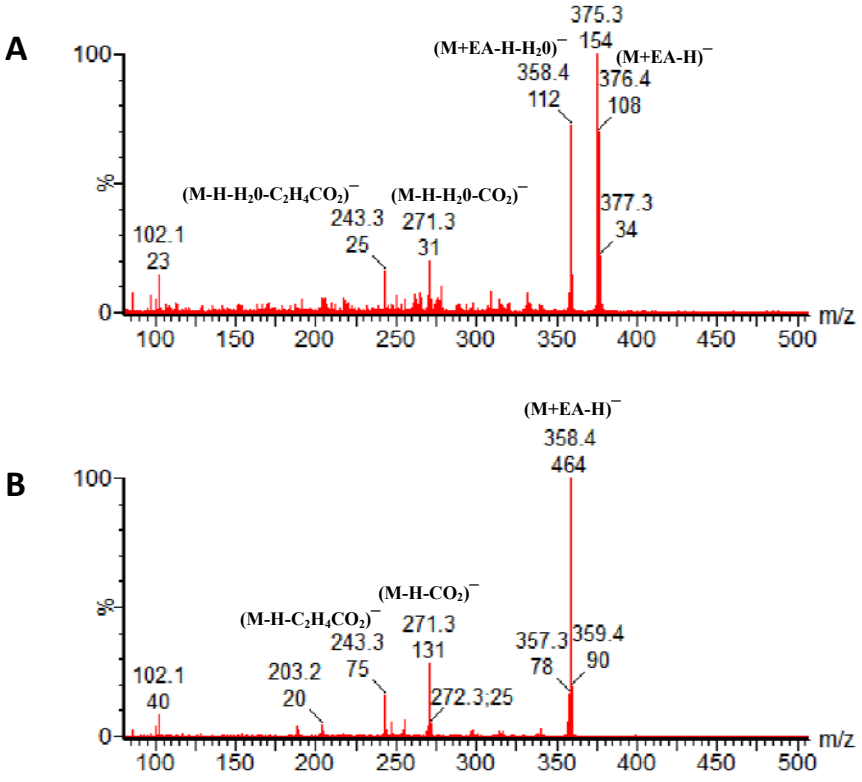


Figure 5.1.4 LC-ESI-MS/MS analysis of the parent ion A) m/z 376 B) m/z 358 identified as the ethanolamide conjugates of J-series in AEA treated sample.

The CID spectra shown in Figure 5.1.4 (A) of the parent ion peak (*m/z* 376) shows the relevant product ions at *m/z* 358 $[M+EA-H-H_2O]^-$, 271 $[M-H-H_2O-CO_2]^-$ and 243 $[M-H-H_2O-C_2H_4CO_2]^-$. Similarly, the CID spectrum of the parent ion (*m/z* 358) shows the product ion peaks at *m/z* 271 $[M-H-CO_2]^-$ and 243 $[M-H-C_2H_4CO_2]^-$. The observed product ions were consistent with the fragmentation pattern demonstrated by the J-series prostaglandins discussed earlier in chapter 4.2. For example, the product ion at m/z 271 was identified in the CID spectrum of the both the parent ions (*m/z* 376 and 358) which was also previously identified as the product ion of the J-series prostaglandins. Hence, the ion corresponding to m/z 376 is structurally confirmed as the deprotonated molecule of the ethanolamide conjugates of the PG-J_2 or /Δ^{12}-PG-J_2. The table below summarizes the *m/z* of the CID fragments identified by LC-ESI-MS-MS analysis of the ethanolamide conjugates.

As the *m/z* 358 was identified as one of the product ions in the CID spectrum of m/z 376, it was essential to investigate the source of this ion shown in the Figure 5.1.3 (B). One of the possibilities is that it could have been resulted from the fragmentation of *m/z* 376 during ion transmission in the LC-ESI-MS analysis. Another possibility is that it is 15-deoxy$\Delta^{12,14}$ PG-J_2 and was synthesized by the cells. To investigate this, LC-ESI-MS-MS analysis of *m/z* 376 was done performed at the same low collision energy, 5 eV, which was the typical operating collision energy during an LC-ESI-MS analysis. The mass spectrum obtained was compared to the one obtained from an LC-ESI-MS analysis. The objective of the analysis was to compare the intensities of the parent ion (m/z 376) and the product ion (m/z 358) with their corresponding intensities shown in the mass spectrum obtained by LC-ESI-MS analysis (Figure 5.1.5 B).

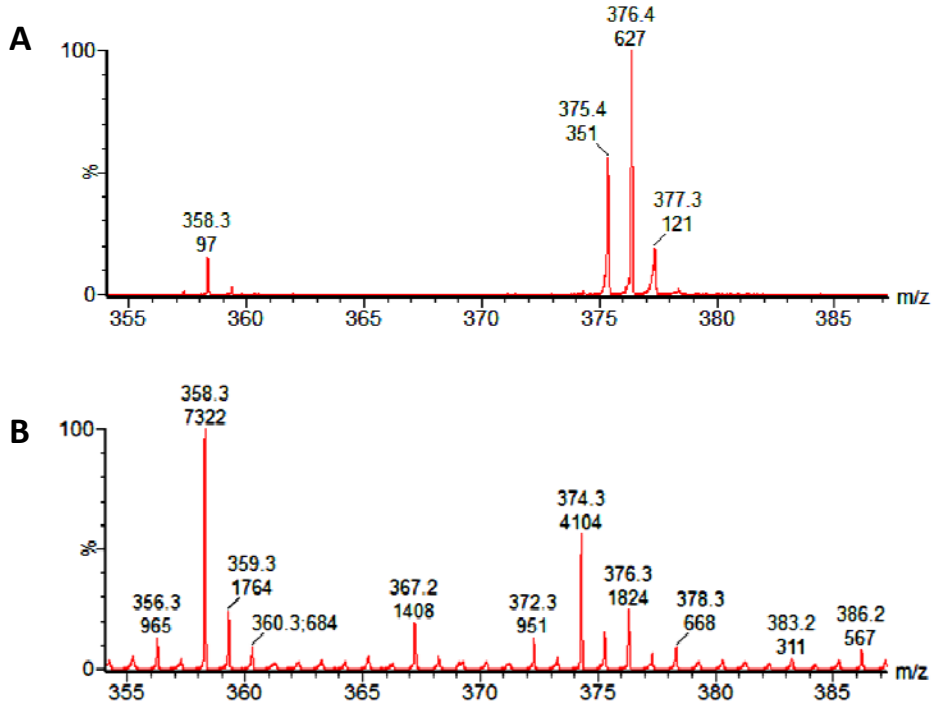


Figure 5.1.5 A) LC-ESI-MS/MS mass analysis of the parent ion (*m/z* 376) at collision energy 5eV B) LC-ESI-MS analysis of AEA treated sample at collision energy 5eV.

Figure 5.1.5A illustrates that the product ion (*m/z* 358) occurs to a limited extent at 5 eV collision energy (~15% relative intensity). On the other hand the mass spectrum obtained during an LC-ESI-MS analysis (Figure 5.1.5 B) demonstrates that the signal intensity of the *m/z* 358 is three fold higher the intensity of *m/z* 376. The high intensity of *m/z* 358 in the mass spectrum could not have been formed by CID of m/z 376 in the mass spectrometer Thus, the majority of signal from *m/z* 358 corresponds to 15-deoxy$\Delta^{12,14}$PG-J_2 that was synthesized by the cells.

Summarizing all the above data it can be concluded that AEA gets metabolized to both 15-deoxy$\Delta^{12,14}$ PG-J_2 ethanolamide and Δ^{12} PG-J_2/PG-J_2 ethanolamide by the JWF-2 cells. Besides the J-series ethanolamide prostglandins, the PGE_2-EA/PGD_2-EA and $PGF_{2\alpha}$-EA were

identified at m/z 394 and 396 respectively. However, MS/MS studies were not carried out for further confirmation.

5.2 Analysis of the effect of increasing dose of AEA on the production of the ethanolamide J-series PGs by LC-ESI-MS.

The goal of this experiment was to study the effect of increasing concentrations of AEA on the production of the ethanolamide J-series prostaglandins by the JWF-2 cells. As we did not use an internal standard in this experiment we were not able to quantify the ethanolamide J-series prostaglandins but we saw a reproducible pattern of how the dose of AEA affected the production of these prostaglandins.

On the day of experiment, cells were treated with 10, 20 and 30μM AEA or ethanol for 5 hours followed by extraction and concentration. The samples were analyzed using LC-ESI-MS. To monitor the intensity of the prostaglandin-related peaks in the LC-MS data, extracted ion chromatograms of m/z 358 and 376 were generated. Figure 5.2.1 shows these plots for m/z 358 for the control and for cells dosed with 10, 20, and 30μM AEA. The plots are normalized against the highest signal intensity of *m/z* 358 which was observed in the 30μM AEA-treated sample, to illustrate the increase in the signal intensity of *m/z* 358 with increasing AEA dosage.

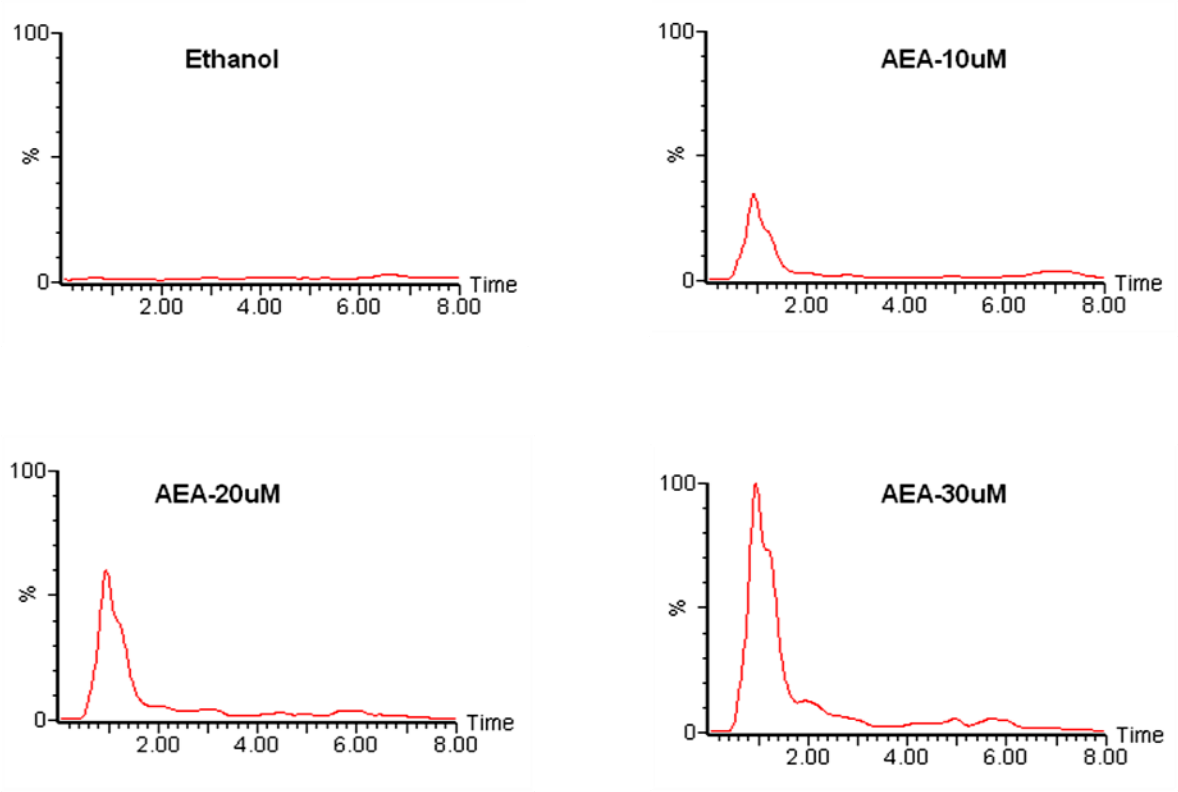


Figure 5.2.1: Normalized extracted ion chromatograms of AEA-treated samples showing the effect of increasing concentration of AEA on the levels of 15-deoxy $\Delta^{12,14}$ PG-J_2 ethanolamide(m/z 358)

The chromatograms indicate a direct correlation between the intensity of the molecular ion(*m/z* 358) of 15-deoxy $\Delta^{12,14}$ PG-J_2 ethanolamide and the dose of AEA. The experiment was done in duplicates and the same pattern was observed for the intensity of ethanolamide-conjugated 15-deoxy $\Delta^{12,14}$ PG-J_2 and Δ^{12} PG-J_2/PG-J_2. The statistics will be shown later in chapter 6.

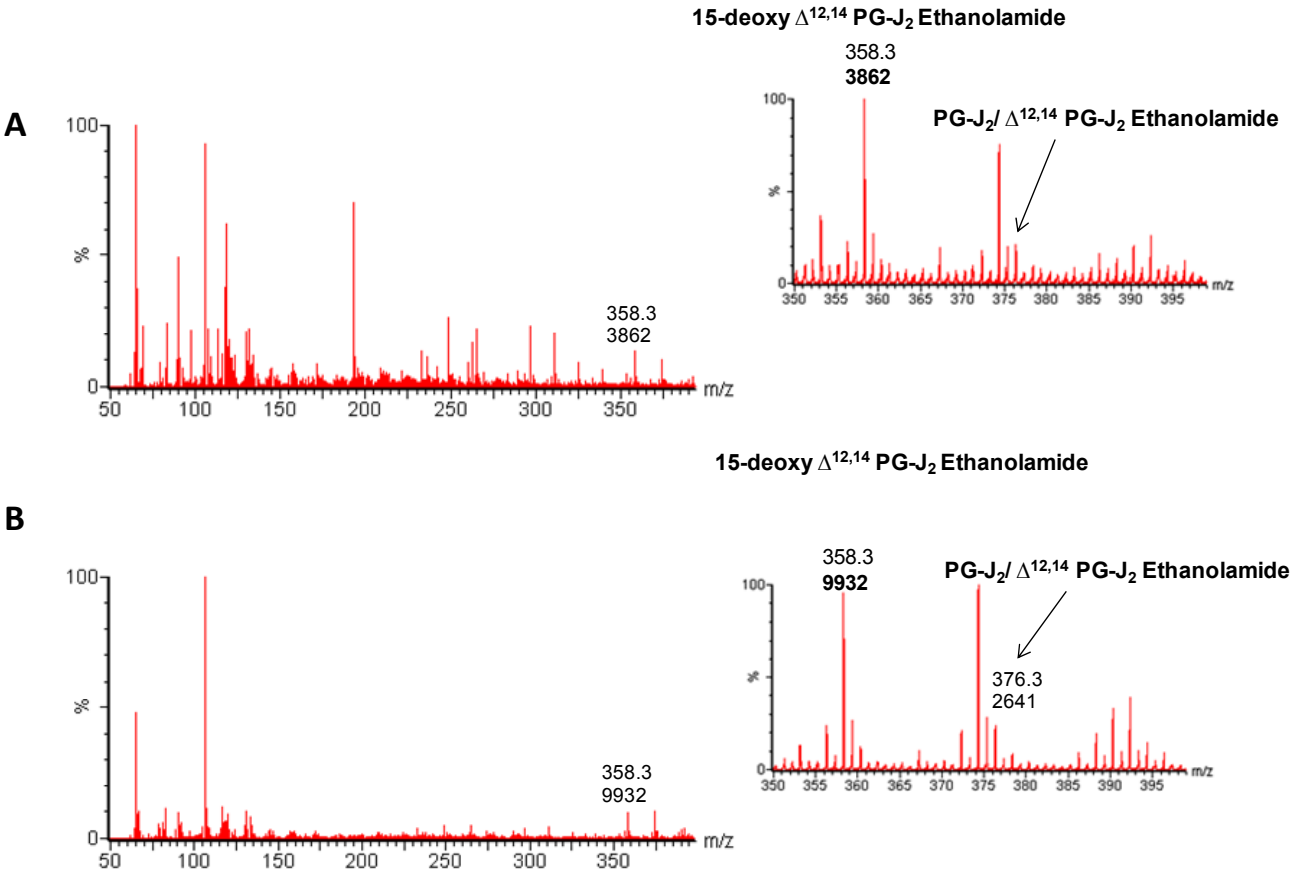


Figure 5.2.2 LC-ESI-MS analysis of A) 10µM B) 30µM AEA treated sample. The number on the top is the *m/z* of the depronated molecule of the ethanolamide J-series prostaglandins and the number below the *m/z* indicates the absolute intensity of the molecular ion.

The mass spectra obtained by scanning the chromatographic peak eluting at 0.5-3.0 minutes from LC shown in Figure 5.2.2 demonstrates the effect of an increasing dose of AEA treatment. The spectrum shows the relative and absolute intensities of both the ethanolamide J-series prostaglandins. It was observed that absolute intensity of the ethanolamide conjugates of the J-series increased with increase in the dose of AEA. Hence, the ethanolamide J-series prostaglandins were found to be produced in a dose-dependent manner.

5.3 Analysis of the N-acetyl cysteine conjugates of ethanolamide J-series PGs by LC-ESI-MS

N-acetyl cysteine(NAC) is an antioxidant that is known to minimize oxidative stress inside the cells similar to that of the glutathione. It was reported that by pretreating the cells with N-acetyl cytsteine followed by treatment with AEA or ethanol, the apoptosis induced by AEA was minimized[6]. The objective of this experiment was to investigate the mechanism by which NAC effects AEA induced apoptosis and determine if it was related to the production of NAC conjugates of the ethanolamide prostaglandins. This was achieved by subjecting the treated media for the by LC-ESI-MS analysis. The cells were treated as follows:

a) Water (pretreatment) and Ethanol(treatment)
b) NAC (pretreatment) and Ethanol(treatment)
c) Water (pretreatment) and AEA(treatment)
d) NAC (pretreatment) and AEA(treatment)

The treatments (a) and (b) are the controls used to monitor apoptosis by either the solvents or the NAC.

Table 5.3 Possible Mass spectral fingerprints of the NAC conjugates of the ethanolamide J-series prostaglandins

Molecule	$(M+EA-H)^-$	$(M+EA+NAC-H)^-$
PG-J_2	376.2	539.3
15-deoxy-$\Delta^{12,14}$-PG-J_2	358.2	521.3
Δ^{12}-PG-J_2	376.2	539.3

Table 5.3 lists the possible *m/z* ratios of the NAC conjugates of the ethanolamide conjugates. Spectra were obtained by averaging the scans across the chromatographic peak eluting around 0.5-3.0minutes from LC. The mass spectrum shows a peak at *m/z* 539 corresponding to the NAC conjugate of the Δ^{12} PG-J_2/PG-J_2 ethanolamide (Figure 5.3.1 B). The peak identified as NAC conjugate of Δ^{12} PG-J_2/PG-J_2 ethanolamide was subjected to LC-ESI-MS-MS analysis to obtain more specific structural information. This was essential to confirm the identity of the peak as the deprotonated molecule of the NAC conjugate of Δ^{12} PG-J_2/PG-J_2 ethanolamide.

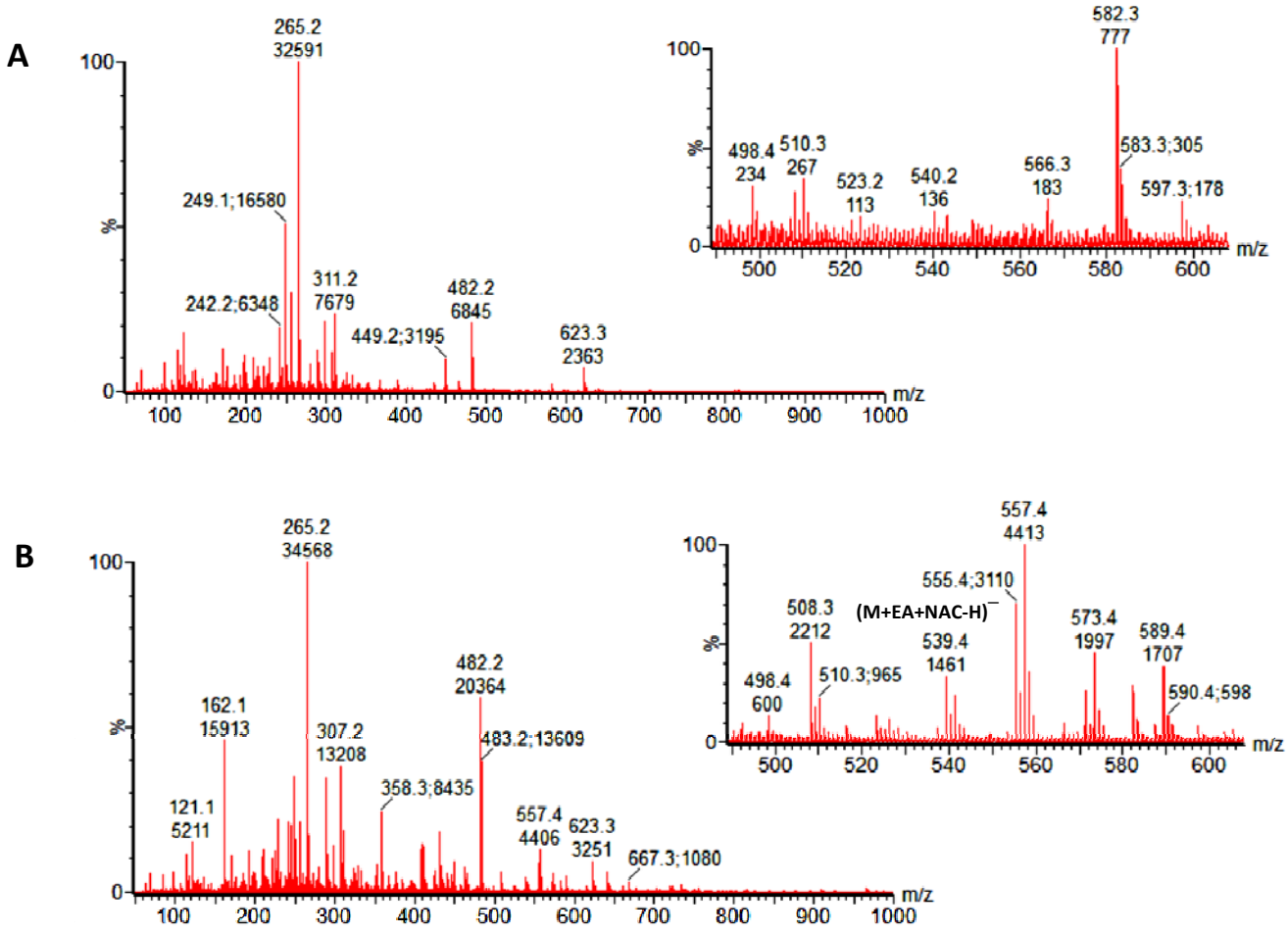


Figure 5.3.1 The LC-ESI-MS analysis of A) NAC and ethanol treated B) NAC and AEA treated samples.

The following is the CID spectrum (Figure 5.3.2) obtained for the parent ion (*m/z* 539) by scanning across the chromatographic peak eluting around 0.5-3.0 minutes. While the largest intensity product ion, m/z 162, is the deprotonated molecule of NAC, the spectrum shows product ions which are identified as prostaglandin-related peaks, such as *m/z* 358 and 271. Hence, the NAC conjugate of Δ^{12} PG-J_2/PG-J_2 ethanolamide has been identified and confirmed. The NAC conjugate of the 15-deoxy $\Delta^{12, 14}$ PG-J_2 ethanolamide was identified at *m/z* 520 in the mass spectra scanned across the chromatographic peak eluting at 4.0-5.0 minutes. However, the

LC-ESI-MS-MS analysis of the parent ion peak did not yield product ion peaks that could be attributed to 15-deoxy $\Delta^{12, 14}$ PG-J_2 ethanolamide. Hence, *m/z* 520 was not confirmed as a NAC conjugate of15-deoxy $\Delta^{12, 14}$ PG-J_2 ethanolamide.

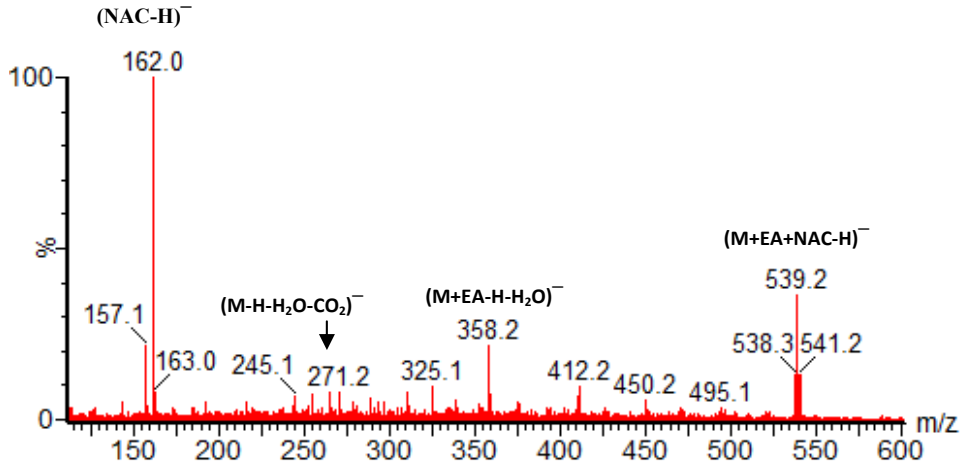


Figure 5.3.2 The LC-ESI-MS-MS analysis of the parent ion (m/z 539) of NAC and AEA treated samples.

5.4 .Analysis of the effect of COX-2 inhibition on production of the ethanolamide J-series PGs by LC-ESI-MS experiments

As mentioned in Chapter 1.3.1, the enzyme COX-2 plays a very important role in the metabolism of AEA to the pro-apoptotic ethanolamide J-series prostaglandins. The goal of this experiment was to investigate the role of the enzyme COX-2 in the production of the ethanolamide conjugates of the J-series prostaglandins by inhibiting this enzyme with a COX-2 inhibitor. A commercially available COX-2 inhibitor is NS-398.

The treatment was done as follows:

a) DMSO pretreatment and Ethanol treatment

b) NS-398(25uM) pretreatment and Ethanol treatment

c) NS-398(50uM) pretreatment and Ethanol treatment

d) DMSO pretreatment and AEA treatment

e) NS-398(25uM) pretreatment and AEA treatment

f) NS-398(50uM) pretreatment and AEA treatment

The treatments a, b and c were the controls used to monitor cell death induced by the inhibitor or the solvent. It was observed that the either the inhibitor or the solvent by itself did not induce any cell death. The mass spectrum obtained by the LC-ESI-MS analysis for treatment (d) showed a peak at m/z 358 and 376(data not shown), corresponding to the ethanolamide conjugates of the J-series prostaglandins. Alternately, the mass spectra obtained for the treatment (e) and (f) by the LC-ESI-MS analysis did not show any peaks related to the ethanolamide conjugates of the J-series prostaglandins. This indicates that, when COX-2 was blocked by a selective COX-2 inhibitor, the AEA was not metabolized to the ethanolamide prostaglandins. Hence, COX-2 is required for the production of ethanolamide conjugates of J-series prostaglandins. The experiment was done in duplicate and the data is found to be reproducible. The statistics will be discussed in Chapter 6.

Chapter 6: Results and Conclusions

6.1 AEA is metabolized to ethanolamide conjugates of J-series prostaglandins

Although it has been reported that AEA is metabolized to J-series prostaglandins by Van Dross, et al[6], the pathway of its metabolism was uncertain. To investigate the pathway, the samples were analyzed using LC-ESI-MS. Mass spectrometry can distinguish between the possible prostaglandins based on the *m/z* ratios of the ions, and MS/MS spectra can be used to verify the identity of the prostaglandins.

It was found that when JWF2 cells are treated with AEA, the cells metabolize AEA to ethanolamide conjugates of the J-series prostaglandins in the presence of the enzyme COX-2, as illustrated in the Figure 5.1.3(B). This enzyme plays a key role in metabolizing AEA to cytotoxic ethanolamide J-series prostaglandins which selectively induce apoptosis in tumor cells. The presence of the ethanolamide conjugates of the E-,F- and D-series and the absence of the unconjugated J-series prostaglandins confirms that AEA is metabolized to ethanolamide prostaglandins in pathway B, shown in Figure 6.1. Thus, AEA-induced cytotoxicity in tumor cells is mediated by the production of ethanolamide conjugates of J-series prostaglandins. This is the first time the production of ethanolamides of J-series prostaglandins has been reported.

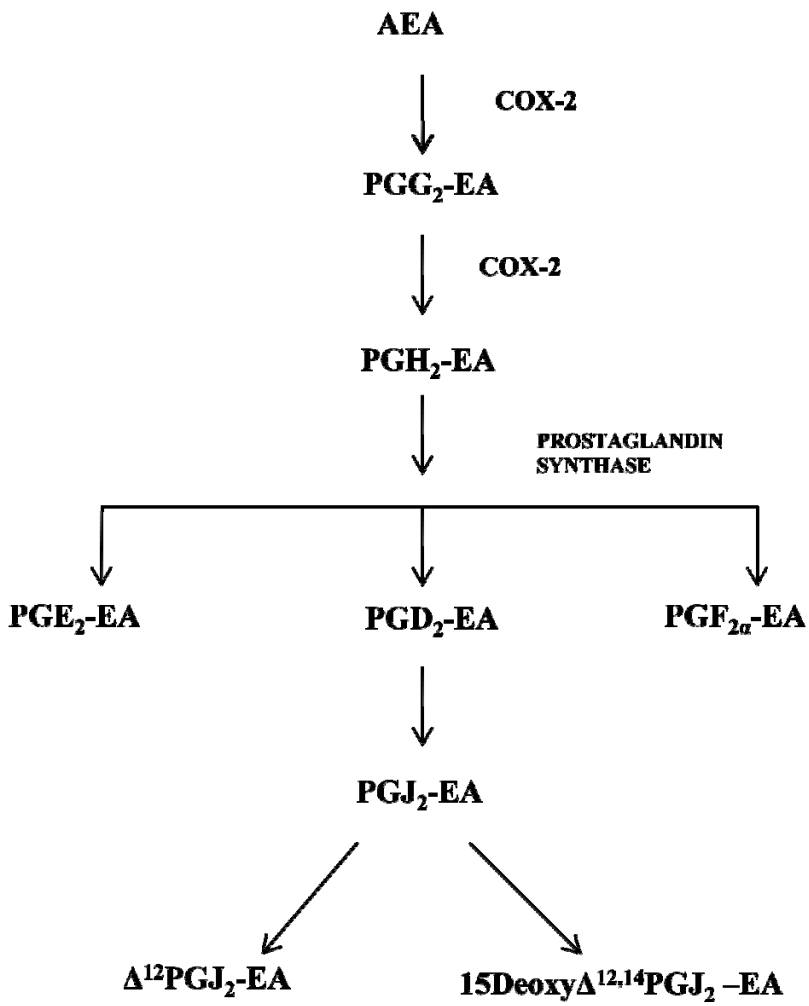


Figure 6.1 AEA is metabolized by COX-2 followed by prostaglandin synthase D to D-series ethanolamide prostaglandins, which undergoes dehydration to ethanolamide conjugates of J-series prostaglandins

6.2 AEA is metabolized to ethanolamide conjugates of J-series prostaglandin in a dose-dependent manner

It was observed that with increasing concentration of AEA, the absolute mass spectral intensity of both the Δ^{12} PG-J_2/PG-J_2 (*m/z* 376) and 15-deoxy$\Delta^{12,14}$ PG-J_2 (*m/z* 358) ions increased. Recall that *m/z* 358 is a possible product ion from *m/z* 376, but under the conditions used in a typical LC-ESI-MS experiment, the production of *m/z* 358 from *m/z* 376 is small.

Thus, the intensity of *m/z* 358 may be identified as 15-deoxy$\Delta^{12,14}$ PG-J_2 and monitored as direct evidence of the effects of increasing AEA dosage.

Figure 6.2 illustrates increase in mass spectral intensity of *m/z* 358 and 376 with increasing AEA dosage. Error bars represent standard deviations of two measurements. The trend of increased prostaglandin production from increased AEA concentration is present in all experiments, but the standard deviations were large. This may be because biological systems often fluctuate in terms of their growth and the physiological response to a drug. The nature of response can be consistent but the extent of response may differ. In addition, mass spectral intensity varies as a function of the efficiency of ionization, transmission, and detection, all of which may not be reproducible among experiments. Hence, it can be concluded that AEA metabolizes to ethanolamide J-series prostaglandins in a concentration dependent manner.

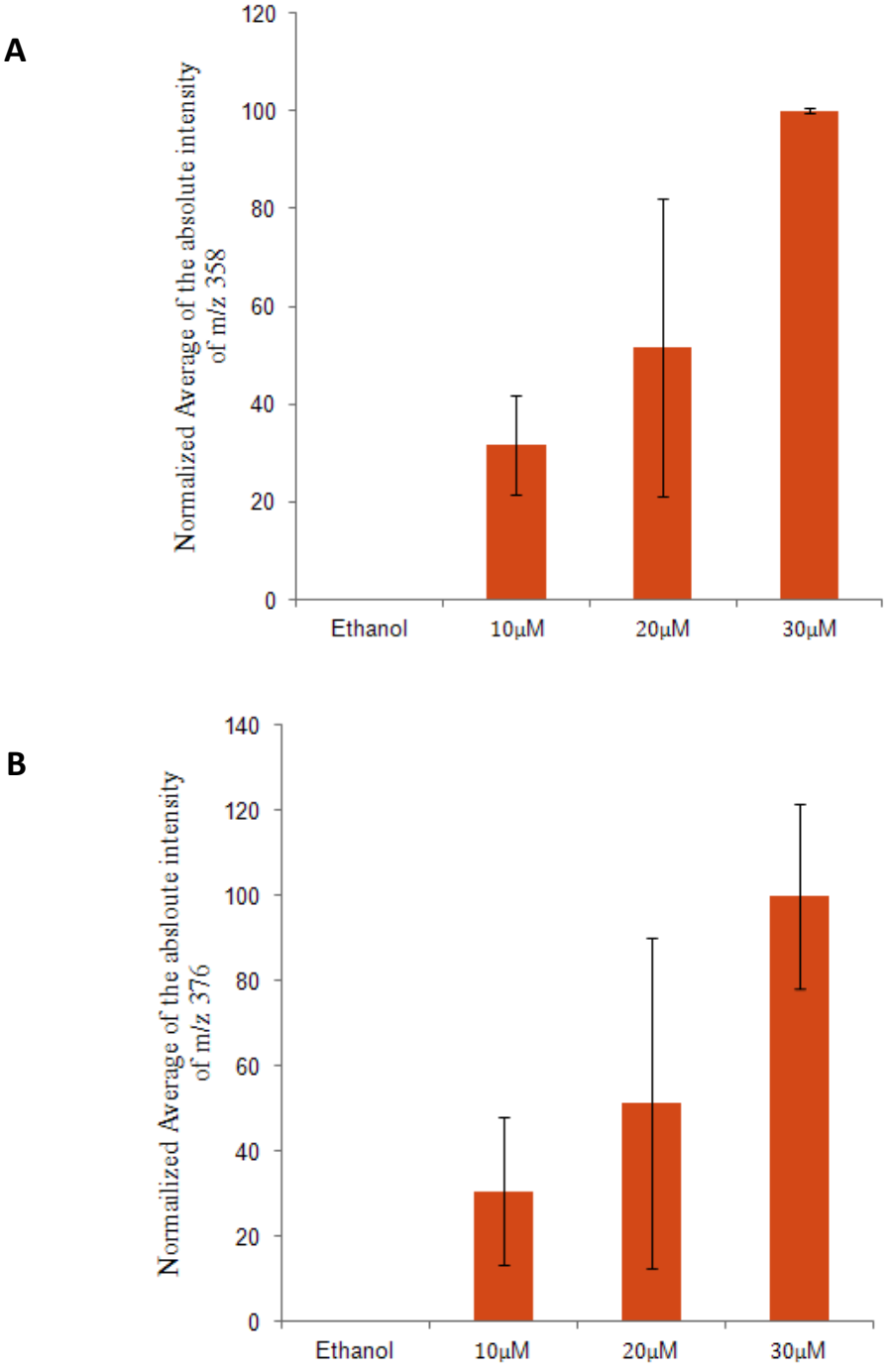


Figure 6.2 Effect of increasing concentration of AEA on the relative intensity of ion (m/z 358) of 15-deoxy $\Delta^{12,14}$ PG-J_2 ethanolamide (A), (m/z 376) of the Δ^{12} PG-J_2/PG-J_2 ethanolamide (B).

6.3 N-acetyl cysteine conjugates with PG-J_2 / Δ^{12} PG-J_2 ethanolamide.

It was reported that when the cells were pretreated with NAC followed by AEA, that NAC inhibited AEA induced apoptosis[6]. As discussed earlier in Chapter 5.3, NAC was found to conjugate with the PG-J_2 / Δ^{12} PG-J_2 ethanolamide as illustrated in the Figure 5.3.2(B).It can be concluded that NAC inhibits AEA induced apoptosis by conjugating with PG-J_2 / Δ^{12} PG-J_2 ethanolamide.

6.4 Metabolism of AEA to Ethanolamide J-series Prostaglandins is inhibited by NS- 398(COX-2 inhibitor)

NS-398 is a selective inhibitor of COX-2 which plays a key role in the metabolism of AEA to ethanolamide conjugates of J-series prostaglandins. It is observed that when the cells were pretreated with NS-398 followed by AEA, the production of the prostaglandins was inhibited. Fig. 6.4.1 illustrates this by plotting the absolute signal intensity of the m/z 358 against the treatment.

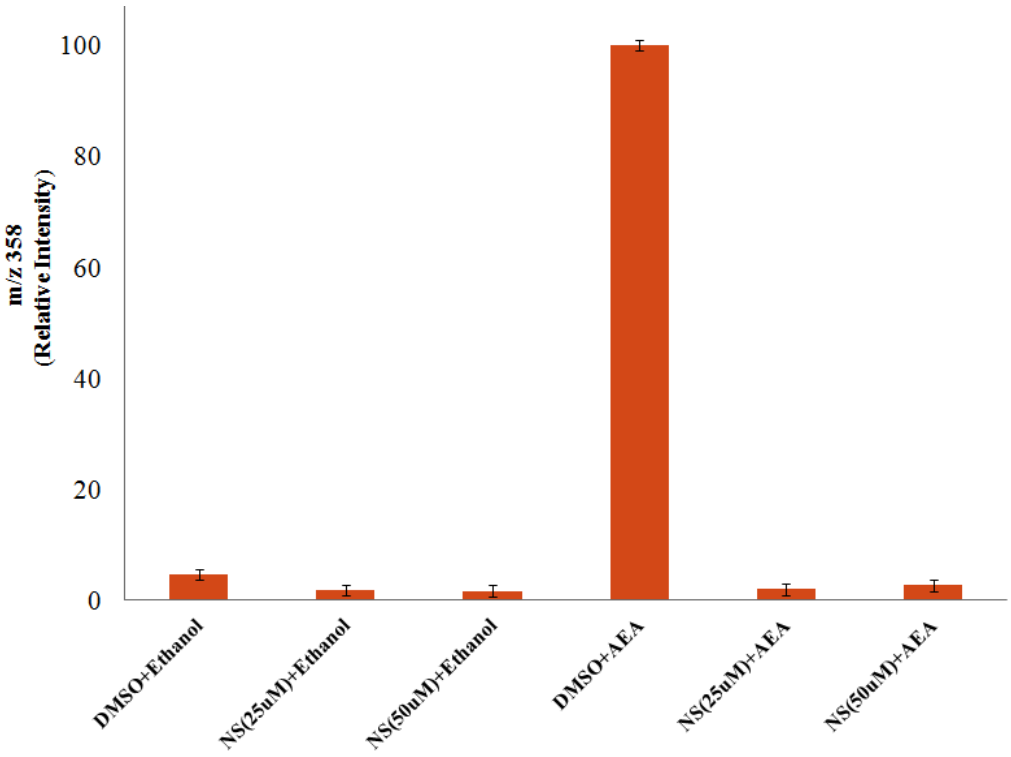


Figure 6.4.1 Effect of COX-2 inhibitor on the relative intensity of the molecular ion peak (m/z 358) of 15-deoxy $\Delta^{12,14}$ PG-J_2 ethanolamide

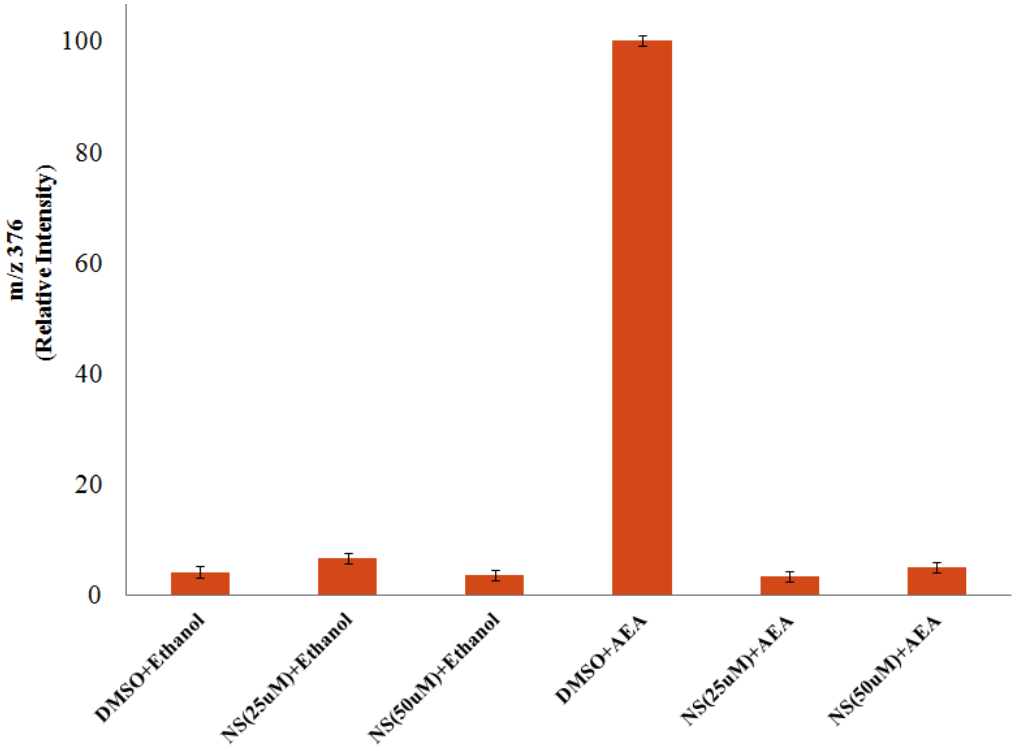


Figure 6.4.2 Effect of COX-2 inhibitor on the relative intensity of the molecular ion peak (m/z 376) of PG-J_2 / Δ^{12} PG-J_2 ethanolamide.

These results indicate that when the enzyme COX-2 is inhibited, AEA does not get metabolized to ethanolamide prostaglandins of J-series. Hence, AEA gets metabolized to ethanolamide conjugates of J-series prostaglandins in COX-2 dependent manner.

Chapter 7: Future Work

7.1 Quantification of the ethanolamide J-series Prostaglandins

Although we could identify the AEA metabolites as ethanolamide conjugates of the J-series prostaglandins, quantifying them using either ELISA or LC-ESI-MS is still a big challenge. As the conjugates are not available commercially, a calibration curve cannot be generated by either technique for quantification. Custom ethanolamide derivatives can be synthesized from the related carboxylic acid and amine compounds using standard coupling reagents in the laboratory of colleague Dr. Colin Burns in the Department of Chemistry at ECU. This synthesis was attempted in 2010, but only very small volumes of reactants were available which evaporated very quickly before mixing and reacting. If future attempts are successful, the synthesized ethanolamide conjugates along with a deuterated internal standard to monitor chromatographic performance can be used to quantify ethanolamide conjugates with LC-ESI-MS[19,20].

7.2 Investigation of the intracellular levels of ethanolamide J-series Prostaglandins

The ethanolamide conjugates of the J-series prostaglandins released into the media were extracted and analyzed successfully by LC-ESI-MS. But, the actual amount of these ethanolamide conjugates synthesized by the cells and the extent to which they are released into the media is not known. This can be studied by extracting the cells for prostaglandins followed by LC-ESI-MS analysis. For analyzing the intracellular levels of ethanolamide conjugates, developing an efficient extraction protocol would be the most challenging part because of the interfering cellular matrix. Although difficult, if the intracellular levels of these prostaglandins are determined it would really help understand the mechanism of action of AEA more clearly.

7.3 Investigation of the production of glutathione conjugates of the ethanolamide J-series prostaglandins

Many studies have reported that when J-series prostaglandins are incubated with Glutathione under physiological conditions[21,22], it resulted in the formation of glutathione conjugates of the J-series prostaglandins. Also, Murphey, et al, have demonstrated that when breast cancer cells were treated with15-deoxy $\Delta^{12,14}$ PG-J_2, the glutathione conjugate of 15-deoxy $\Delta^{12,14}$ PG-J_2 was produced by the intact cells. Hence, the formation of glutathione conjugates of the J-series prostaglandins has been reported both in vivo and vitro. The ethanolamide conjugates of J-series prostaglandins also have an α, β-unsaturated carbonyl moiety that can readily conjugate with the thiol groups on glutathione similar to the unmodified J-series prostaglandins. Thus, the production of glutathione conjugates of the ethanolamide J-series prostaglandins can also be investigated similarly by incubating the cells with AEA and glutathione under physiological conditions. The cells after incubation can be extracted for the glutathione conjugates of the ethanolamide prostaglandins followed by LC-ESI-MS analysis.

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