UNIVERSITÉ DU QUÉBEC À MONTRÉAL

DEVELOPMENT OF ANALYTICAL METHOD FOR TRACE LEVEL ANALYSIS OF RETINOIC ACID

MASTER THESIS
IN PARTIAL FULFILMENT OF REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE IN CHEMISTRY

BY
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DÉVELOPPEMENT D'UNE MÉTHODE D'ANALYSE DE TRACE DE L'ACIDE RÉTINOÏQUE

MÉMOIRE

PRÉSENTÉ
COMME EXIGENCE PARTIELLE
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PAR
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AVERTISSEMENT

AVANT PROPOS

Ce projet mis en avant par un groupe de recherches de l'UQAM vise à évaluer le dosage des rétinoïdes par la chromatographie liquide à haute performance (CLHP) et il est repris par les équipes de recherche de professeur Breau et de professeur Tra du département de chimie de l'UQAM.

Plus précisément, l'objectif de mon projet était l'analyse au niveau de trace le tout-*trans* acide rétinoïque (***tt*AR**) et ses métabolites dans un milieu physiologique (milieux de culture de cellules P19 de carcinome embryonnaire de souris). Bien que les résultats n'aient pas atteint le niveau attendu et que certaines limites expérimentales aient été rencontrées, cette étude a permis la mise sur pied d'un protocole de quantification au niveau de trace par la méthode sensible de CLHP-ESI-SM/SM et aussi un protocole d'extraction optimisé dans un milieu physiologique simulé. Ce protocole pourra être utilisé pour étudier l'effet de concentration de l'acide rétinoïque sur sa voie métabolique et les anomalies résultant d'un manque ou d'un excès de rétinoïde qui ont été observées chez les animaux ou chez l'homme. Cette méthode fournirait une sensibilité nécessaire pour la quantification de l'**AR** afin élucider la fonction de l'**AR** dans la neurogénèse, la morphogénèse et la contribution des altérations de l'homéostasie de l'**AR** telles que celles observées dans la maladie d'Alzheimer, le diabète du type 2, l'obésité et le cancer.

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LIST OF ABREVIATION SIGLES AND ACRONYMES

at	all-*trans* Isomer
αMEM	Alpha Minimum Essential Medium
ACS	American Chemical Society
ACN	Acetonitrile
HOAc	Acetic Acid
B.C.	Buffer Capacity
CE	Collision Energy
CID	Collision-Induced Dissociation
DISP	Direct Infusion Syringe Pump
ESI	Electrospray Ionization
EtOAc	Ethyl Acetate
FBS	Fetal Bovine Serum
GC-MS	Gas Chromatography Mass Spectroscopy
HCl	Hydrochloric Acid
Hex	Hexane
HPLC	High Performance Liquid Chromatography
HPLC-UV	High Performance Liquid Chromatography with Ultra Violet Detector
HPLC-FLU	High Performance Liquid Chromatography with Fluorescence Detector
HPLC-ESI-MS/MS	High Performance Liquid Chromatography with Tandem Mass Spectroscopy Using Electrospray Ionization
ID	Internal Diameter
LLE	Liquid-liquid Extraction
LOD	Limit of Detection
MS	Mass Spectroscopy
MW	Molecular weight
P/D	Particular Diameter

RA	Retinoic Acid (Acide Rétinoïque)
RAL	Retinal
RAR	Retinoic Acid Receptor
RXR	Retinoid X Receptor
NH_4OAc	Ammonium Acetate
L/h	Litres per hour
NaOAc	Sodium Acetate
S/N	Signal-to-Noise

LIST OF SYMBOLS

A.U.	Arbitrary Unit
eV	electron Volt
%	Percentage
°	Degree
°C	Degree, Celsius
C	Concentration
D	Density
g	gram
µL	Microlitre
µg	Microgram
M	Molar
mg	Milligram
min	Minute
mL	Milliliter
mB	Millibar
SD	Standard Deviation
V	Volt
v	volume
v/v	volume per volume
fmole	Femto Mol (10^{-15} mol)
ppb	Part Per Billion
ppm	Part Per Million
m/z	Mass to Charge
N	Normality

RÉSUMÉ

L'acide tout-*trans* rétinoïque (*tt***AR**) est un métabolite actif de la vitamine A qui a de nombreuses fonctions physiologiques importantes. Le *tt***AR** contrôle la prolifération et la différenciation cellulaire, il est essentiel pour le développement et la croissance des organismes. Il agit sur l'expression génique.

Le *tt***AR** a plusieurs propriétés pharmacologiques et physiologiques utiles en dermatologie et dans le traitement de différents cancers sensibles aux rétinoïdes. Par exemple, la leucémie promyélocytaire aiguë est sensible au *tt***AR** avec un taux de réponse de 90%. Un niveau anormal du *tt***AR** peut permettre ou provoquer la dégénérescence épithéliale, comme la xérophtalmie et des troubles neurologiques comme dans la schizophrénie et les maladies, d'Huntington, de Parkinson et d'Alzheimer.

Une méthode sensible pour la quantification au niveau de trace du *tt***AR** dans le milieu aqueux et un milieu de culture cellulaire supplémenté en sérum a été développée avec succès. La méthode consiste en un chromatographe liquide à haute performance (CLHP). La quantification a été effectuée sur une colonne de séparation C18 avec élution isocratique dans un temps total d'élution de 25 minutes avec un ionisateur par l'électronébulisation (ESI) en mode négatif et couplée à un analyseur comprenant deux spectromètres de masse triple quadripolaire en tandem (SM/SM). Un mélange d'acétonitrile et de tampon d'acétate d'ammonium 0.1 M a été utilisé comme phase mobile pour faciliter la formation de paires d'ions au cours de l'électronébulisation.

Dans les conditions optimales, le *tt***AR** génère un ion abondant correspondant à l'anion carboxylate $[M-H]^-$ de *m/z* 299 et un ion moins abondant correspondant à $[M-CO_2]^-$ de *m/z* 255. La limite de détection de la méthode d'analyse est de 1 ng/mL à un rapport S/N de 3 pour le milieu aqueux analysé par CLHP-ESI-SM/SM et de 10 ng/mL analysé par CLHP-UV. La méthode CLHP-ESI-SM/SM est 10 fois plus sensible que la méthode CLHP-UV.

L'extraction liquide-liquide a été appliquée à la séparation, pré-concentration et détermination du *tt***AR** pour des échantillons dans les milieux mentionnés. Les paramètres d'extraction ont été optimisés pour la récupération maximale *tt***AR**. La limite d'extraction avec le facteur de pré-concentration de 2 est de 5 ng avec un taux de récupération de 34 % pour le milieu aqueux analysé par CLHP-ESI-SM/SM et la limite de l'extraction pour le milieu de culture cellulaire supplémenté en sérum est inconnue à cause de limitation du temps du projet. Un essai d'extraction effectué sur 50 ng a donné un taux de récupération de 60% lorsqu'analysé par la méthode CLHP-UV. La méthode pourrait être raffinée pour détecter les métabolites du *tt*RA.

Mot clé : L'acid tout-*trans* rétinoïque (*tt***AR**), analyse de trace, CLHP-ESI-SM/SM, CLHP-UV, milieu aqueux, milieu de culture cellulaire supplémenté en sérum αMEM: FBS (90:10, v/v), extraction liquide-liquide.

ABSTRACT

All-*trans* retinoic acid (*at***RA**) is an active metabolite of vitamin A which has important physiological functions. *at***RA** controls cellular proliferation and differentiation, is essential in development and growth of organisms and acts on genes expression. *at***RA** has several pharmacological and physiological properties useful in dermatology and in the treatment of several cancers that are sensitive to retinoids. For example, acute promyelocytic leukemia is particularly sensitive to *at***RA** with response rate of 90%. Abnormal *at***RA** levels may cause or permit epithelial degeneration such as in xerophthalmia and neurological disorders such as in Schizophrenia, Parkinson's, Huntington's and Alzheimer's diseases.

A sensitive method was successfully developed for the quantification of *at***RA** present at trace level in aqueous and serum supplemented cell culture media. The method uses an HPLC coupled to triple quadruple mass spectrometer using electrospray ionisation interface source (ESI) in negative mode. The separation was done on a C18 column using an isocratic elution time of 25 min. A mixture of acetonitrile and aqueous ammonium acetate 0.1M buffer was used as mobile phase, employed to facilitate ion pair formation in mobile phase during negative ion electrospray. *at***RA** formed an abundant ion $[M-H]^-$ of m/z=299 for the **RA** carboxylate anion and a less abundant ion for $[M-CO_2]^-$ m/z=255. Under optimal conditions, the instrumental detection limit of *at***RA** were 1 ng/mL at S/N ratio of 3 for aqueous media analysed by HPLC-ESI-MS/MS and 10 ng/mL when analysed by HPLC-UV. HPLC-ESI-MS/MS method was found to be 10 times more sensitive than HPLC-UV method for aqueous solution.

Liquid-liquid extraction was applied for separation, pre-concentration and determination of *at***RA** in aqueous and serum supplemented cell culture media. The extraction limit with a pre-concentration factor of 2 in aqueous media was 5 ng with a 34% of recovery rate analysed by HPLC-ESI-MS/MS. As the result of time limitations, the extraction limit for serum-supplemented cell culture media αMEM: FBS (90:10, v/v) was not determined. An extraction was performed at the quantity of 50 ng with a recovery rate of 60% analysed by HPLC-UV. The method could be refined to detect *at***RA** metabolites.

Key words: all-trans Retinoic Acid (*at***RA**), Trace level analysis, HPLC-ESI-MS/MS, HPLC-UV, Aqueous media, Serum-supplemented cell culture media αMEM: FBS (90:10, v/v), Liquid-Liquid extraction.

INTRODUCTION

Vitamin A is a fat-soluble vitamin and supports a wide range of biological actions (Chapman, 2012). Three forms of vitamin A and derivatives (Retinol, Retinal and Retinoic acid) are active in the body with their specific physiological roles (Tatum and Chow, 2005). Vitamin A doesn't have a direct physiological function *in vivo* but rather serves as precursor for the biologically active retinoids (Kane *et al.*, 2008). Retinoids are natural or synthetic derivatives of vitamin A (De Luka, 2003). Retinoids are important micronutrients in human diet and is taken up as retinol (Ruhl, 2006). Retinol is a form of vitamin A that is found in animal food sources (Chapman, 2012). Retinoids include vitamin A compounds such as retinol, retinal and retinoid derivatives and related molecules that bind to retinoid receptors which are subsequently responsible for specific biological response such as embryonic development, differentiation, proliferation and apotosis (Bushue and Yvonne, 2010 ; Van Breemen *et al.*, 1998). Retinoic acid **(RA)** is produced when retinol is initially oxidized to retinal **(RAL)** by retinol dehydrogenase enzymes and then irreversibly converted to **RA** by retinal dehydrogenase enzymes (Gundersen, 2005). The most important biological activity occurs when retinol is transformed into **RA** by oxidation (McCaffery *et al.*, 2002). **RA** is a ligand for nuclear retinoic acid receptors **(RAR)** and retinoic X receptors **(RXR)**, which mediate its biological effects. An excess of, or a deficiency in **RA** can cause malformation during embryonic development (Gundersen, 2005 ; Söderlund, Fex and Nilsson-Ehle 2005). **RA** can isomerize at several of its double bonds into various isomers. Among these, all-*trans* retinoic acid *(at***RA**, tretinoin) and 9-*cis*-**RA** (alitretinoin) will bind and activate **RAR** and **RXR.** These receptors act as transcription factors controlling the multiplication of normal and cancerous cells. These two isomers are the most biologically active retinoids. In addition 13-*cis*- **RA** (isotretinoin) is anti-inflammatory and act as an anti-tumour agent (Bushue and Yvonne, 2010). Three **RA** isomers (*at***RA**, 13-*cis*-**RA** and 9-*cis*-

RA) are used clinically in dermatology and oncology (Cox, Muccio and Hamilton, 2013). *at***RA** and 9-*cis*-**RA** are only minor metabolites of retinol and beta-carotene. However, they show 100 to 1000 fold higher biological activity than either precursor. *at***RA** binds to **RAR** while 9-*cis*-**RA** binds to both **RAR** and **RXR** (Roos *et al.*, 1998). Unfortunately, *at***RA** is only present in very small quantity in human serum or plasma (Eckhoff and Nau, 1990).

P19 stem cells are an embryonic carcinoma cells which can be easily cultivated, growing continuously in serum-supplemented media (McBurney, 1993). These cells are useful for the study of the early stages of neuronal differentiation *in vitro*. Stem cells have been found to provide an attractive alternative for the research and treatment of neurodegenerative diseases such as Parkinson's, Alzheimer's and Huntington's disease (Wu *et al.*, 2010). It was also shown that P19 stem cells provide a useful model for physiological study of **RA** activity (Solari *et al.*, 2010). For example, exposing P19 cells to **RA** will promote their aggregation and induces their differentiation in neuronal cells (Jones-Villeneuve *et al.*, 1983).

Three important factors for method development are quantity level, resolution and media of analysis. In order to address these factors, the experiments were divided into two main groups related to the concentration and quantity range:

- Micro molar concentration or pharmacological level.
- Nano molar concentration or physiological level (Marill *et al.*, 2003).

The words high concentration related to pharmacological level and low concentration related to physiological level.

The development of highly sensitive methods of *at***RA** quantification (μg to ng) is required for the following reasons:

- **RA** isomerization occurs at very low concentrations. Furthermore, **RA** isomers have been shown to be active at nano molar concentration *in vivo* in many tissues (Van Merris *et al.*, 2002).
- Different doses of ***at*RA** act differently on cells (Bidad *et al.*, 2011).
- At pharmacological concentration (μM), which is relatively high for ***at*RA,** there is the decrease in the viability of immature dendrite cells (Bidad *et al.*, 2011).
- **RA** is only useful in specific concentration and an overdose of vitamin A is associated with toxicity (Van Breemen and Huang, 1996).
- Pharmacokinetic delay to ***at*RA** was observed in oral administration of patients (Idres, Gerald and Flexor, 2001; Marill *et al.*, 2003).
- Cellular intake resistance was observed in presence of high concentrations (μM) of **RA** *in vitro* (Marill *et al.*, 2003).
- A rapid decrease of ***at*RA** concentration in plasma was observed in oral administration (Choi *et al.*, 2003).
- An increase in the concentration of the **RA** isomers causes a dramatic shift in the metabolic pathway of these retinoids (Van Merris *et al.*, 2002).
- Biological effects on cell proliferation similar to those of ***at*RA** were observed at higher concentration *in vitro* for more polar and less active metabolites such as 4-*oxo*-**RA** and 13-*cis*-**RA** (Van Merris *et al.*, 2002).

  Information on the physiologic concentrations of all-*trans* and 13-*cis*-**RA** is lacking for newborns and pregnant women (Söderlund, Fex and Nilsson-Ehle, 2005).
- **RA** is highly soluble oil which can diffuse into cell membranes (Kam *et al.*, 2012).

Quantification of **RA** relies heavily on resolution because the desired **RA** isomers have different polarities and, therefore, different affinities for **RAR** and **RXR.** This

causes them to act differently *in vivo* (Lanvers *et al.*, 1996; McCaffery *et al.*, 2002 ; Van Merris *et al.*, 2002).It is beneficial to initiate study of ***at*RA** *in vitro* since it has recently been shown that ***at*RA** metabolites are active *in vitro* against human leukemia cells, inhibiting their growth and maturation (Idres *et al.*, 2001).

Among a series of isomers, ***at*RA** was chosen as an analyte of interest in method development because ***at*RA** has a relatively higher plasmatic concentration level compared to other retinoids and is responsible for most of the biological effects of vitamin A. It has a wide spectrum of biological activities such as cell growth, differentiation and morphogenesis. Furthermore, it is a precursor of several metabolites such as 4-*OH*-**RA**, 4-*oxo*-**RA**, 18-*OH*-**RA** and 5,6-*epoxy*-**RA**. ***at*RA** can also be isomerised to 9-*cis*-**RA** and 11-*cis*-**RA**. Therefore, the study of ***at*RA** has priority over the other isomers for the development of a sensitive and selective method for the trace analysis of ***at*RA** and its metabolites in physiological media.

To meet this goal the following steps were planned:

Extraction and trace analysis of ***at*RA** in aqueous media was optimized and then its concentration was quantified by HPLC-UV (high performance liquid chromatography with ultra violet detector) and HPLC-ESI-MS/MS (High Performance Liquid Chromatography with Tandem Mass Spectroscopy Using Electrospray Ionization).

Extraction and trace analysis of samples consisting of serum-supplemented cell culture αMEM: FBS 90:10, v/v (Alpha Minimum Essential Medium: Fetal Bovine Serum) to which was added ***at*RA** and quantification by HPLC-UV and HPLC-ESI-MS/MS.

CHAPTER I
RETINOIC ACID CHEMISTRY

1.1 History of Vitamin A and the Retinoids

Vitamin A was probably known by ancient Egyptians at least 3500 years ago. However, the modern investigation of the vitamin A began around 130 years ago (Wolf, 1996).

- In 1816, François Magendie conducted nutritional experiments with dogs.
- In 1880, Nicolai Lunin showed that there was a substance in milk that was essential for growth and Carl Socin discovered that the same substance, present in the yolk, was essential for chick growth.
- In 1906, Frederick Gowland Hopkins suggested that there were unsuspected dietetic factors necessary for life.
- In 1911, Wilhelm Stepp demonstrated that the essential substance in milk was also fat soluble.
- In 1913, Elmer McCollum and Marguerite Davis, Thomas Osborne and Lafayette Mendel showed that butter and egg yolk were superior to lard and olive oil in supporting the growth and survival of rats.
- In 1918, the growth-supporting factor became known as fat-soluble vitamin A.
- In 1932, Paul Karrer described the chemical structure of vitamin A.
- In 1937, Harry Holmes and Ruth Corbet isolated and crystallized vitamin A.
- David Adrian van Dorp and Jozef were the first who applied the methods for the synthesis and characterization of vitamin A.
- Research on the role of vitamin A in immunity and child survival continued into the 1990s (Wolf, 1996).

Vitamin A Derivatives (The Retinoids)

The retinoids are natural and synthetic derivatives of Vitamin A. They are divided into three categories (Chapman, 2012 ; Roos *et al.*, 1998):

- The non-aromatic retinoids such as β-carotene, ***at*RA** and 13-*cis*-**RA**, the two latter are naturally occurring retinoids formed from the metabolism of retinol. They are present in small amounts in the body and were first synthesized in 1955.
- Non-aromatic retinoids such as 9-*cis*-**RA**, the etetinate and the acitretin.
- Polyaromatic retinoids (the arotinoids) such as adapalene, tazarotene, and bexarotene

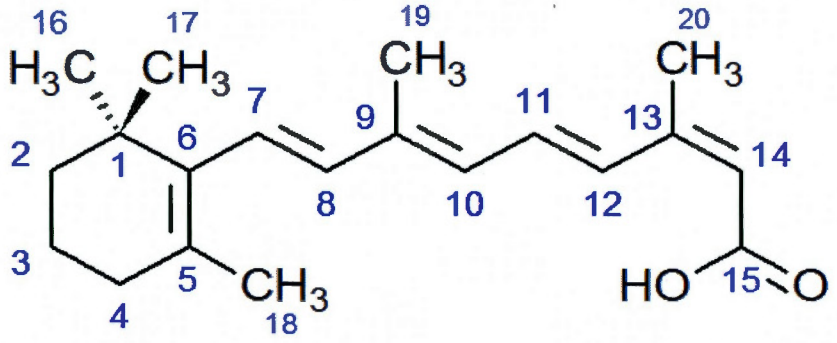
1.2 Structures, Nomenclature and Classification of the Retinoids.

1.2.1 Structure

Structurally, the retinoids consist of four isoprenoid units attached head to tail, with each unit having a ($H_2C=C(CH_3)-CH=CH_2$) structure. The molecule can be considered to be divided into three parts, a trim ethylated cyclohexene ring, a conjugated tetraene side chain and a carbon-oxygen functionality (Barua and Furr, 1998 ; Cox, Muccio and Hamilton, 2013 ; Luterotti, Sikovec and Bicanic, 2000).

1.2.2 Nomenclature

The numbering of the carbons of the main retinoid chain, according to IUPAC, is shown in Figure 1.1 (IUPAC, 1983).



all-*trans* Retinoic Acid (*at***RA**)

Figure 1.1: *at***RA** Chemical Structure (Cox, Muccio and Hamilton, 2013).

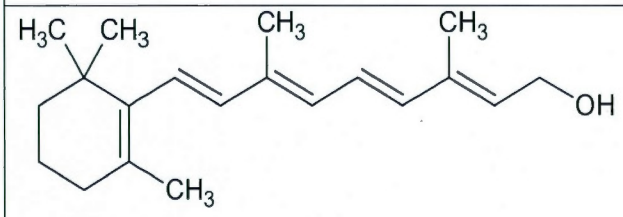
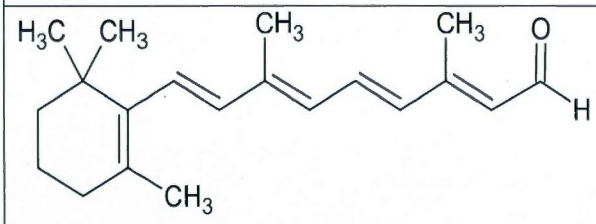
1.2.3 Classification

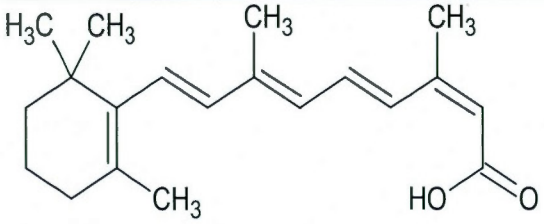
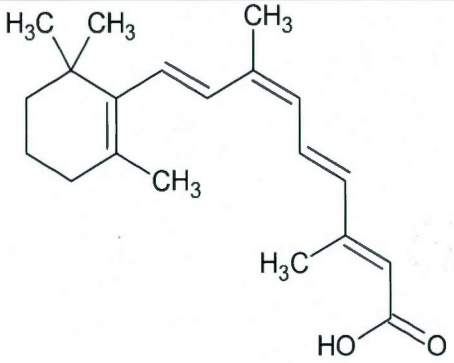
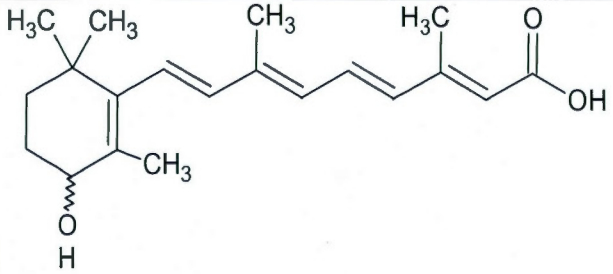
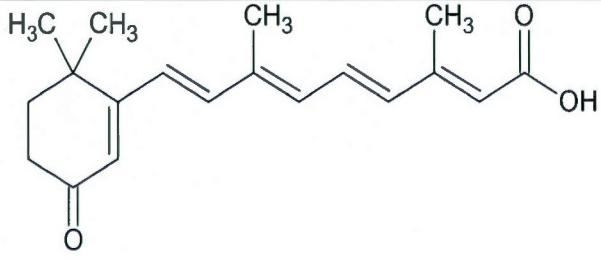
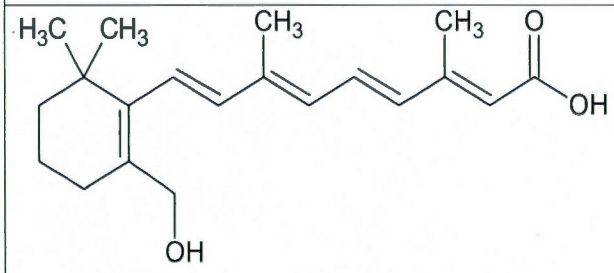
The retinoids can be classed by origin, i.e. natural or synthesis. The naturally occurring retinoids include vitamin A and its physiological metabolites e.g. retinaldehyde, *at***RA** and 9-*cis*-**RA**, all of which are provided to the human organism by the diet and their general chemical structure is shown in Figure 1.2.

From 1967 onwards, synthetic retinoids were developed by changes in the nucleus and the side chain, as well as in the terminal group. Synthetic derivatives of vitamin A are less toxic than the naturally-sourced vitamin and are used at present as therapeutics. The synthesis of retinoids has helped the advancement of the topical treatment forms of comedonal acne. Since 1968, more than 2000 molecules have been synthesized however, only a few are currently being used clinically. Among aromatic retinoids, etretinate was used clinically but now it has been replaced by acitretin. Adapalene was the first retinoid of a new class to be discovered. It is used in the topical treatment of acne. The main structural features of synthetic retinoids are shown in Figure 1.2.

1.3 Isomerization and Degradation Products

There are several reports on the formation of photoisomers of **RA** after exposing solution of the latter to light for short time (Urbach and Rando, 1994). As shown in Figure 1.1, each of double bonds in the conjugated polyene chain can undergo isomerization to give both mono *cis* and multiple *cis* isomers. However, due to steric hindrance, some of the isomers are not thermodynamically stable at room temperature and thus quickly isomerize to more stable forms. Mono *cis* isomers of retinoids play important biological roles. There are differences in receptor binding and thus, biological activity, for these isomers. Furthermore, some of isomers of **RA** are teratogenic. Therefore, determination of the exact isomerization products obtained from synthesis is essential (Ruhl, 2006). The chemical structures of these retinoid isomers are shown in Figure 1.2.

Structure	Products
	Retinol
	Retinaldehyde

	13-*cis*-**RA**
	9-*cis*-**RA**
	4-*OH*-**RA**
	4-*oxo*-**RA**
	18-*OH*-**RA**

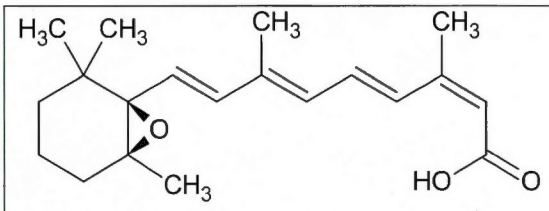
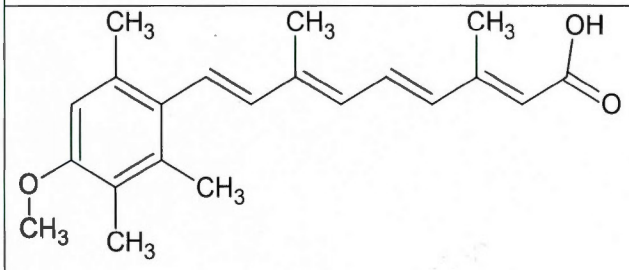
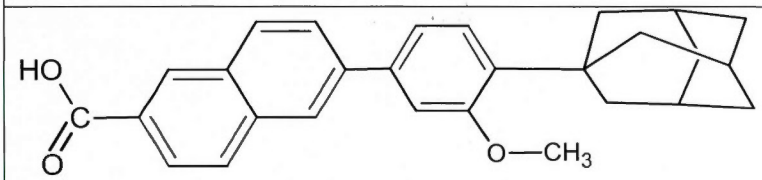
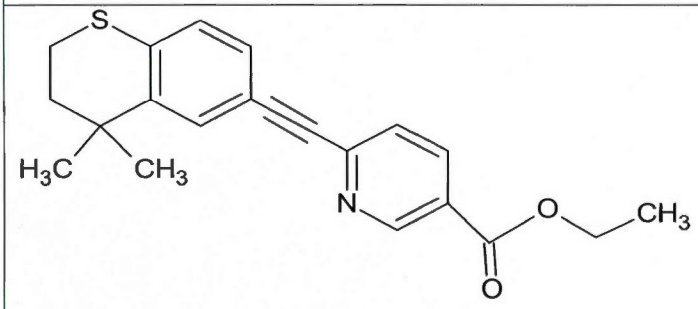
	5,6-*epoxy*-**RA**
	Acitretin
	Adapalen
	Tazarotene

Figure 1.2: Chemical Structures of Natural and Synthetic Retinoids and Derivatives.

1.4 Source of Vitamin A

Retinol and the carotenoids are the two main forms of vitamin A that exist in the human diet. Retinol is commonly found in animal foods like fish, meat and particularly in liver at high concentration. The carotenoids are organic hydrocarbons

composed of eight isoprene units, which act as pigments in plants, bacteria and algae. They are synthesized by photosynthetic microorganisms and plants. There are two types of carotenoids:

- The xanthophylls (having oxygen).
- The carotenes (not having oxygen).

The carotene is plant-, not animal-sourced. Beta-carotene is probably the best known of these retinoids providing the red, orange and yellow pigments found in many fruit, vegetables and green leafy plants (Chapman, 2012 ; Luterotti, Sikovec and Bicanic, 2000).

1.5 Physiological Roles of Vitamin A

1.5.1 Vision

Night vision or dark adaptation is a physico-chemical phenomenon associated with vitamin A. The presence of a photosensitive pigment called rhodopsin in the rod cells of the retina, allows for this phenomenon. Rhodopsin, a derivative of vitamin A, is synthesized from 11-*cis*- retinal with a protein opsin. When rhodopsin is exposed to light of low intensity, 11-*cis*-retinal is isomerized to *trans*-retinal (Perlman, Tamarhaim and Schramek, 1983 ; Sieving *et al.*, 2001).

1.5.2 Embryonic Development

*at***RA** is the major retinoid needed for the early stages of embryonic development. It regulates the formation of the germ layers and body axis and is involved in neurogenesis. It is important for the development the pancreas, lung and eye organs

and is a critical element for vision (De Luca, 1991 ; Kam *et al.*, 2012 ; Sharon *et al.*, 2000). It can also induce carcinogenesis if present in unadequate concentrations.

1.5.3 Cellular Differentiation

RA and its isomers influence many physiological processes and act as hormones to regulate gene expression. **RA** is the most potent factor for cell differentiation. *at***RA**'s ability to differentiate cells is the main focus of research into the development of therapies for prevention and treatment of various human cancers (Idres *et al.*, 2001)

1.5.4 Reproduction

*at***RA** supports both male and female reproduction and is also essential for the maintenance of male genital tracts and for spermatogenesis. Vitamin A is involved in the development of spermatozoa, ovaries and placenta in addition to the growth of the embryo and in the differentiation and proliferation of epithelium cells. Low serum vitamin A in the mother is associated with infant morbidity or, indeed mortality (Clagett-Dame and Knutson, 2011).

1.5.5 Immune System

Vitamin A, used as dietary supplement, is believed to enhance resistance to infection. It is known as an anti-infection vitamin because it is essential for the proper functioning of the immune system. It plays an important role in the development and differentiation of white blood cells and the activation of T-lymphocytes via the binding of *at***RA** to the **RAR** (Bidad *et al.*, 2011).

1.5.6 Oncology

The role of vitamin A (through **RA**) in cell differentiation, growth and control makes it very important in oncology. Many retinoids have been used alone or in combination for the treatment of several cancers such as basal cell carcinomas, squamous cell carcinoma of the skin, melanoma, cell lymphoma, acute promyelocytic leukemia (APL), ovarian, lung, breast, prostate, mouth and renal cell carcinomas and squamous cell head and neck syndrome. Vitamin A and its esters are capable of inhibiting carcinogenesis.

1.5.7 Dermatology

The importance of retinoids in dermatology was discovered by Wallach and Howe in 1925 (Roos *et al.*, 1998). This research group identified the epidermal changes which occur in abnormal keratinization in vitamin A-deficient animals. Vitamin A is presently a molecule of interest in dermatology because its effect on the differentiation and proliferation of epithelium cells. The mechanisms for action of retinoids on skin are still the subject of research. Nonetheless, it is known, from the study of epithelial morphogenesis rebuilding at a low dose *at***RA** allows for the optimal formation of the epidermis. As the active precursor in the skin, *at***RA** is converted in the epidermis and probably in the dermis into 13-*cis*-**RA**, 9-*cis*-**RA** and 4-*OH*-**RA**.

1.6 Metabolic Pathway of *at*RA

The metabolism of *at***RA** is complex and currently is only partially understood, the metabolism has different mechanisms depending on whether it is analysed *in vivo* or

in vitro. A few enzymes are known to participate in **RA** metabolism. The latter may play a role in cancer cell response or resistance to retinoid therapy (Marill *et al.*, 2003). *at***RA** can be oxidized by P450 cytochrome to different metabolites and these metabolites may be further metabolised to water soluble glucuronides (Idres *et al.*, 2001). The metabolism of *at***RA** occur in two phases:

- First Phase *in vivo*

*at***RA** can be metabolized by isomerase into several isomers which can undergo phase 1 metabolism into oxidized metabolites: 4-*oxo*-**RA**, 4-*OH*-**RA**, 18-*OH*-**RA** and 5,6-*epoxy*-**RA** while these isomers are believed to be less biologically active and more polar than *at***RA**.

- Second Phase

The second phase of retinoids metabolism involved the production of conjugated metabolites of *at***RA.** The oxidized metabolites produced in phase 1 can undergo glucuronidation in animals and humans. Thus, *at*-retinol β-glucuronide, 13-*cis*-retinol β-glucuronide, *at*-4-*oxo*-retinol β-glucuronide, 13-*cis*-4-*oxo*-retinol β-glucuronide are all conjugated metabolic products. **RA** metabolism reduces biopotency and results into an auto-regulatory loop. Several groups have observed that *at***RA** metabolites can stop the differentiation and growth of several cancer cell lines. The analysis of glucuronides products has shown that *at***RA**-4-*oxo*-**RA** and 5-6 *epoxy*-**RA** are carboxyl-linked glucuronides whereas 4-*OH*-**RA** was glucuronidated *via* the hydroxyl group (Marill *et al.*, 2003). Another interesting observation is that **RA** glucuronide shows activity *in vitro* and but not *in vivo*. The glucuronide derivatives doesn't bind

to **RAR** due to their polar nature which facilitates their excretion (Marill *et al.*, 2003). Thus the glucuronides are less interesting for use in cell differentiation studies.

CHAPTER II
METHODOLOGY

The choice of the method for *at***RA** trace quantification was based on several considerations, i.e. desired level of concentration, the resolution and the nature and effect of the sample matrix. Furthermore, the speed and cost of analysis must be optimized.

2.1 Reported Methods for RA Analysis

Analytical methods for retinoids have been developed using various high performance liquid chromatography-diode array detection (HPLC-DAD) (Van Merris *et al.*, 2002). HPLC coupled to thermal lens detection (Luterotti, Sikovec and Bicanic, 2000), HPLC coupled with ultra violet detector (HPLC-UV) (De Leenheer, Lambert and Glaeys, 1982 ; Gundersen and Blomhoff, 2001 Klvanova and Brtko, 2002 ; Schmidt, Brouwer and Nau, 2003 ; Tatum and Chow, 2005), HPLC coupled to particle beam mass spectrometry (Lehman and Franz, 1995), electroionisation mass spectroscopy (Bempong, Honigberg and Meltzer, 1995), HPLC coupled with atmospheric pressure chemical ionisation tandem mass spectrometry (HPLC-APCI-MS/MS) (Gundeson, Bastani and Blomhoff, 2007 ; Kane *et al.*, 2008 ; Wang *et al.*, 2001) and HPLC-ESI-MS/MS in both positive and negative modes (Lin, Jing and Jianping, 2011 ; Van Breemen and Huang, 1996) have also been reported.

2.2 Categorisation for RAs

Two different methods, routine and high sensitivity, were developed based on the required resolution, their applicability to research and the quantity to be analysed.

- The routine method involves high performance liquid chromatography coupled with ultra violet detector (HPLC-UV), as is generally required for pharmacological level (μg) scale analyses (Marill *et al.*, 2003).
- The high sensitivity method of high performance liquid chromatography coupled with electrospray ionization mass spectrometer (HPLC-ESI-MS/MS) is more appropriate for ng scale quantification. This method is suitable for researchers who work in the field of embryonic development (Marill *et al.*, 2003) and may be applied to the physiological level quantification.

2.2.1 HPLC-UV

High performance liquid chromatography coupled with a UV detector (HPLC-UV) is a powerful method for the analysis of ***at*RA**. The main advantages of HPLC-UV method are :

- Rapid, low cost, accurate, accessible and suitable for routine analysis (Barua, 2001).
- ***at*RA** has a specific UV absorption. Retinoids absorb light in the high UV (λ_{max}= 325 to 370 nm) as the result of their conjugated polyene chains (Van Merris *et al.*, 2002).
- UV detection allows the possibility of simultaneous quantitative and qualitative testing (Van Merris *et al.*, 2002).
- No interfering peaks with similar retention times were observed in chromatogram of extracted ***at*RA** in both aqueous solution and serum-supplemented cell culture media.
- This method is not selective nor can one detect low or trace level concentrations with it.

2.2.2 High Sensivity Method (HPLC-ESI-MS/MS)

High performance liquid chromatography electrospray ionization coupled with tandem mass spectrometry (HPLC-ESI-MS/MS) is a flow analysis technique for rapid and high through-put analysis (Ho *et al.*, 2003). This method is one of the most powerful tools in modern analytical chemistry. It is particularly useful in trace analysis. HPLC-ESI-MS/MS is very powerful tool capable of analysing both small and large molecules of varying polarity, e.g. in complex biological mixtures (Banerjee and Mazumdar, 2012).

ESI-MS has also become an important technique in clinical laboratories (Ho *et al.*, 2003). Most HPLC-ESI-MS/MS analyses are carried out using reverse phase liquid chromatography with a non-polar C18 silica bonded stationary phase. The molecule of interest must be able to undergo charge changes *via* protonation and deprotonation.

The main advantages of this method are:

- It is capable of providing both qualitative (structure) and quantitative (molecular mass or concentration) results for analytes (Ho *et al.*, 2003).
- It is a valuable method for complicated matrix analysis (Wu *et al.*, 2004).
- It is the most sensitive analytical method for structural characterization of a molecule (Banerjee and Mazumdar, 2012).
- It is the most powerful method for target compound characterization.
- It allows for the possibility of obtaining the entire molecular mass by selecting the appropriate parent ion peak.
- Finally, it is a time saving method when a large number of samples must be analysed.

Limitations of HPLC-ESI-MS/MS are:

- The selectivity and sensitivity of HPLC-ESI-MS/MS analysis not only depends on the ionization technique and mass spectrometer used, but also on the chromatographic technique chosen (Kostiainen and Kauppila, 2009).
- The optimization of chromatographic and mass spectrometric conditions is required while maintaining a balance between sensitivity and resolution (Kane *et al.*, 2005).
- The mobile phase composition for the HPLC must be compatible with electrospray mass spectrometry. For example, nonvolatile ion pair reagents and/or buffers must be excluded to prevent precipitation in the ion source (Van Breemen and Huang, 1996).
- Ionization suppression can occur when mass spectrometric analysis is carried out at atmospheric pressure (Manuur *et al.*, 2011).
- The signal response depends on many parameters: sample concentration, sample matrix, analyte characteristics, buffer, organic solvent and/or curtain gas purities as well as collision cell parameters and eluent composition.
- There is no possibility to apply non-volatile additives and/or buffers in mobile phase as this can causes excessive background noise and/or rapid contamination of the interface source (Kostiainen and Kauppila, 2009).

A summary of the numerous advantages using a high sensitivity method for analysis of ***at*RA** are as follows:

- There is a favorable ionization efficiency due to conjugation and the carboxylic acid group in ***at*RA** (Hung, 1996).
- Greater sensitivity allows for greater signal intensity and lower background noise (Hung, 1996).

- Creating **RA** derivatives is not required for this method in comparison with other methods like Negative Ion Electron Capture Chemical Ionization (Hung, 1996).
- This method has higher selectivity and does not require prolonged sample preparation in comparison of GC-MS (Peng *et al.*, 2014).

2.3 High Sensitivity Instrumentation (HPLC-ESI-MS/MS)

The figure 2.1 represents three components of a mass spectrometer :

1. An ion source for producing gaseous ions from the sample in ionization compartment.
2. An analyser for resolving the ions into their characteristics mass components according to their mass to charge ratio.
3. An ion detector system which measures the relative abundance of each of the resolved ionic species and converts the ions into electrical signals.

A sample introduction system is necessary to introduce the samples into the ion source such as in an HPLC. All mass spectrometers operate under vacuum allowing ions to reach the detector without colliding with the other gaseous molecules or atoms (Kang, 2008).

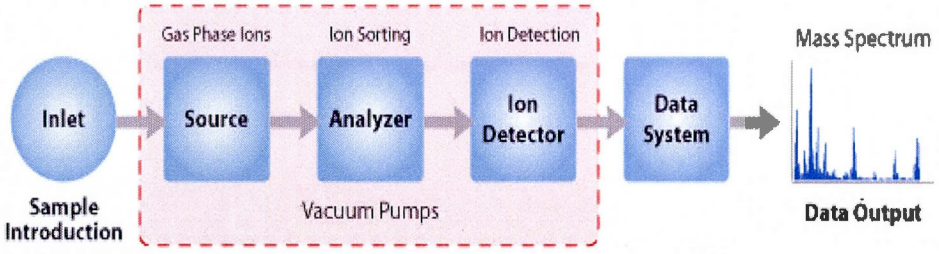


Figure 2.1: Components of a Mass Spectrometer (www.premierbiosoft.com).

2.3.1 Ion Source and Ionization Technique

Electrospray Ionization (ESI) is conducted at atmospheric pressure ionization technique, and it is used to produce polar and ionic species in gas-phase ions at high voltage (Tang, Bruce and Hill, 2006). John Fenn, the first person to develop this method, shared the 2002 Nobel Prize in chemistry for the crafting ESI-MS.

ESI promotes electrostatic dispersion and creation of aerosols and is sometimes improperly called electro-hydrodynamic atomization (Kebarle and Verberk, 2010). This ionization technique has totally replaced other earlier ones such as continuous Fast Atom Bombardment (FAB), Thermospray and Particle Beam Ionization (PBI) (Kostiainen and Kauppila, 2009).

Mechanism of Action

All stages of the ESI occur in atmospheric pressure regions of the machine and the sample proceeds through three major steps in the conversion from electrolytes in solution to gas phase ions (Figure 2.2):

1- Initial production of charged droplets in very small diameter ESI capillary tips.

2- Shrinkage of the charged droplets due to solvent evaporation.

3- Gaseous ion formation.

A voltage of 2-5 kV is applied across the capillary spray tip, and a high electric field at the capillary tip causes polarization of the solvent. The solution supplied to the capillary must be polar and one in which the analyte is soluble: (ex: MeOH or MeOH/H_2O; and ACN or ACN/H_2O).

In the presence of an electrolyte, the solution must be sufficiently conducting and the positive and negative ions in solution sufficiently labile under the influence of the

field. A common electrolyte used in these solutions is ammonium acetate which provides an excess, at the surface, of NH_4^+ as the positive ion.

The droplets initially produced are expected to be approximately of similar size and positively charged due to an excess of positive electrolyte ions at the surface of the cone and the cone jet. The mode of charging, which depends on the positive and negative ions traveling in opposite directions under the influence of the electric field, has been called the electrophoretic mechanism. The droplets migrate downfield towards the oppositely charged electrode. Solvent evaporation at constant charge causes droplet shrinkage and an increase in the repulsion between the charges. Increasing repulsion overcomes the surface tension of the droplet and thus columbic fission can occur. Formation of a cone jet splits the charged droplet into numbers of smaller ones while further evaporation of the parent droplet leads to repeated fissions and generation of smaller droplets.

In 1882, Lord Rayleigh theoretically estimated the maximum amount of charge a droplet can carry which is called the Rayleigh limit. Evaporation of droplet's solvent increases the charge on the analyte until Rayleigh limit is achieved. At this point, the droplet breaks apart and the analyte is accelerated toward the counter electrode and subsequently into mass spectrometer.

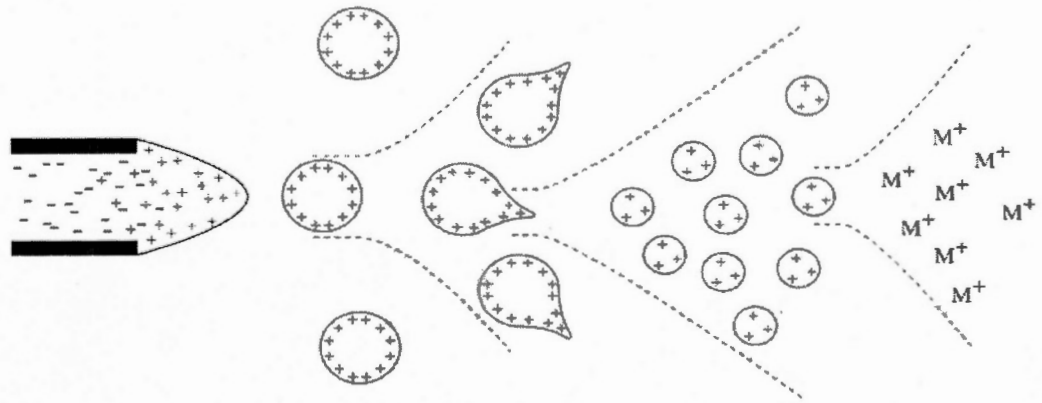


Figure 2.2: Mechanism of Electrospray Ionization (Ho *et al.*, 2003).

Sensitivity is an important aspect of ESI-MS/MS, and depends on several factors including:

- Ionization efficiency, defined as the method's effectiveness in producing gaseous ions.
- Ion transmission efficiency, defined as the fraction of electrospray current which enters the mass spectrometer and the ability to transfer the charged ions from an area of atmospheric pressure to the vacuum of the mass spectrometer.

Limitations in ionization and transmission efficiencies lead directly to a reduction in the ESI-MS sensitivity (Page *et al.*, 2007). Several factors affect the ionization efficiency:

- Interface design.
- Flow rate. A lower flow rate provides better ionization efficiencies because it generates smaller initial droplets that require less solvent evaporation which offers a better sensitivity (Page *et al.*, 2007 ; Tang, Bruce and Hill, 2006).
- The chemical and physical properties of the analyte (pKa, hydrophobicity, surface activity, ion solvation, and proton affinity).
- Eluent composition and volatilities, surface tensions, viscosities, conductivities, ionic strengths, dielectric constants, electrolyte concentrations, pH and potential gas-phase ion-molecule reactions (Kostiainen and Kauppila, 2009).
- Solvent charge separation (cation *vs* anion). The conductivity of the solvent must be sufficient to achieve good sensitivity along with good stability.
- Polarity of solvent. A more polar solvent better stabilizes multiply charged ions in solution.
- Sensitivity is improved when higher proportions of organic solvents are used. The signal response in ESI increases with increased amount of organic solvent used (Kostiainen and Kauppila, 2009).

- Smaller initial droplet size increases the amount of charge per analyte molecule and improves the ionisation efficiency as well as the surface activity.
- Instrumental conditions and ion source voltages.

ESI has numerous benefits:

- It is an excellent method for the detection of analytes generated by HPLC. Furthermore, ESI coupling with an HPLC is relatively easy (Page *et al.*, 2007).
- It is a soft ionization technique which can produce the gas phase ions of large, thermally liable supermolecules (Banerjee and Mazumdar, 2012).
- ESI is sensitive, robust and reliable tool for the study of femtomole quantities of analytes in microliter volumes (Ho *et al.*, 2003).
- Using ESI increases the speed of analysis for a variety of analytes of differing polarities such as organic or inorganic species, polymers, nucleic acids or, indeed, very high mass proteins in physiological matrices (Ho *et al.*, 2003 ; Kebarle and Verberk, 2010).
- ESI allows for creation of intact multiply charged gas-phase biomolecules (Page *et al.*, 2007), protonated and deprotonated molecular ions from compounds with high molecular weights (130 KDa) as well as from thermostable compounds.
- ESI is particularly effective for analytes with a polar functionality (Banerjee and Mazumdar, 2012), like *at***RA** which has an acidic carboxylic moiety.
- ESI is less affected by salt contaminations of the analyte solution than the other ionization techniques.

ESI Ionization Mode (Negative)

Negative ion mode was seldom used in the past due to irreproducibility arising from several factors:

- It is more difficult to obtain a stable signal in negative *vs* positive mode because the electrical discharge occurs at lower electric field (Kostiainen and Kauppila, 2009).
- In negative ion mode mass spectroscopy, the ion sources are operated at pressure of up to 133Pa, producing a high concentration of low energy electrons which may directly react with sample molecules to form negative molecular daughter ions.
- Mass spectra in negative mode generally exhibit less fragmentation than in positive mode.
- The sensitivity of electron attachment process is very high for electronegative elements, and precursors which react as Bronsted based frequently give $[M-1]^-$ ions, which commonly undergo electron capture or anion attachment (Pavani *et al.*, 2012).

Nonetheless reagent gases may be used to generate negative ions and acids, phenols and nitro compounds which are easily ionized in negative mode by ESI (Kebarle and Verberk, 2010).

Mass spectra resulting from production of negative ions *via* ESI are more efficient, sensitive and selective than mass spectra produced in positive mode. Furthermore, the presence of acetic acid improves the signal response for ESI in negative mode (Wu *et al.*, 2004). In the current context, ***at*RA** has a medium polarity, having a non-polar hydrocarbon chain and a polar acidic group which can be ionized *via* negative mode ESI.

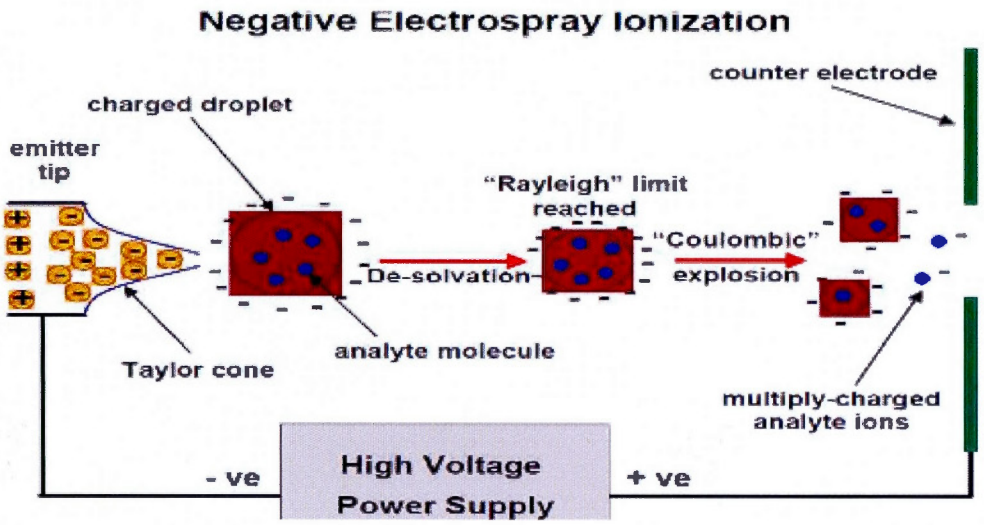


Figure 2.3: Diagram of Negative Electrospray Ionization (Pavani *et al.*, 2012).

2.3.2 Mass Spectrometer

Quadrupole mass spectrometers sort ions according of their mass-to-charge ratio (Miller and Denton, 1986). They consist of four circular sections arranged with their axes in a square array. The quadrupoles have a tuneable and a variable band pass region. At present, the most widely used tandem mass spectrometer is the quadrupole mass spectrometers. Only the first and third quadrupole scan and analyse the ions.
The performance of the mass analyser is defined by such characteristics as accuracy, resolution of the mass range as well as the scan speed. Selected Ion Monitoring (SIM) is the most selective mode used for single quadrupole MS, while triple quadrupole is used for enhanced sensitivity and selectivity in multiple reaction monitoring (MRM). Tandem mass spectrometry, having two stages of mass analysis, includes a product and a constant natural loss scan.

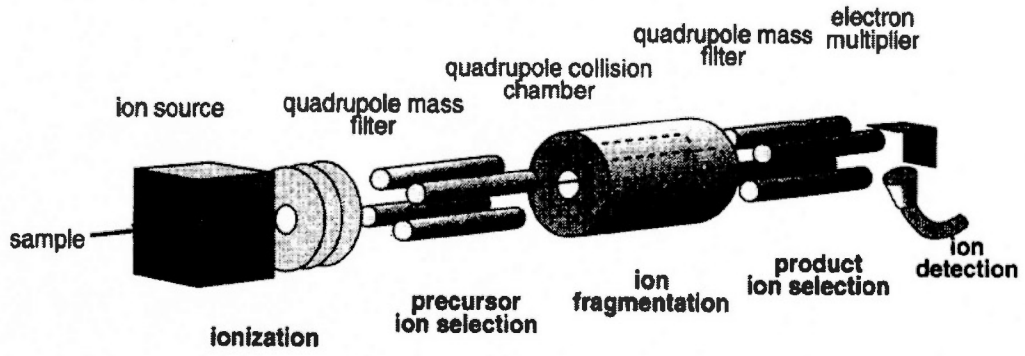


Figure 2.4: Schematic Diagram of a Triple Quadrupole Mass Spectrometer (Edmond de Hoffmann, 1996).

Significant Advantages of the Quadrupole

- The shorter distance between the ion source and the detector having a relatively low pressure (5×10^{-5} Torr), allows for the coupling of a HPLC to the mass spectrometer (Miller and Denton, 1986).
- Quadrupole MS resolves ions on the basis of *m/z* instead of kinetic energy momentum (Miller and Denton, 1986).
- The quadrupole is relatively low cost while having bulk weight due to its mechanical simplicity. It also avoids the slow scan speeds associated with magnets (Miller and Denton, 1986).

Advantages of the Triple Quadrupole

- Triple quadrupole MS/MS offers higher selectivity due to double mass filtering (Schreiber, 2010).

- This allows for quantification of many targeted analytes in a single experiment.
- Higher selectivities result in less interference from co-eluting compounds and the matrix (Schreiber, 2010).
- The quadrupole collision chamber and the mass analyser allow for high efficiency fragmentations.
- The triple quadrupole MS/MS is capable of analysing up to *m/z* 4000 with a precision of *m/z* 0.1-0.2.
- The triple quadrupole allows for better accuracy and reproducibility especially at low concentrations.
- This technique has a better signal to noise ratio (S/N) allowing for a lower limit of quantification (Schreiber, 2010).
- Speed of such scans are up to 5000 *m/z* per second.
- Triple quadrupole MS/MS tolerates relatively lower pressures.

2.4 Buffer Choice

2.4.1 Strategies for Choosing the Right Buffer

Small changes in the mobile phase pH have a dramatic effect on *at***RA** analysis (Kaushal and Srivatara, 2010). Therefore, a buffer is used to maintain the pH of the solution for analysis within a narrow range. The nature of the buffer is determined by its buffer capacity, i.e., its resistance to change in pH when strong acids or bases are added. The closer the pKa of the buffer is to the desired pH, the greater is its buffer capacity and the better it controls the pH of the analysis, an important factor for the results (www.bartek.on.ca) ; one of the analyte should have a pH of 1.5 units above or below that of the mobile phase. A lower pH decreases the solubility of **RA** in

water and thus its analysis requires a mobile phase with a higher proportion of organic solvent for elution under reverse phase conditions. The buffer, therefore, must be chosen based on ability to maintain analyte ionization at the MS interface.

An ammonium acetate-acetic acid buffer was used in the present study. Acetic acid is a monoprotic acid.

Samples containing ionizable compounds are influenced by the pH of the mobile phase. ***at*RA** is ionizable and strongly influenced by the pH of the mobile phase (Heyman and Henry, www.westerm analytical.com).

HPLC separation and ESI-MS detection depend upon the buffer and other additive species. Depending on the analytical method used, two types of buffers were employed as shown in Table 2.1.

Table 2.1: List of Buffers Used for ***at*RA** Method Development.

Buffer	Ion	Type	Effective pH Range	pH	Tested Method
Sodium Acetate 0.01M	CH_3COO^-	Non Volatile	4.8	5.2	HPLC-UV HPLC-FLU
Ammonium Acetate 0.01 M and 0.1M	CH_3COO^-	Volatile	4.3-5.3	5.2	HPLC-UV HPLC-MS/MS

2.4.2 Advantages of the Presence of a Volatile Buffer in the Mobile Phase

- This method employed an acidic, buffered mobile phase, which offers a rapid separation and detection of **RA** at the most sensitive wavelength (Tatum and Chow, 2005).

- Ammonium acetate is a volatile buffer which doesn't precipitate in the ion source of an MS (Stering *et al.*, 2010).
- Addition of ammonium acetate can improve signal stability and reproducibility (Kostiainen and Kauppila, 2009 ; Stering *et al.*, 2010).
- Ammonium acetate removes other non-volatile salts and/or detergents *via* a buffer ion exchange in the cell culture medium (Stering *et al.*, 2010).
- Ammonium acetate is an effective buffer for obtaining ESI signals for complex media that require a high concentration of essential salts (Stering *et al.*, 2010).
- High concentrations of a volatile buffer can reduce the adverse effects of contamination in ESI (Stering *et al.*, 2010).
- Ammonium acetate is highly soluble in an aqueous-organic eluent at pH 5 (Schelinger and Carr, 2004).

2.5 Extraction Method

Biological samples are extremely complex matrices which contain many components that can interfere with the analysis of the target. Such samples cannot normally be injected directly into the spectrometer without sample extraction. Liquid-Liquid Extraction (LLE) is the most common technique for separation of organic products from a biological matrix (Waters, 2008). LLE separates the analyte from other interferences by partitioning the sample between two immiscible phases. The ability of LLE to separate a mixture depends on the relative solubility of each compound in the two immiscible solvents. The objectives of extraction of *at***RA** from a physiological matrix are as follows:

- Isolate *at***RA** and related metabolites from any interfering compounds.
- Dissolve *at***RA** and pre-concentration in suitable solution.

- Achieve high extraction efficiency and low handling loss.

LLE Advantages and Limitations

- This technique is simple and low cost effective.
- In comparison to other direct methods, LLE is more time consuming.
- LLE requires an evaporation step for removal of excess organic solvent before analysis.
- The formation of an emulsion is possible when two immiscible phases are used in the extraction procedure.
- The extracted volume of *at***RA,** after filtering, was about 100 µL and each injection consumed 20 µL of this. The number of injections was, thus, limited as was the study of the stability of the extracted solution.

All extractions were performed according to the protocol of (Bérubé *et al,* 2005) using the following media:

- Acidic media since a change in the pH of the solvent can considerably change the solubility of an acidic organic compound and, therefore, affect the amount of recovered.
- A mixture of 50:50 v/v polar and non-polar organic extraction solvents was used (EtOAc and acidified hexanes in volumetric proportions) since *at***RA** contains a polar functional group (carboxylic acid) and a non-polar side chain. A polar molecule requires a more polar organic solvent (EtOAc) for a high yield extraction from aqueous solution. The LLE steps will be shown in Chapter 4 (Method Development).

2.6 Challenges Encountered in Trace Analysis Method Development for *at*RA

- A reasonable run time with a balance of sensitivity and selectivity is required.
- Successful separation methods for less polar metabolites generally fail to separate more polar ones (Van Merris *et al*, 2002). Since the retinoids have a wide polarity range, separation of particular samples can be difficult.
- Some retinoids have a long retention times when isocratic elution is used (Tatum and Chow, 2005).
- The method suggested for the quantification of **RA** uses a gradient elution which requires additional time for column re-equilibration (Tatum and Chow, 2005).
- The carboxylic acid functional group in **RA** shifts to its non-ionized form when the pH drops. A change in the concentration of ammonium acetate or the pH of the eluent influences retention times and thus it is very important to control the mobile phase pH (Eckhoff and Nau, 1990 ; Van Merris *et al,* 2002).
- An adjustment of buffer solubility in the aqueous-organic eluent is required to prevent the loss of electrospray ionization efficiency.
- There is a wide variation in physical and chemical properties of *at*RA and its isomers. For example, the solubility varies from soluble to insoluble in polar aqueous solvents and vice versa in non-polar solvents like hexanes.
- An adjustment in pH is also required for the optimization of an **RA** liquid-liquid extraction when water-immiscible solvents such as *n*-hexane and ethyl acetate are used.
- Since it has four double bonds, **RA** is able to undergo isomerization and oxidation in air when exposed to the UV light. Sample preparation, handling, extraction and analysis must be performed under yellow light.

In addition, the following elements have been considered:

- An efficient protocol, the maintenance and control of the instrumentation (HPLC-MS) requires an optimized condition for day-to-day routine analysis to prevent lack of sensitivity in quantification of **RA**.
- Chemical contamination of the ESI probe should be monitored to avoid loss of sensitivity.
- The presence of acidic and catalytic components must be monitored during sample analysis (Gundeson, Bastani and Blomhoff, 2007).
- The development of anammox bacteria in the presence of ammonium acetate should be avoided during method development.

CHAPTER III
EXPERIMENTAL SECTION

3.1 Physico-Chemical Properties of *at*RA

***at*RA** is yellow powder with an orange cast having the molecular formula of $C_{20}H_{28}O_2$, a molecular weight of 300.4 and a melting point of 180-182°C. Acid or alkali impurities in the solvent can affect the spectroscopic parameters. The molar absorptivity of ***at*RA** in ethanol is 44300 at 351 nm and in methanol is 45000 at 350 nm (Aldrich Sigma).

3.2 Handling, Storage and Laboratory Precaution

- ***at*RA** should be kept frozen at below -20°C.
- ***at*RA** can be expected to be stable for at least one year when stored at -20°C in an unopened ampule (Barua and Furr, 1998).
- All experiments (extraction and instrumental analysis) were carried out in dim yellow light since the retinoid molecule does not absorb light in the yellow range of the spectrum, that is, 570 nm-585 nm (Ruhl, 2006).
- Installation of black curtains was used to cover doors and windows in laboratories while working with the retinoids.
- Since some retinoids are highly teratogenic and can cause skin irritations, it is recommended that gloves be worn while working with them (Barua and Furr, 1998).

3.3 The Polarity and Solubility of Retinoids

The polarity and therefore the solubility of the retinoids varies from very soluble to insoluble in polar solvents like water and *vice versa* in non-polar solvents like hexanes. The retinoids can dissolve in chlorinated hydrocarbons such as dichloromethane and chloroform, however, the latter is acidic and should be avoided because it produces free radicals. Furthermore, care should be taken with dichloromethane because retinoids isomerize in chlorinated solvents (Barua and Furr, 1998). The solubility of individual retinoids in organic solvents depends on the terminal functional group of the side chain. *at***RA** has a carboxylic acid function and, thus, is soluble in methanol and ethanol as well as in acetonitrile but its solubility in water is low. Spectrophotometric and radioactive techniques were used to measure the water solubility of **RA** under physiological conditions. Hydration decreases the molar extinction coefficient of **RA** and shifts its absorption bathochromically (Szuts and Harosi, 1991).

The maximum absorption wavelength is observed from 337 nm to 350 nm for **RA** dissolved in 1 ml methanol containing 2 drops of 0.01 N NaOH. The maximum wavelength shifted to about 350 nm upon addition of the same mentioned volume and concentration of HCl to 1 mL of the above *at***RA** solution. The presence of conjugated double bonds in retinoids renders the molecular structure unstable in the presence of an oxidant, a strong acid and/or in a solvent which contains dissolved oxygen or peroxides (Aldrich Sigma).

3.4 Chemical Products and Solvents

- Purified *at***RA** was provided by an organic laboratory (Pr. Breau's Laboratory, UQAM, Canada).
- Commercial *at***RA**, glacial acetic acid (99.99%) and hexanes (HPLC grade) were obtained from Sigma Aldrich (Toronto, Ontario, Canada).
- Sterilized mixtures of serum-supplemented cell culture media αMEM:FBS (90:10, v/v) were prepared by Pr. Paquin's Laboratory, UQAM, Canada.
- Ammonium acetate (ACS grade, 97%) and sodium acetate was obtained from Anachemia (Toronto, Ontario, Canada).
- Acetonitrile (HPLC grade) and hydrochloric acid (37%) were obtained from Caledon Laboratory (Toronto, Ontario, Canada).
- Ethyl acetate (ACS grade) and methanol (HPLC grade) were obtained from the EMD Laboratory (Toronto, Ontario, Canada).
- Water (Nanopure grade) was obtained by filtering distilled water using a Thermo Fisher water purification apparatus (Branstead model) (MA, USA).

3.5 Solution Preparation

3.5.1 Stock, Mother and Standard Solutions

The *at***RA** was dissolved in MeOH, producing a stock solution of exactly 1000 µg/mL and then the solution was stored at -20°C. A mother solution of 10.0 µg /mL in MeOH was produced by diluting the stock solution with MeOH. The standard solutions were prepared freshly on the day of use by dilution of the mother solution with the proper volume of the desorption solution to obtain a range of concentrations between 10 to 100 ppb

3.5.2 Buffer Preparation

Two buffers were prepared: a sodium acetate solution (0.01 M) and an ammonium acetate one (0.1 M). Both had a pH 5.2 and the method was adapted from a standard analytical method.

- Sodium Acetate 0.01M

The buffer solution for the HPLC-UV was sodium acetate (0.01 M, pH 5.2). 0.01M Acetic acid solution was prepared by diluting glacial acetic acid (0.57 mL, 17.4 M) with nanopure water to a final volume of 1L. The sodium acetate solution (0.01M), was prepared by dissolving of sodium acetate (0.8203g, MW: 82.03 g/mol) in water (Nanopure 1L). The sodium acetate buffer solution (0.01M, pH 5.2) was prepared by mixing acetic acid (267 mL, 0.01M) with sodium acetate solution (733 mL, 0.01M)

- Ammonium Acetate 0.1M

A suitable buffer solution for the high sensitivity method was found to be ammonium acetate (0.1 M, pH 5.2). This buffer can also be used in a HPLC-UV. The acetic acid solution (0.1M) was prepared by diluting acetic acid (2.8 mL, 0.35 M) in nanopure water (10 mL). The ammonium acetate solution (0.1 M) was prepared by dissolving ammonium acetate (0.1927g) in nanopure water (25 mL). The ammonium acetate buffer (pH 5.2, 0.1M) was prepared by mixing acetic acid (5.3 mL, 0.1M) with ammonium acetate (14.7 mL, 0.1M). Ammonium acetate solution (pH 5.2, 0.1M) was used in the mobile phase as well as the desorption solvent (pH 5.2, 0.15 M).

- Preparation of Acidified Hexanes

The acidified hexanes was prepared by mixing glacial acetic acid (25 µL, 17.4 M) to hexanes (10 mL HPLC grade).

3.6 Instrumentation

UV detection was performed at wavelength 343 nm with a sensitivity of 0.2000 AUFS. Fluorescent detection was performed at an excitation wavelength of 340 nm and emission at 470 nm. Chromatograms were recorded by the physico software (Pr. Pichet, UQAM, Canada, 2003) *via* a data acquisition interface. Table 3.1 shows the instruments used in this work.

Table 3.1: List of Instruments Used for *at***RA** Method Development.

Type	Manufacturer	Model
HPLC Pump	Thermo Scientific	Spectra P4000
HPLC Pump	Waters	590
HPLC Pump	Waters	2695
Mass Spectrometer	Waters	Micromass Quattro LC
UV Dual Detector	Waters	2487
FLU Multi Detector	Hitachi	L-7480
FLU Spectrometer	Hitachi	F2000
Pump Syringe	Harvard	-

CHAPTER IV

METHOD DEVELOPMENT, CLASSIFICATION AND OPTIMIZATION

Method development is classified according to quantification of *at***RA** at either the pharmacological level (μg) or the physiological level (ng). The aim of this project is the quantification of *at***RA** in physiological level (ng); however, before attempting to create a method at this level, some preliminary pharmacological level information must be obtained. Based on the quantity of *at***RA**, the method development was divided into two stages, initial and final. As shown in Table 4.1, the initial step focused on the quantification of *at***RA** at a high mobile phase flow rate (1.5 mL/min) in which *at***RA** was analyzed and quantified by HPLC-UV and HPLC-FLU. In its final stage, the study focused on low mobile phase flow rates (0.2 mL/min) at which *at***RA** was analyzed and quantified by HPLC-UV and HPLC-ESI-MS/MS.

Table 4.1: Defined Concentrations and Quantities in Method Development.

Method Development	Concentration Range (μg/mL)	Extraction Quantity (μg)	Category	Detection and Analytical Methods
Initial	1-15	10.0-0.2	Pharmacological Level	HPLC-UV HPLC-FLU
Final	1.00- 0.002	0.200 –0.005	Physiological Level	HPLC-UV HPLC-ESI-MS/MS

Note: Ranges are stated from lowest to highest

4.1 Initial Method Development and Optimization (*at*RA Pharmacological Study)

The detection methods used at the pharmacological level are HPLC-UV and HPLC-FLU. These methods are less sensitive but more rapid. Nonetheless, they are useful for the following reasons:

- It allows for the selection of the better method: HPLC-UV *vs* HPLC-FLU, based on analytical parameters such as the limit of detection, the linearity range and the quantification domain and limit.
- It is possible to study *at*RA at high or low flow rates without any restrictions.
- It is possible to apply non-volatile or volatile buffer in the mobile phase for the study of their effect on extraction recovery rate.
- It is possible to study the effect of flow rate on the extraction recovery rate as well as the analytical parameters in different mobile phases (i.e. ACN *vs* MeOH).
- It allows for a study of the efficiency of an extraction protocol at high or low flow rates in aqueous and serum-supplemented cell culture media.

Pharmacological level quantification in early method development was aimed at:

- Collecting information about the physico-chemical properties of the analyte of interest (*at*RA) *via* a spectroscopic study.
- Determining the stabilities of the *at*RA mother and standard solutions.
- Determining the *at*RA's light sensitivity
- Comparing evaporation and calibration curves to evaluate the loss of analyte *via* evaporation.
- Determining the efficiency of extraction and analysis at high flow rates in aqueous and serum-supplemented cell culture media.

4.1.1 Ultra Violet (UV) and Fluorescent Spectroscopy Studies

The UV spectroscopy study was performed with a UV visible spectrometer using *at***RA** solution in MeOH (15μg/mL). As shown in Figure 4.1, the maximum absorbance was found at 343 nm.

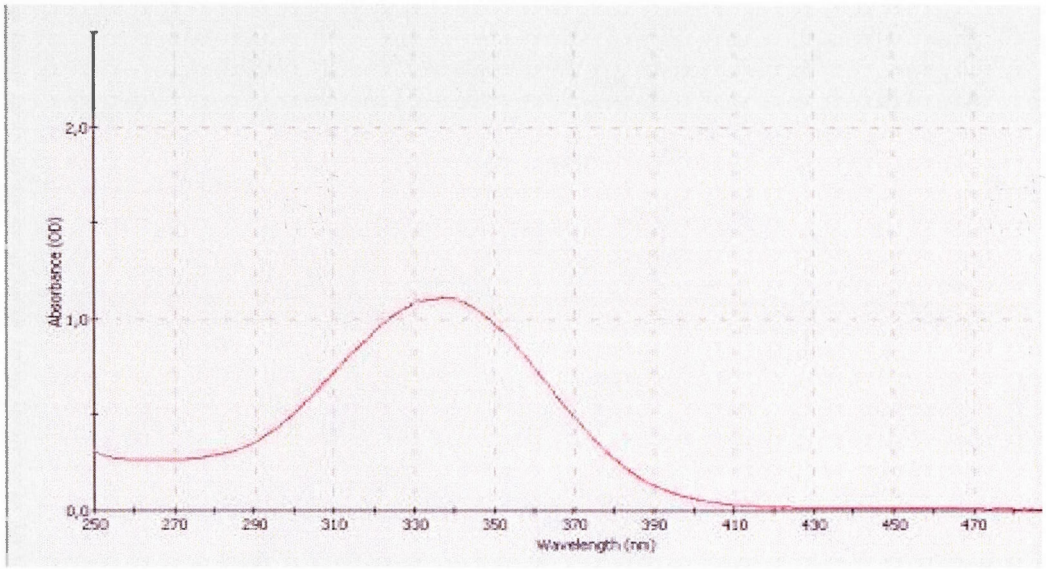


Figure 4.1: UV Absorbance Spectrum of *at***RA** Recorded between 250 to 470 nm at a Concentration of 15 μg/mL in MeOH.

The fluorescence spectroscopy of an *at***RA** solution in MeOH (15μg/mL) was recorded over the range of 340-640 nm with a Hitachi fluorescent spectrometer F2000. Excitation was determined to be 343 nm. As demonstrated in Figure 4.2, 470 nm appears to be the optimal emission wavelength for the detection of *at***RA**.

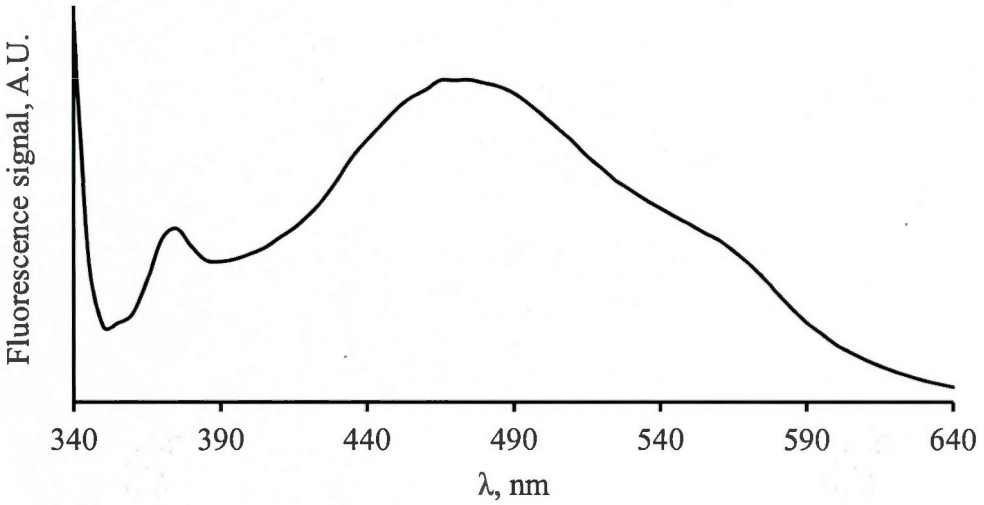


Figure 4.2: Fluorescence Emission Spectrum of *at***RA** at a Concentration of 15μg/mL in MeOH Using 343nm at the excitations wavelength.

UV and FLU spectroscopic characteristics of an *at***RA** solution are summarized in Table 4.2.

Table 4.2:Spectroscopic Characteristics for a Solution of *at***RA** in MeOH (15 μg /mL)

UV	$\lambda_{Optimal}$: 343 nm
FLU	$\lambda_{Excitation}$: 343 nm Scan from 340 to 640 nm $\lambda_{optimal}$: 470 nm

It should be mentioned that the results presented in the following sections are for the pharmacological level which was analyzed by HPLC-UV using a HPLC pump spectrometer P4000, mobile phase MeOH:NaOAc 0.01M (85:15, v/v) at a flow rate

of 1.5 mL/min. A Waters 2487 UV detector and a Phenomenex Luna C18(2) separation column, (5 μm, 150mm × 4.60 mm ID) were also used.

4.1.2 Evaluation of *at*RA Mother Solution Stability

A mother solution (10 μg/mL) was prepared in MeOH from stock solution. It was monitored by HPLC-UV at intervals of 24 hours for sixteen days. As shown in Figure 4.3, the results demonstrate that there was no significant change in the *at*RA signal intensity during this period. Therefore, a 16d interval was chosen for the preparation of fresh mother solution.

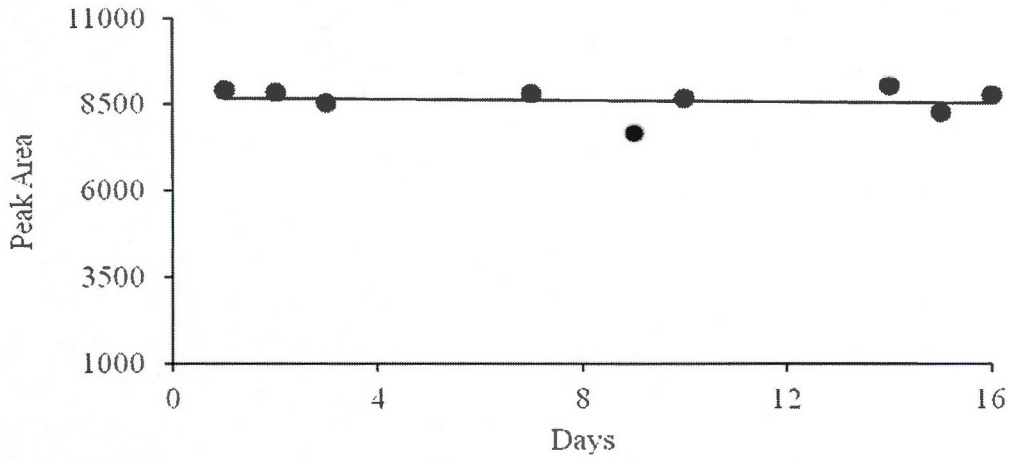


Figure 4.3: Stability Evaluation of *at*RA by HPLC-UV Analysis of a Mother Solution (10 μg/mL) Over 16 Days.

4.1.3 *at*RA Light Sensitivity Study

*at*RA is a light sensitive compound, therefore, appropriate storage and handling of the solution is very important. To study *at*RA photo-isomerization and the effect of light

on the rate of transformation; transparent and amber glass vials were chosen for this study, as indicated in Table 4.3. This study was performed using the two types of vials, under different conditions, that is,

- Without or with an exposition to light for 25 min,
- The presence or absence of aluminums foil around the vials.

Table 4.3: Type of Vial and Presence or Not of Light for Solution of *at***RA**. (Entries 1 to 6 are for Figures 4.4 and 4.5)

Entry	Vials Containing *at***RA** Solution	Light (25 min)
1	Transparent glass vial not wrapped with aluminum foil	Exposed
2	Transparent glass vial not wrapped with aluminum foil	Not Exposed
3	Amber glass vial not wrapped with aluminum foil	Exposed
4	Amber glass vial not wrapped with aluminum foil	Not Exposed
5	Amber glass vial wrapped with aluminum foil	Exposed
6	Amber glass vial wrapped with aluminum foil	Not Exposed

The transformation rates were determined by variations in the signal intensities of the HPLC-UV chromatograms. Figure 4.4 shows the chromatograms for *at***RA** in MeOH (10μg/mL). The dotted trace resulted from the use of a transparent glass vial which was not wrapped by aluminum foil and exposed to light for 25 min (Entry 1) and the solid lined one resulted from use of amber glass vials wrapped with aluminum foil and unexposed to light for 25 min (Entry 6). Each chromatogram has two peaks, one representing *at***RA** and the other representing isomerization products. The transformation rate was calculated by dividing the measured area of the first peak (attributed to unknown isomerization products) by the area of the two peaks.

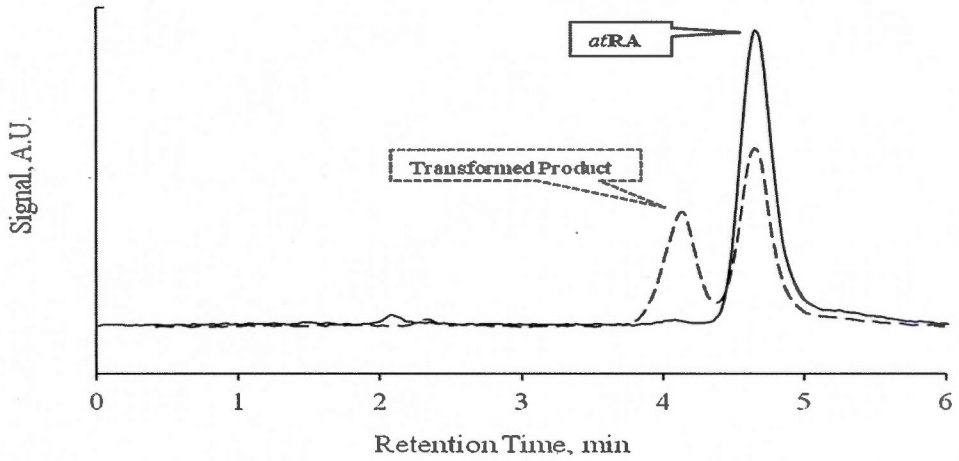


Figure 4.4: Comparison of HPLC-UV Chromatograms of *at*RA and Isomerization Products obtained from an Exposed (Table 4.3 Entry 1; dashed line) and Unexposed (Table 4.3 Entry 6; solid line), mother solution (10 µg/mL).

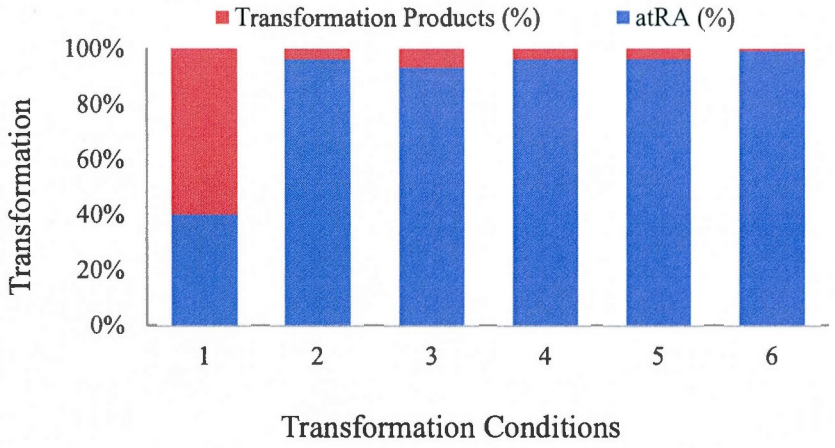


Figure 4.5: Comparison of *at*RA (Blue) *vs* Products Generated from Varied Exposures to Light (Red).

These results demonstrate that *at*RA is a light sensitive. Therefore, it is recommended that amber glass vials wrapped with aluminum foil be used for handling and storage of stock and standard solutions.

4.1.4 Comparison between Evaporation and Calibration Curves for *at*RA

In order to evaluate the loss of analyte during the evaporation process, the evaporation and calibration curves were compared. Figure 4.6 shows the change in peak area *vs.* the concentration of *at*RA. The slopes of calibration and evaporation curves were 7845 and 7503, respectively, that is, they were quite close. This indicates that using a stream of nitrogen would not result in a significant loss of *at*RA.

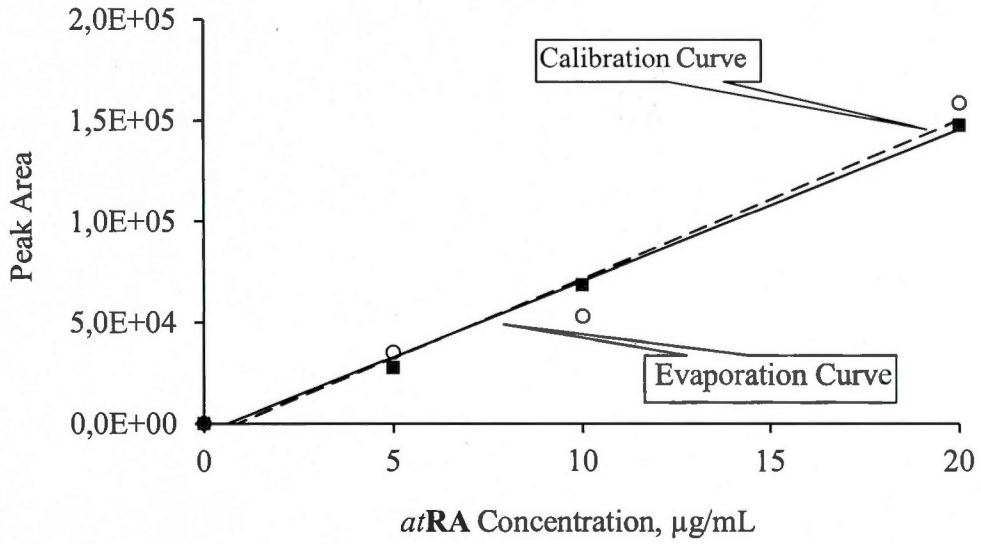


Figure 4.6: Comparison of Evaporation and Calibration Curves for *at*RA

4.1.5 *at*RA Pharmacological Level Extraction at Buffered Mobile Phase

The extractions of *at*RA were performed in µg quantities (at a pharmacological level) in aqueous and serum-supplemented cell culture media. The effect of different extraction parameters on the recovery rate were determined. A HPLC-UV was used at a high flow rate (1.5 mL/min) with MeOH:NaOAc 0.01M (85:15, v/v) as the mobile phase.

- *at***RA** Extraction in Aqueous Media

The extraction procedure followed was that of Bérubé *et al.*,2005 with the following modifications:

- The mobile phase was MeOH:NaOAc 0.01 M (85:15, v/v) and the desorption solvent was MeOH.
- The acidified hexanes was prepared by adding 2 drops of glacial acetic acid to hexanes (10 mL) *via* a Pasteur pipette. The resultant solution was mixed for 15s.
- The volume of organic solvent (EtOAc: Acidified Hexanes, 50:50, v/v) used for the extraction was 5 mL.
- Volume of desorption solvent (V_2) used varied from 0.2 to 5 mL.

Extraction Protocol

1- The *at***RA** mother solution of 10 µg/mL in MeOH was freshly prepared by dilution of the stock solution (1000 µg/mL) on the day of analysis after having been warmed to room temperature.

2- The aqueous solutions of *at***RA** were prepared by dilution of the mother solution in water (C_1) and a volume (V_1, 1 to 5 mL) of this solution was transfered to glass a tube.

3- The same volume of MeOH was then added to each tube.

4- A solvent mixture (5 mL) composed of EtOAc:Acidified hexanes 50:50 v/v was added to each tube.

5- The solution was mixed for 5 min and left to rest until phases separated (approximately 1 min).

6- The upper organic phase was tranferred to a glass test tube and evaporated almost to dryness under nitrogen at room temperature.

7- A second extraction was obtained in the same manner (i.e. steps 4 to 6).

8- On the other hand, a third extraction was performed as before but with the use of acidified hexanes (5 mL) as the extracting solvent.

9- The combined extracts was evaporated to dryness at room temperature under a gentle stream of nitrogen gas.

11- The residue was dissolved in MeOH (V_2, 200 µL).

12- A volume of 20 µL was injected into the HPLC.

13- The peaks of ***at*RA** were identified by comparing with *at*RA dissolved in MeOH and not subjected to extraction. Blank extraction solution was injected prior of the extraction solution and the absence of ***at*RA** signal was verified.

14- The extraction recovery rate was determined from the area ratio of the extraction solution *vs* a standard solution at the same concentration.

The extraction protocol is shown in figure 4.7

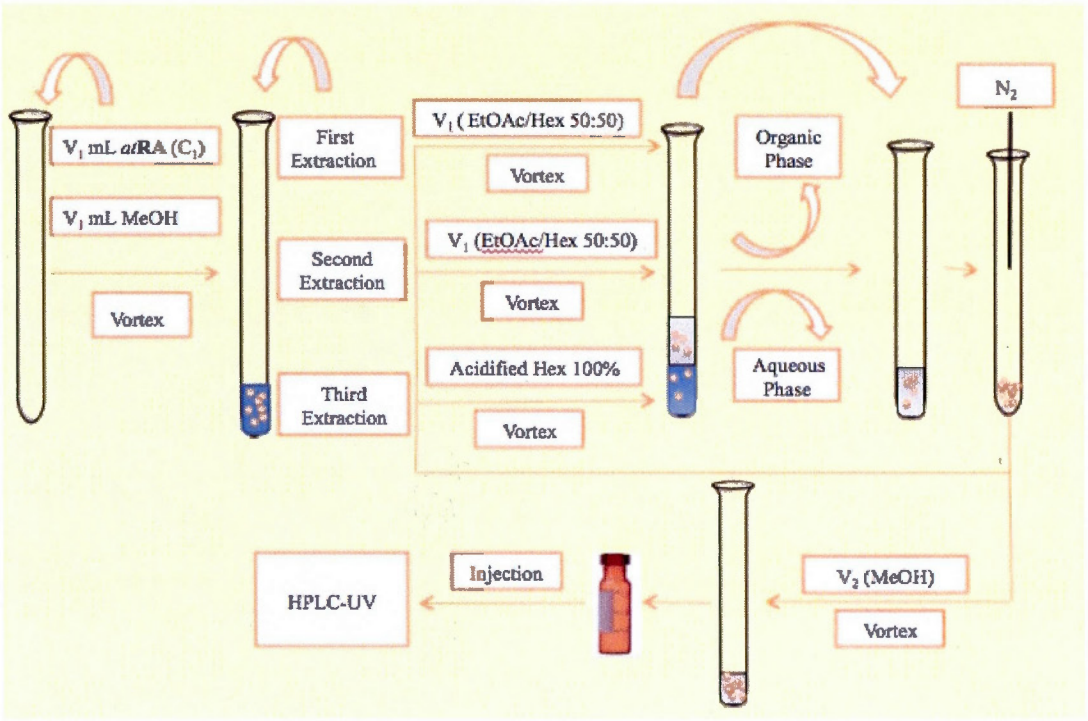


Figure 4.7: Schematic of *at***RA** Liquid- Liquid Extraction.

➢ Effect of *at***RA** SampleVolume on Extraction Recovery Rate

The effect of sample volume on extraction recovery rate from aqueous media was determined using a solution *at***RA** (C_1, 10 µg/mL). Different volumes of the solution (1 to 5 mL) were used in the above extraction protocol. The results are shown in Table 4.4 and figure 4.8 which demonstrate that the recovery rate slightly decreases with an increase of sample volume.

Table 4.4: *at***RA** Extraction Parameters for Samples Volumes and Extraction Recovery Rate.

C_1 (µg/mL)	V_1 (mL)	Quantity (µg)	V_2 (mL)	C_2 (µg/mL)	PCF [a]	Recovery Rate (%)
10	1	10	0.2	50	5	62
10	2	20	0.2	100	10	59
10	3	30	0.2	150	15	42
10	5	50	0.2	250	25	41

[a]: Pre-Concentration Factor

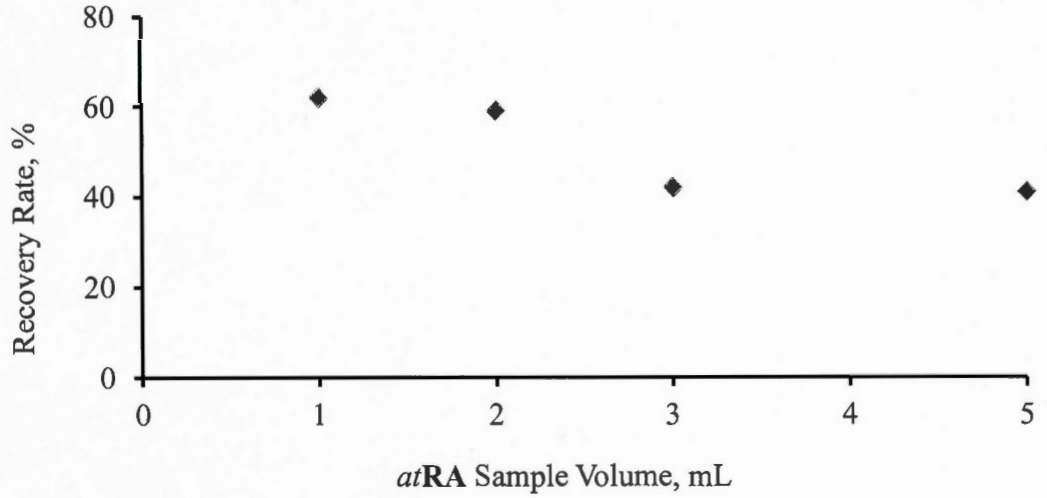


Figure 4.8: Effect of *at***RA** Sample Volume on Extraction Recovery Rate.

- Effect of *at***RA** Sample Concentration on Extraction Recovery Rate

The effect of sample concentration on extraction recovery rate from aqueous media was determined using a fix *at***RA** sample volume (V_1) of 1mL. Different concentrations of *at***RA** from (C_1, 0.2 to 10 µg/mL) were used in the extraction and

the results are shown in Table 4.5 and Figure 4.9. The results demonstrate that the extraction recovery rate was optimal at 2 µg/mL.

Table 4.5: *at***RA** Extraction Parameters for the Study of Effect of Sample Concentration on Recovery Rate

C_1 (µg/mL)	V_1 (mL)	Quantity (µg)	V_2 (mL)	C_2 (µg/mL)	PCF	Recovery Rate (%)
0.2	1	0.2	0.2	1	5	51
2	1	2	0.2	10	5	80
10	1	10	0.2	50	5	62

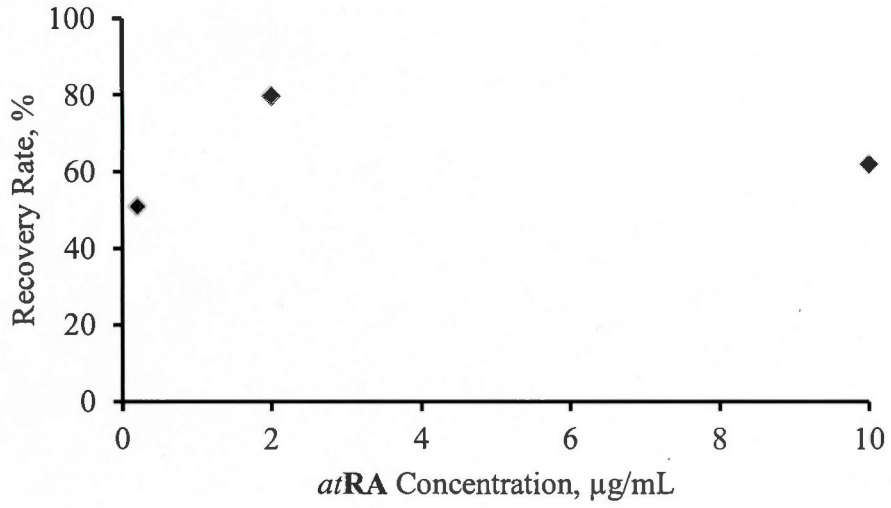


Figure 4.9: Effect of *at***RA** Sample Concentrations on Extraction Recovery Rate.

- *at***RA** Extraction in Serum-Supplemented Cell Culture Media αMEM:FBS (90:10, v/v).

The extraction protocol was the same as that used for *at***RA** in aqueous solution. The extraction of *at***RA** using serum-supplemented cell culture media was performed and then repeated 3 times. The results show an average recovery rate of 73±10%

4.2 Final Step of Method Development (*at***RA** Physiological Level)

This development step was time-consuming because two of the major parameters required optimization. The decrease in the quantity of *at***RA** from a pharmacological to a physiological amount requires a more sensitive method. Thus additional optimization was required for the transition from HPLC-UV to HPLC-ESI-MS/MS. Nevertheless, preliminary knowledge gathered from the HPLC-UV method was subsequently used for the development of the HPLC-ESI-MS/MS method. To this end, the following modifications were required: a reduction in the flow rate of the mobile phase from high to low (1.5 to 0.2 mL/min) and a modification of the buffer from non-volatile to volatile. Initially, HPLC-UV was used to determine these parameters while selecting a mobile phase.

4.2.1 Qualitative Assay Using HPLC-ESI-MS/MS

Qualitative assays were investigated with the idea of developing a specific HPLC-ESI-MS/MS method. Also, optimal conditions were needed to be found and a study of the effect of collision energies and gas on the formation of an intense specific ions (255 *m/z*) for *at***RA** were pursued. For the development of the highly sensitive HPLC-ESI-MS/MS method, qualitative assays were performed using a syringe pump fitted directly onto the MS section and the data acquisition was set in continuum mode.

Qualitative Assays with Buffered Mobile Phase MeOH:NH_4OAc (0.01 M 85:15, v/v)

- Analysis in Scan Mode

A solution of 10 µg/mL of *at***RA** dissolved in the mobile phase was injected and eluted with MeOH:NH_4OAc 0.01M (85:15, v/v) as mobile phase at flow rate of 0.1 mL/min. Analysis in scan mode was performed using mass detection ranging from 80-320 *m/z* . As shown in figure 4.10, two abundant ions were identified at (*m/z* 255) and (*m/z* 299) in the following mass spectra.

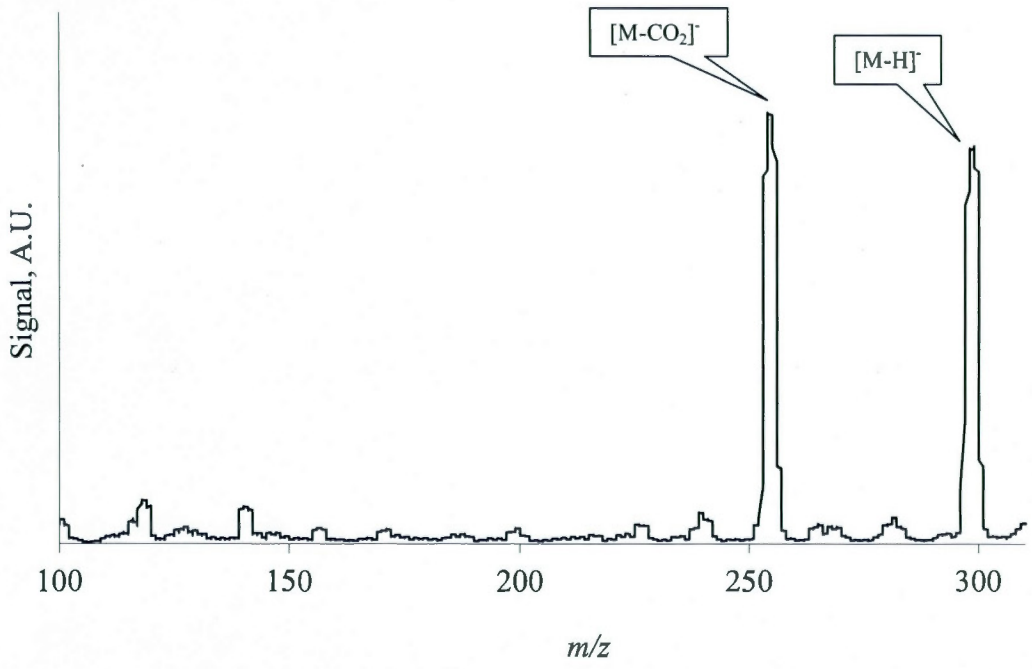


Figure 4.10: Mass Spectrum in Continuous Scan Mode Observed for an *at***RA** Solution of 10 µg/mL Dissolved in MeOH:NH_4OAc 0.01M (85:15, v/v).

The same assay, but with a lower concentration of *at***RA** (100 ng/mL), was performed and is shown in Figure 4.11. The result demonstrates that the two aforementioned peaks are present in trace amounts.

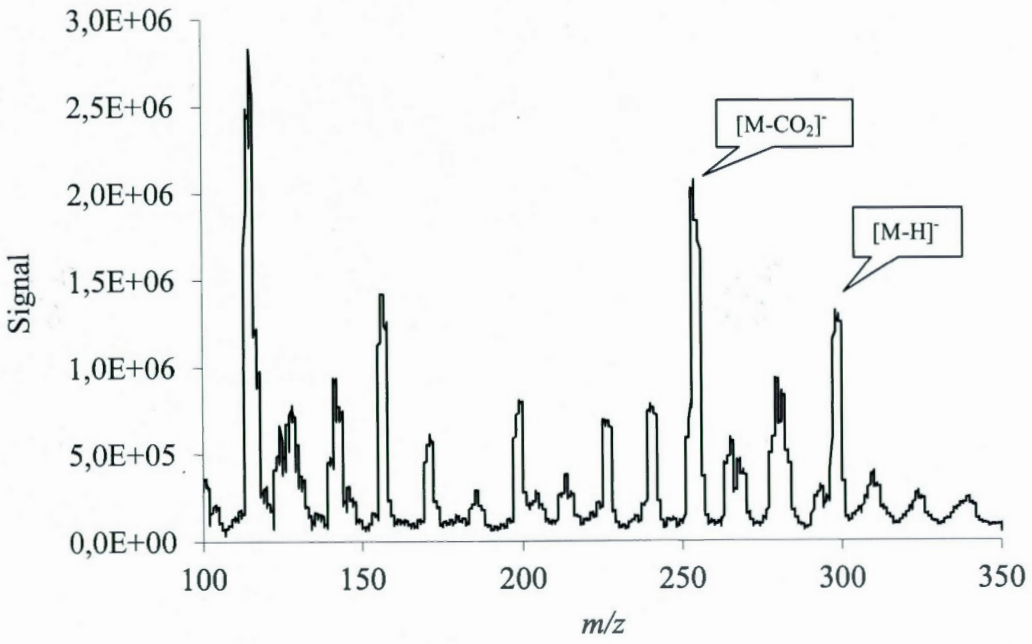


Figure 4.11: Mass Spectrum in Continuous Scan Mode Obtained for an *at***RA** Solution of 100 ng/mL Dissolved in MeOH:NH_4OAc 0.01M (85:15 v/v).

➢ Analysis Using Tandem Mass Spectroscopy (MS/MS)

MS/MS analysis was performed in daughter ion mode with an *at***RA** solution of 100 ng/mL at the flow rate of 0.1 mL/min. The pressure of the collision gas and its energy were set at 17×10^{-3} mbar and at -29 eV, respectively. As shown in Figure 4.12, an important peak is obtained for *at***RA** precursor ion of *m/z* 299 corresponds to $[M-H]^-$. Also observed was a less important peak at *m/z* 255, identified as $[M-CO_2]^-$. The relative abundance of the *m/z* 255 ion peak to that of

the precursor ion is about 3%. Like the other reported methods using MS, no major fragmentation of *at***RA** was observed by ESI in negative mode with a buffered mobile phase (Van Breemen and Huang, 1996).

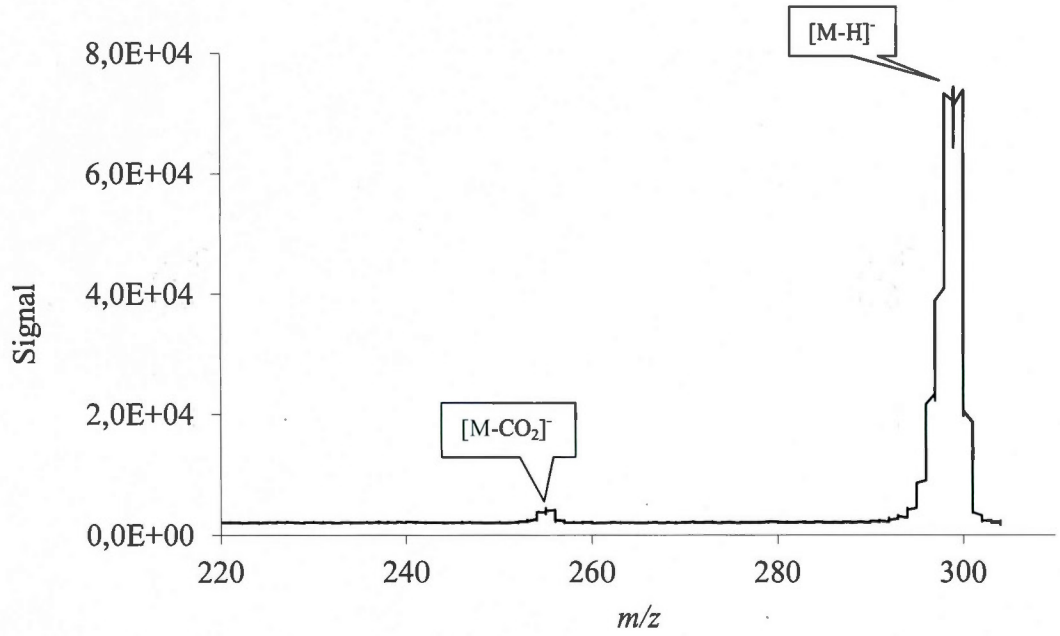


Figure 4.12: MS/MS Mass Spectra in Daughter Mode of an *at***RA** Solution of 100 ng/mL Dissolved in MeOH:NH_4OAc 0.01M (85:15, v/v).

- Effect of Collision Energies on the Fragmentation of the Precursor Ion in Tandem Mass Spectrometry

Tests were performed with different collision energies so as to optimize the system. The result demonstrates that changing the collision energy in the range between -29 to -60 eV did not have a significant effect on the production of the specific ions *m/z* 255 for this type of mobile phase. As shown in Table 4.6 and Figure 4.13, *at***RA** does

not generate an intense daughter peak using this buffered mobile phase. A strong daughter ion peak is required for a high sensitivity analysis.

Table 4.6: Signal Intensity of Daughter Ion/ Precursor Ions Ratio *vs* Collision Energies Using the Buffered Mobile Phase MeOH:NH_4OAc 0.01M (85:15, v/v).

Collision Energy (eV)	Precursor Ion (*m/z* 299)×10^3	Daughter Ion (*m/z* 255)×10^3	*m/z* (255/299) (%)
-29	49.0	1.4	3
-40	5.9	3.4	57
-50	8.4	4.5	54
-62	6.5	2.4	37

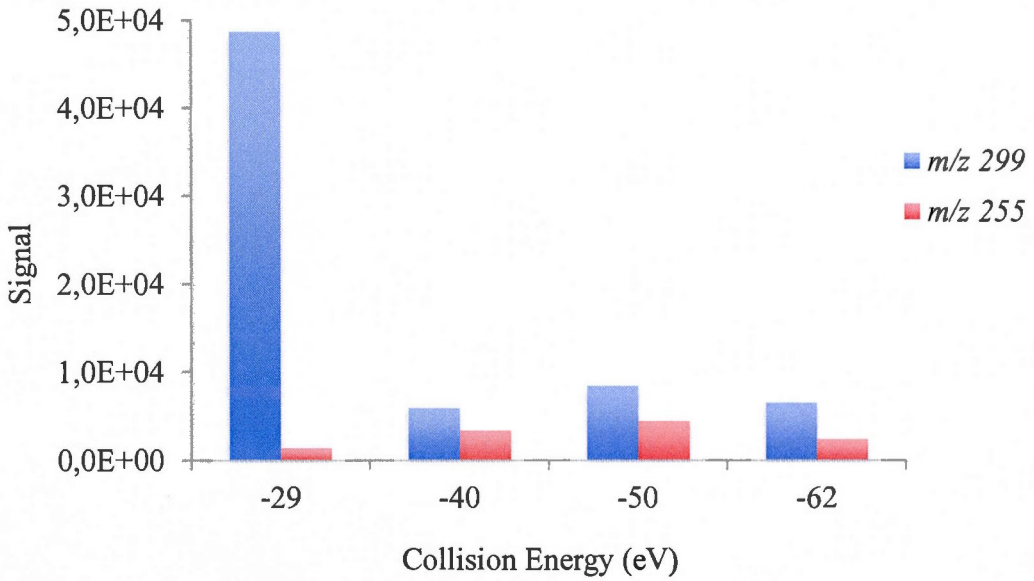


Figure 4.13: Effect of Collision Energies on Signal Intensity of the Precursor (blue) and Daughter (red) Ions for *at***RA** Solution of 100 ng/mL Dissolved in MeOH:NH_4OAc 0.01 M (85:15, v/v).

- Qualitative Assay with Non-Buffered Mobile Phase MeOH:HOAc 1mM (75:25, v/v)

The signal intensity of specific daughter ion plays a key role in method development for the trace analysis of *at***RA**. As shown in the previous section, the solvent MeOH: NH_4OAc was not suitable for these analyses. Therefore, a non-buffered mobile phase was chosen, as shown in Figure 4.14. This mobile phase allowed for an increase in the ratio of daughter ion to precursor.

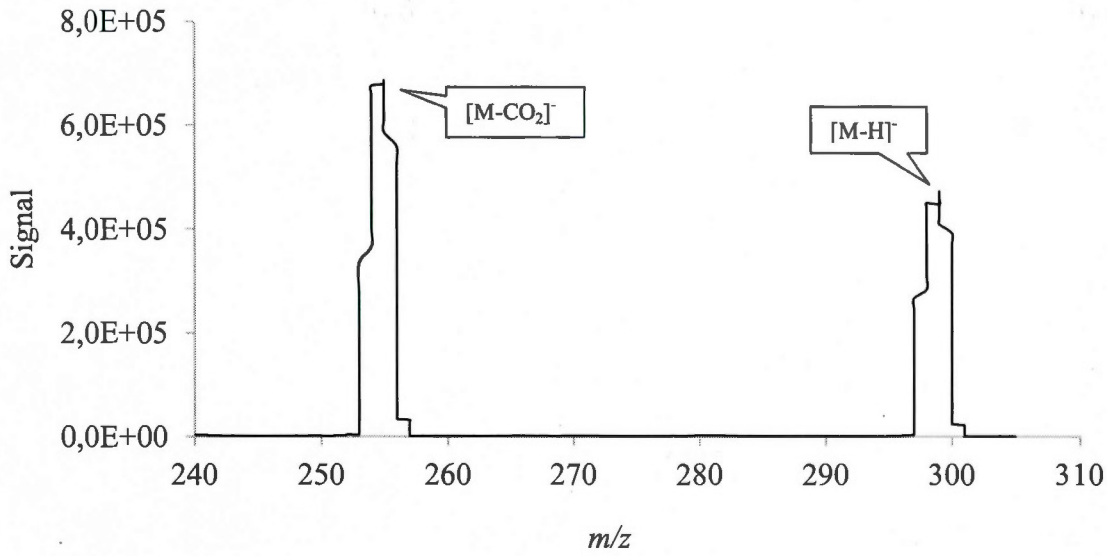


Figure 4.14: MS/MS Mass Spectra in Daughter Mode of an *at***RA** solution of 100 ng/mL Dissolved in MeOH:HOAc 1mM (75:25, v/v).

- Effect of the Collision Gas (Argon) on Fragmentation of *at*RA Using Non-Buffered Mobile Phase

To study the effect of argon as a collision gas for the fragmentation of ***at*RA,** MS/MS analyses were performed in the presence and absence of argon. The results are shown in Figure 4.15 and Figure 4.16, respectively. The results demonstrate that there are no significant changes in the signal intensity of the *m/z* 255 ion in the presence or absence of argon.

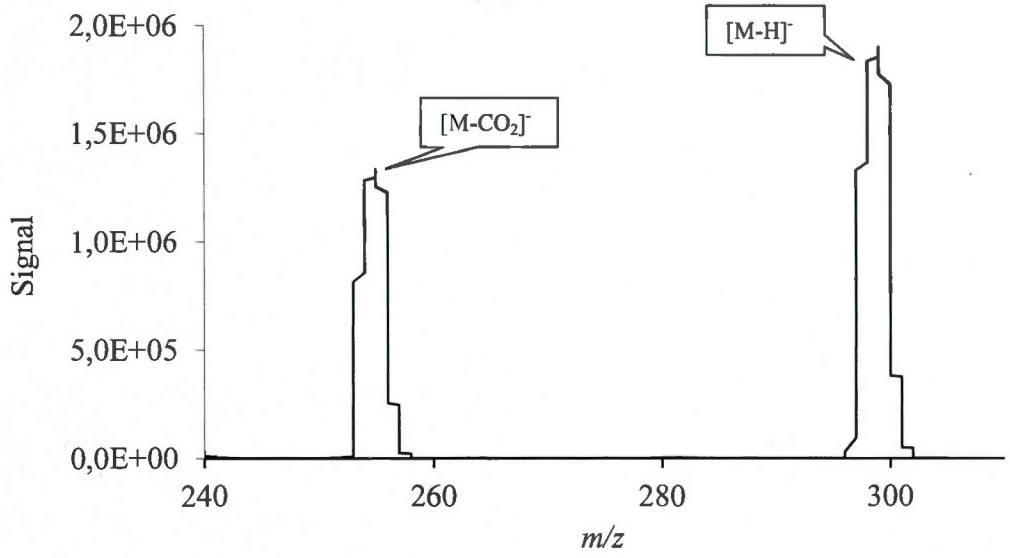


Figure 4.15: MS/MS Spectrum of an ***at*RA** Solution of 1000 ng/mL in the Presence of Argon (1.9 10^{-3} mbar) Dissolved in MeOH:HOAc 1mM (75:25, v/v).

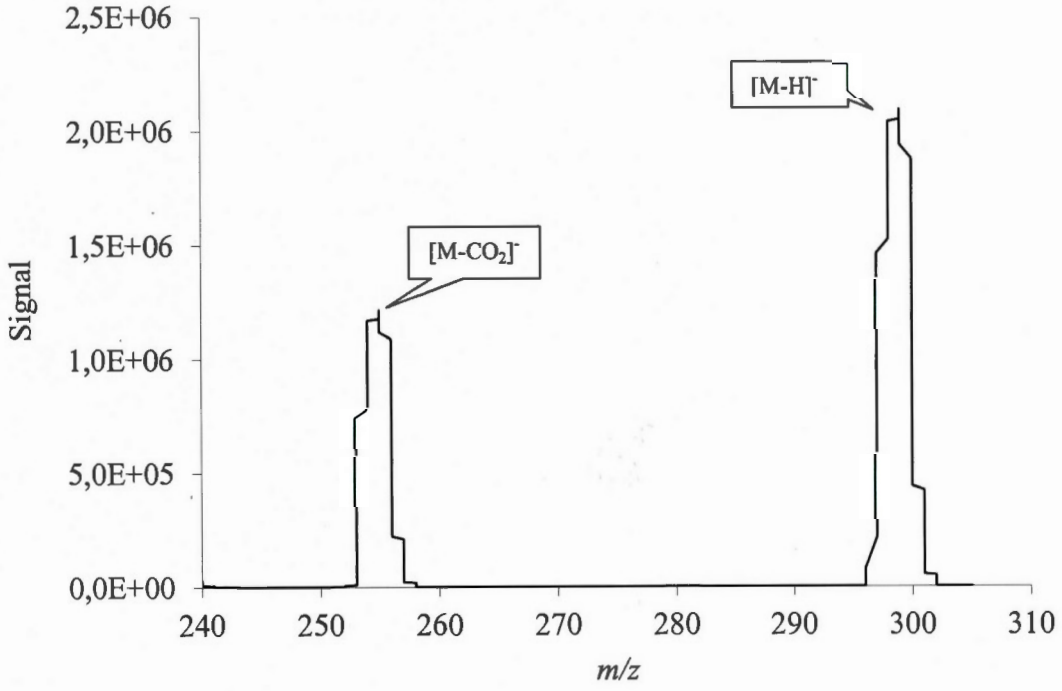


Figure 4.16: MS/MS Spectrum of an *at***RA** Solution of 1000 ng/mL Dissolved in MeOH:HOAc 1mM (75:25, v/v) in the Absence of Argon.

Another test was performed with an increased pressure of argon gas. The result demonstrates that this change did not affect the signal intensity. As shown in Figure 4.17, an acidic non-buffered mobile phase generated abundant specific ions at *m/z* 255 at very low concentration contrary to the buffered one MeOH:NH_4OH 0.01M (85:15, v/v). Therefore, MS/MS analysis were done without argon. However, a filtering mode was used. Thus the first quadrupole mass filter was used for precursor ion selection (*m/z* 299) and the second, again in filtering mode, was used for increased detection and sensitivity allowing for a better limit of detection (LOD).

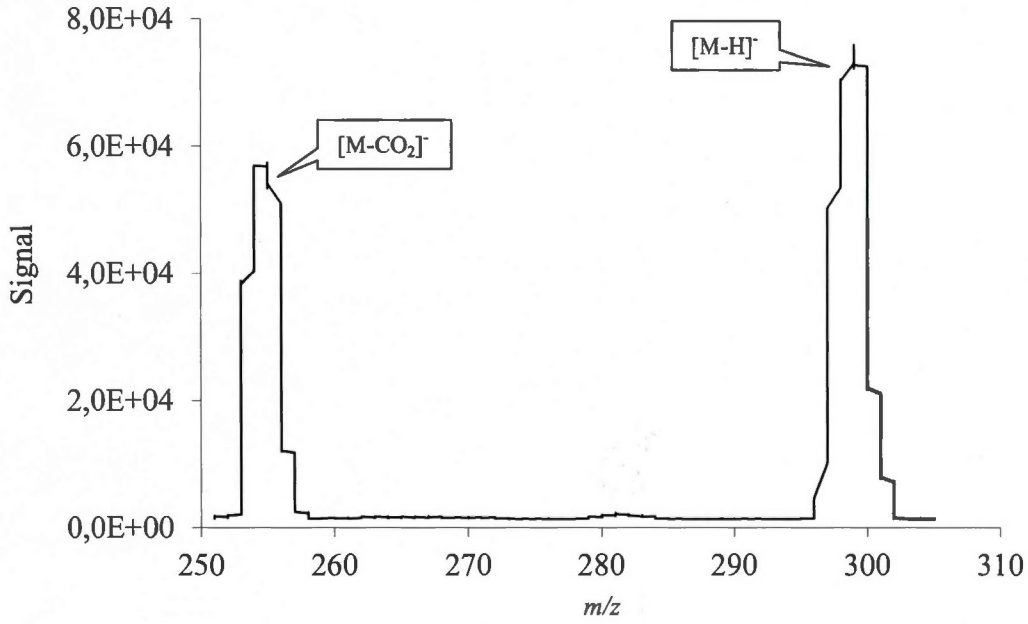


Figure 4.17: MS/MS Mass Spectra of an *at***RA** Solution of 10 ng/mL Dissolved in MeOH:HOAc 1mM (75:25, v/v).

➢ Parameters of ESI-MS/MS

The optimized parameters of mass spectrometer including ESI interface, mass analyzer and aquisition data are summerarized in Table 4.7.

Table 4.7: Parameters for Analysis of *at***RA** by ESI-MS/MS.

	ESI
Capillary	-3.20 kV
Cone	-32 V
Extractor	3 V
RF Lens	0.50 V
Source Block Temperature	140 °C

Desolvation Temperature	250 °C

Mass Spectrometer Parameters

MS_1

Entrance	20 V
Exit	0.0 V
Ion Energy	0.4 V
Ion Energy Ramp	0.0 V
LM Resolution	1.2
HM Resolution	1.2

MS_2

Ion Energy	2.5 V
Ion Energy Ramp	0.0 V
LM Resolution	0.0 V
HM Resolution	5.6 V
Multiplier	650 V

Aquisition Parameters

Start Mass	297.2
Set Mass	299.2
End Mass	301.2
MS Function Type	Daughter of 299
Acquisition Format	Continuous
Slope Gain	500
Inter Scan Time (secs)	0.10

Gas flow

Desolvation Gas Flow	709 L/h
Nebulization Gas Flow	75 L/h

Pressure

Analyser Vacumm	5.29 e^{-6} mbar
Gas Cell	3.7 e^{-5} mbar

4.2.2 Quantitative Assays Using HPLC-ESI-MS/MS

- Assays with Non-Buffered Mobile Phase

The information obtained from the qualitative study was used to perform the first quantitative analysis using ACN:H_2O:HOAc (95:4.8:0.2, v/v) with the extraction of *at***RA** in aqueous media. The second assay was performed by increasing the aqueous portion of the mobile phase while decreasing the HOAc from 0.2 to 0.1 then to 0.01 v/v.

➢ Analysis of *at***RA** Extract by HPLC-ESI-MS/MS Using Non-Buffered Mobile Phase

The extraction protocol was same as that described in above in the method development section. However, several parameters were modified and thus, the revised protocol is described here.

1- Aqueous *at***RA** solutions of various concentrations were prepared from the mother solution of 10 µg/mL in MeOH. A volume (0.4 mL) of each aqueous solution was transferred to a glass tube.

2- HPLC grade MeOH (0.4 mL) was added to the glass tube containing the *at***RA** solution.

3- A solvent mixture 1.3 mL of of EtOAc: Acidified Hexanes (containing 0.90 % v/v of HCl 4N/ Hexane) 50:50 v/v was subsequently added to the tube.

4- The mixture was stirred for 5 min at the speed of 5000 RPM.

5- The organic phase was transferred to a test tube and evaporated almost to dryness.

6- The extraction was performed two times and then another extraction was done using 1.3 mL acidified hexanes instead of 1.3 mL EtOAc:Acidified Hexanes 50:50 v/v.

7- The combined extractions was evaporated to dryness at room temprature under a gentle stream of nitrogen gas.

8- The residue was dissolved in 200 µL of desorption solvent (MeOH).

9- The final extracted solution was filtered on a 0.45 µm filter before injection.

10- A volume of 20 µL of the resultant solution was injected into the reverse phase HPLC system.

- Effect of Presence of Acid in a Non-Buffered Mobile Phase on Extraction Recovery Rate

To study effect of the acidity in a non-buffered mobile phase on extraction recovery rate, the extraction of a fixed quantity of *at***RA** (80 ng) using the protocol described above in aqueous media. Figure 4.18 shows the chromatograms of extracted *at***RA** solutions with different compositions of the mobile phase. The dashed line represents the chromatogram of the extracted *at***RA** solution eluted with ACN:H_2O:HOAc (95:4.8:0.20, v/v) while the solid line represents the extract solution eluted ACN: H_2O:HOAc (95:4.99:0.01, v/v). The latter was the best choice for non-buffered mobile phase since it offered a recovery rate of about 72%. The results also demonstrate that, in a non-buffered mobile phase, the proportion of acid leads to a

wide variation in results and irreproducibility in the extraction recovery rate. The results indicate that ACN:H_2O:HOAc (95:4.9:0.01, v/v) was the best choice of the mobile phase since it offers the best signal intensity for both extraction and standard solutions in comparison to those obtained from more acidic ones.

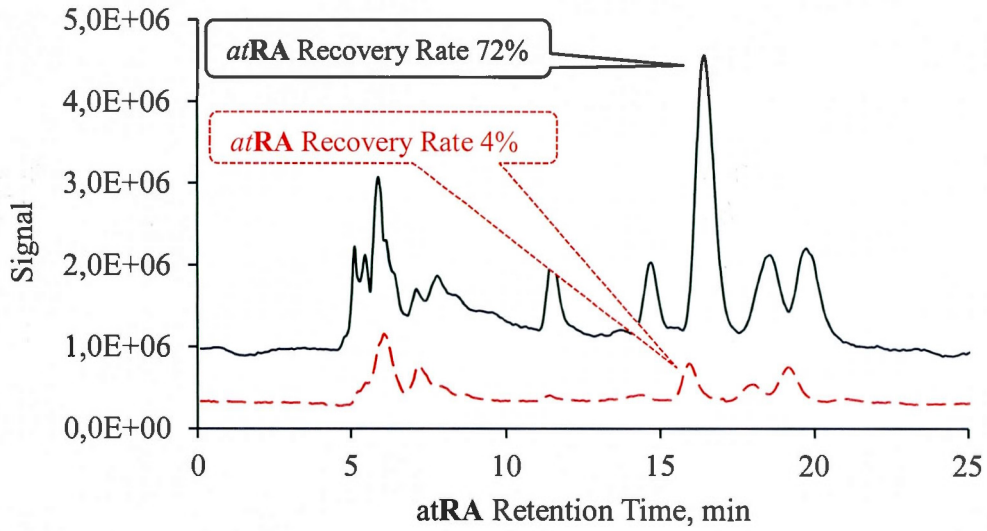


Figure 4.18: Comparison of Extraction Recovery Rate for 80 ng of *at***RA** with Non-Buffered Mobile Phase ACN:H_2O:HOAc (95:4.8:0.2 v/v, dashed line) and ACN:H_2O:HOAc (95:4.99:0.01 v/v, solid line); HPLC Pump: Waters Model 2695, Separation Column Phenomenex Luna C18(2) (3 µm, 100 mm × 4.6 mm ID) and Flow Rate: 0.2 mL/min.

- Effect of Acid in Organic Extraction Solvents (Hexanes) on the Profile of the Extracted *at***RA** spectra

To study the effect presence of acid in the organic extraction solvents, three extractions of 80 ng of *at***RA** were performed with a same volume of acidic organic solvents: EtOAc: Hex 50:50, v/v containing 0.30 %, 0.60 % and 0.90 % HCl (4N) :

Hex v/v. The resulting chromatograms of the extracted *at***RA** are shown in Figure 4.19. They display undesired double peaks eluting at around 18 min. These are present in all extraction profiles and will cause interference because they are very close to the retention time of *at***RA.** These undesired peaks are also present in chromatogram of the blank extraction eluted with non-buffered mobile phase and thus are attributed to the acid (HCl, 4N) used for acidification of the hexanes.

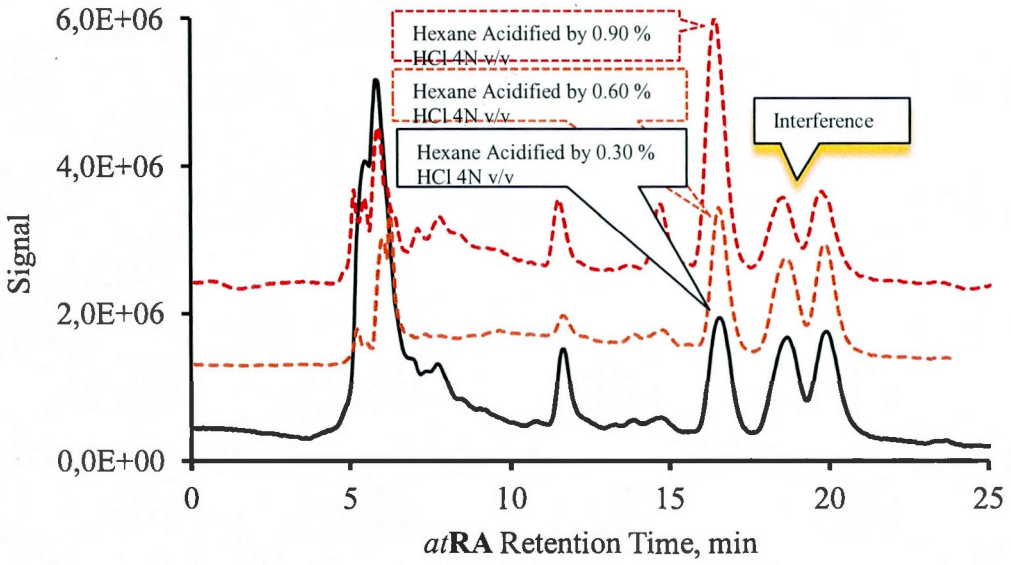


Figure 4.19: Chromatograms of *at***RA** Solutions Extracted with EtOAc: Hex, 50:50, v/v Containing Different Amounts of HCl (4N). HPLC Pump: Waters, Mobile Phase: ACN:H_2O:HOAc (95:4.99:0.01, v/v), Separation Column: Phenomenex Luna C18(2) (3 µm, 100 mm × 4.6 mm ID) and Flow Rate: 0.2 mL/min.

- Results Obtained from Analysis by HPLC-UV and HPLC-ESI-MS/MS

Each *at***RA** extraction was repeated three (3) times (triplicate). The parameters used and the analysis of the extracts by HPLC-UV and HPLC-ESI-MS/MS are given in Tables 4.8 and 4.9, respectively.

Table 4.8: ***at*RA** Extraction Parameters for Analysis by HPLC-UV Using ACN:H_2O:HOAc (95:4.99:0.01, v/v).

C_1 (ng/mL)	V_1 (mL)	Quantity (ng)	V_2 (mL)	C_2 (ng/mL)	PCF	Average Recovery Rate (%)
100	0.4	40	0.2	200	2	47±11
200	0.4	80	0.2	400	2	43±5
300	0.4	120	0.2	600	2	70±5

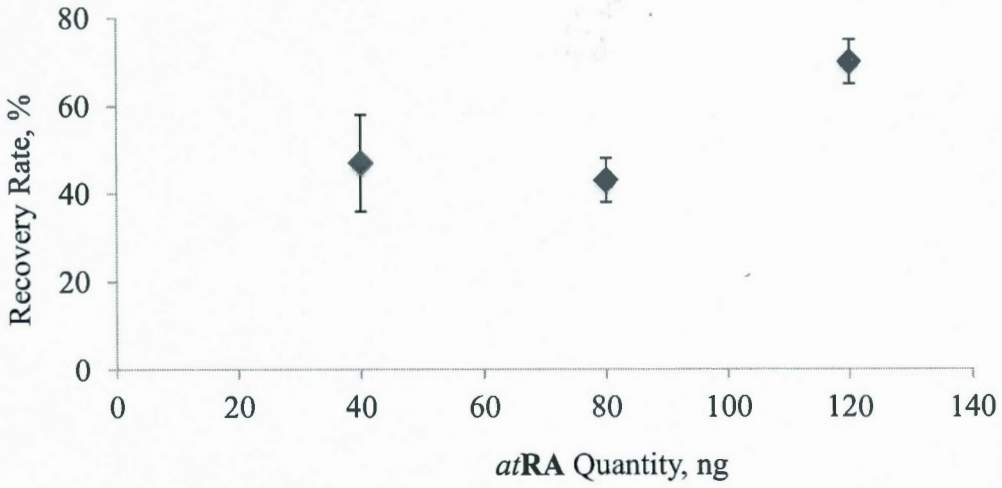


Figure 4.20: Recovery Rate of ***at*RA** from Aqueous Solution and Analysed by HPLC-UV Using a Non-Buffered Mobile Phase ACN:H_2O:HOAc (95:4.99:0.01, v/v) with Low Flow Rate (0.2 mL/min). HPLC Pump: Waters, model 2695 Separation Column : Phenomenex Luna C18(2) (3 µm, 100 mm × 4.6 mm ID).

Table 4.9: *at***RA** Extraction Parameters for Analysis by HPLC-ESI-MS/MS Using ACN:H_2O:HOAc (95:4.99:0.01, v/v).

C_1 (ng/mL)	V_1 (mL)	Quantity (ng)	V_2 (mL)	C_2 (ng/mL)	PCF	Average Recovery Rate (%)
25	0.4	10	0.2	50	2	35±34
50	0.4	20	0.2	100	2	57±32
75	0.4	30	0.2	150	2	80±36
100	0.4	40	0.2	200	2	75±32
200	0.4	80	0.2	400	2	64±9

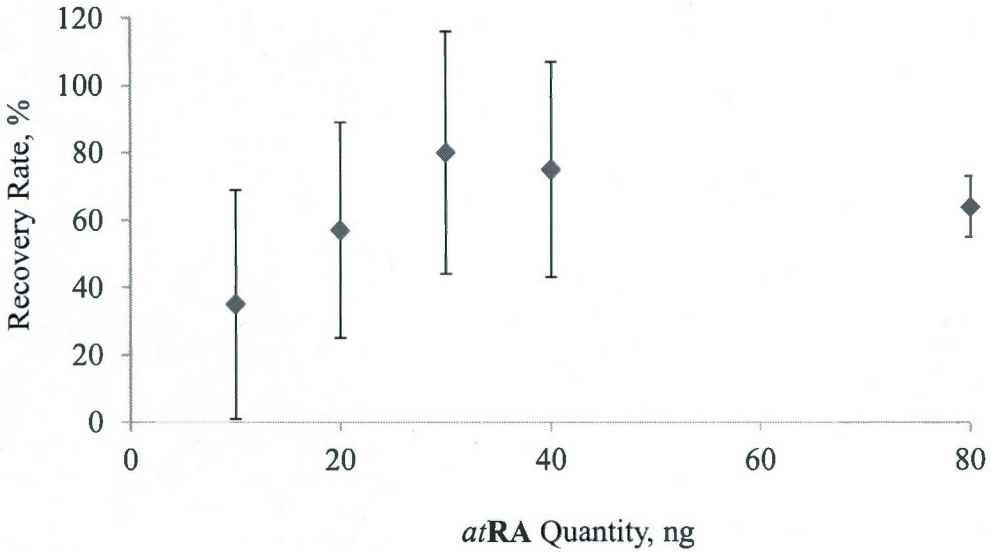


Figure 4.21: *at***RA** Recovery Rate from Aqueous Solution and Analysed by HPLC-ESI-MS/MS at a Low Flow Rate 0.2 mL/min Using a Non-Buffered Mobile Phase ACN:H_2O:HOAc (95:4.99:0.01, v/v). HPLC Pump: Waters, Model 2695 Separation Column: Phenomenex Luna C18(2) (3 µm, 100 mm × 4.6 mm ID).

- Comparsion of *at***RA** Recovery Rate Analysis by HPLC-UV and HPLC-ESI-MS/MS Using a Non-Buffered Mobile Phase

The conditions used in Figures 4.20 and 4.21 were applied to 40 and 80 ng of *at***RA** and the results are compared in Figure 4.22. There are differences in extraction recovery rate when *at***RA** is analysed by the two methods (HPLC-UV and HPLC-ESI-MS/MS), for the same quantity of *at***RA** and the same extraction proceture. The reason for these differences is not known.

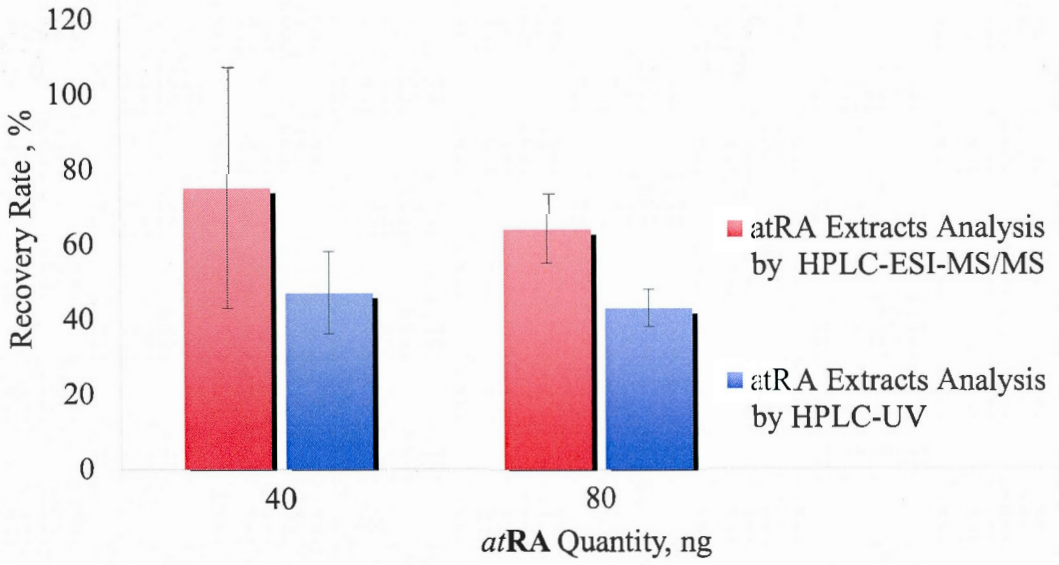


Figure 4.22: Comparison of Extraction Recovery Rates of *at***RA** by Different Analytical Methods (HPLC-UV and HPLC-ESI-MS/MS) for Different Quantities of *at***RA** (40 and 80 ng), Using a Non-Buffered Mobile Phase ACN:H_2O:HOAc (95:4.99:0.01, v/v). HPLC Pump: Waters Model 2695, Separation Column: Phenomenex Luna C18(2) (3 μm, 100 mm × 4.6 mm ID) and Flow Rate: 0.2 mL/min.

It was found that the signal intensity is not stable in a non-buffered mobile phase.

Figure 4.23 shows the fluctuations in signal intensity for standard solutions over 7 days.

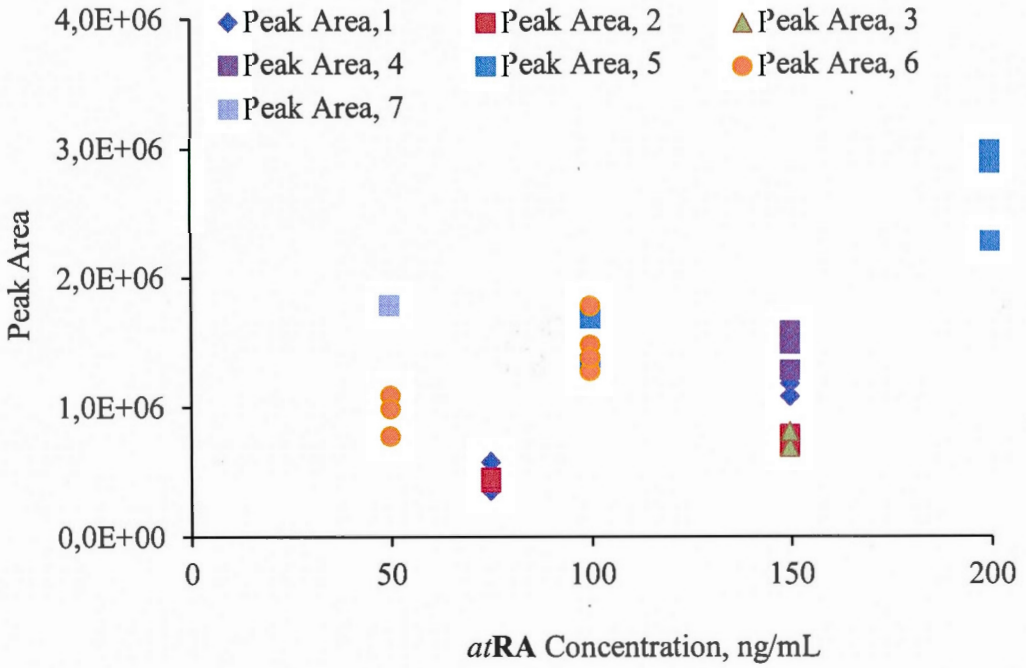


Figure 4.23: *at***RA** Standard Solution Reproducibility Using a Non-Buffered Mobile Phase ACN:H_2O:HOAc (95:4.99:0.01, v/v). HPLC Pump: Waters Model 2695, Separation Column: Phenomenex Luna C18(2) (3 µm, 100 mm × 4.6 mm ID) and Flow Rate: 0.2 mL/min.

As shown in Table 4.10, the signal intensity is not reproducible for standard solutions ranging from 50 to 200 ng/mL. A linear calibration curve was created from average data obtained in this concentration range. The coefficient of variation was 0.591 and slope of curve is equal 11426. There are significant differences between average observed areas and theoretically calculated areas of peaks using a high sensitivity method. The non-reproductibility of signal intensity of the standard solutions in a non-buffered mobile phase resulted in large standard deviations.

Table 4.10: Chromatographic Characteristics of *at***RA** Standard Solutions Using a Mobile Phase of ACN:H_2O:HOAc (95:4.99:0.01, v/v).

Standard (ng/mL)	Average Obtained Peak Area (10^6)	Calculated Peak Area (10^6)
50	1.3 ± 0.47	0.58
75	0.47 ± 0.07	0.87
100	1.5 ± 0.19	1.16
150	1.06 ± 0.30	1.74
200	2.73 ± 0.38	2.34

In conclusion, a buffered mobile phase should be used to achieve a good reproductibility for both standard and extracted *at***RA** solutions if high sensitivity is desired.

- Assay with Buffered Mobile Phase

➢ HPLC

❖ Mobile Phase Optimization

Mobile phase optimization is extremely important for chromatography. Solvent selection is experimentally based which can be very time consuming when applied to complex media. The main objective of mobile phase optimization is to meet the following requirements:

✓ Determination the best retention time. It is preferable to elute *at***RA** later than other polar metabolites in reverse phase so as to maintain sensitivity.

✓ Determining the best separation selectivity while balancing sensitivity.

In our case, more systematic strategies were needed. Retention time and selectivity may be optimized by changing mobile phase composition. The following parameters affect the sensitivity and selectivity and must be optimized

- The mobile phase flow rate.
- The type of organic solvent (MeOH *vs* ACN).
- The proportion of the organic portion of the mobile phase.
- The type and concentration of buffer.
- The type and proportion of aqueous buffer in the mobile phase
- The eluent acidity when dealing with ionisable analytes like ***at*RA**

MeOH was initially used as the organic solvent in the development of the HPLC-UV method for the following reasons:

- MeOH elutes analytes at lower retention time, therefore, it is more efficient for method development.
- MeOH is a less toxic and a more enviromentally friendly solvent than ACN.
- MeOH is less expensive than ACN.
- MeOH decreases the risks of buffer precipitation in the mobile phase.

Some limitations associated with the use of MeOH as the organic solvent are as follows:

- The back-pressure for MeOH increases when mixed with water, thus, using a column with a low back-pressure is recommended. At the same flow rate, ACN-based solutions produce lower pressures in the column.
- The flow rate of the organic solvent should be set lower with MeOH and higher with ACN.
- UV absorption of MeOH is larger than for ACN.
- The viscosity of MeOH is higher than that of ACN, thus, the pressure applied on the column by MeOH: H_2O is higher than that of ACN: H_2O (Aburjai, 2011).

ACN was chosen as the best organic solvent for HPLC-UV method development for the following reasons:

- ACN provides better resolution for many compounds by forming hydrogen bonds which result in better selectivity.
- ACN has a higher ionisation strength than MeOH.
- ACN offers reasonable buffer solubility.
- ACN is 100% miscible with water.
- ACN is transparent to UV light and offers a lower cutoff than MeOH.
- ACN is able to form dipole-dipole type interactions.

Different compositions of the mobile phase were investigated during development as shown in Table 4.11

Table 4.11: Composition of Mobile Phase Study for Method Development.

Organic Solvent Type	Buffer Type	Buffer (M)	pH	O.P [a] % v/v	A. P [b] % v/v	Flow Rate mL/min	Demostrated Method
MeOH	H_2O	-	-	85	15	1.5	HPLC-UV HPLC-FLU
MeOH	NaOAc	0.01	5.2	80	20	1.5	HPLC-UV HPLC-FLU
MeOH	NaOAc	0.01	5.2	85	15	1.5	HPLC-UV HPLC-FLU
MeOH	NaOAc	0.01	5.2	90	10	1.5	HPLC-UV
MeOH	NaOAc	0.01	5.2	95	5	1.5	HPLC-UV
MeOH	NaOAc	0.1	5.2	95	5	1.5	HPLC-UV
MeOH	NH_4OAc	0.1	5.2	95	5	0.2	HPLC-UV HPLC-ESI-MS/MS

MeOH	NH_4OAc	0.15	5.2	95	5	0.2	HPLC-UV HPLC-ESI-MS/MS
ACN	NH_4OAc	0.1	5.2	95	5	0.2	HPLC-UV HPLC-ESI-MS/MS
ACN	NH_4OAc	0.15	5.2	95	5	0.2	HPLC-UV HPLC-ESI-MS/MS
ACN	H_2O	-	-	95	5	0.2	HPLC-UV HPLC-ESI-MS/MS

[a]: O. P: Organic Portion.
[b]: A. P: Aqueous Portion

❖ Effect of Proportion of Buffer in the Mobile Phase on Sensitivity.

To study the effect of the proportion of buffer in the mobile phase on sensitivity and retention time, a solution of 1μg/mL of *at***RA** was dissolved in MeOH to with two different volumes of buffer (MeOH: NH_4OAc, 0.01M, 90:10 v/v; MeOH: NH_4OAc, 0.01M; 80:20, v/v) were added. The data are summarized in Table 4.12 and the chromatograms obtained are shown in Figure 4.24. *at***RA** elutes later with MeOH: NH_4OAc 0.1M (80:20, v/v) than with MeOH:NH_4OAc 0.1 M (90:10, v/v) using the same stationary phase and flow rate. The results demonstrate that the buffer composition of the mobile phase does not significantly affect the intensity of the signals but it does have an effect on the retention time for *at***RA**.

Table 4.12 Chromatographic Characteristics Obtained for *at***RA** Eluted With MeOH: NH_4OAc 0.01M (80:20, v/v) *versus* MeOH: NH_4OAc 0.01M (90:10, v/v) Using a Waters X-Terra MS Column

Mobile Phase Composition	Area	Height	RT(min)
MeOH: NH_4OAc 0.01M (90:10, v/v)	5796	74	8
MeOH: NH_4OAc 0.01M (80:20, v/v)	5967	63	11

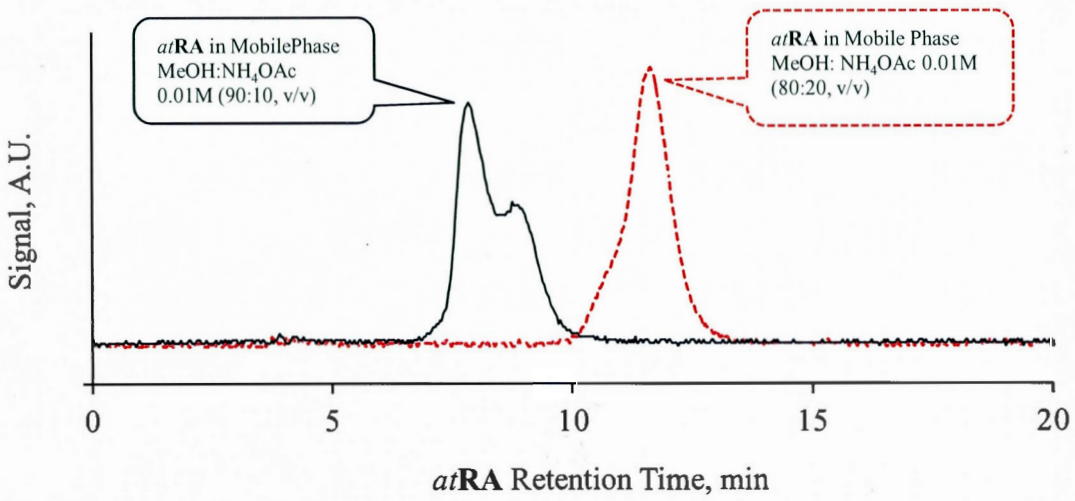


Figure 4.24: Comparison of Chromatograms of 1µg/mL *at***RA** Analysed by HPLC-UV Eluted With MeOH:NH_4OAc 0.01M (80:20, v/v) *vs* MeOH:NH_4OAc 0.01M (90:10, v/v). HPLC Pump: Spectra P4000, UV Detector: Waters 2487, Separation Column: Waters X-Terra MS C18 (2.5 µm, 50mm × 4.60 mm ID).

- ❖ Effect of Different Types of Organic Portion of Mobile Phase on Sensitivity and on Signal Shape

To determine the effect of the type of mobile phase on the sensitivity and retention times, two organic solvents (ACN *vs* MeOH) in the same proportions were used for the same stationary phase. The same flow rate was employed for both. As shown in Table 4.13 and Figure 4.25, the results demonstrate that, with ACN, *at***RA** elutes later than with MeOH and the *at***RA** was detected with a reasonable signal intensity. Furthermore, the chromatogram obtained using MeOH shows two peak. Therefore, ACN was chosen as the organic solvent for the mobile phase as it better corresponds to ideal mobile phase characteristics.

Table 4.13: Chromatographic Characteristics Obtained for *at***RA** Eluted with ACN: NH_4OAc 0.01M (95:5, v/v) *vs* MeOH: NH_4OAc 0.01M (95:5, v/v) Using a Waters X-Terra MS C18 Column.

Mobile Phase Composition	Area	Height	RT (min)
MeOH:NH_4OAc 0.01M (95:5, v/v)	4266	337	3.9
ACN:NH_4OAc 0.01M (95:5, v/v)	6285	110	5.4

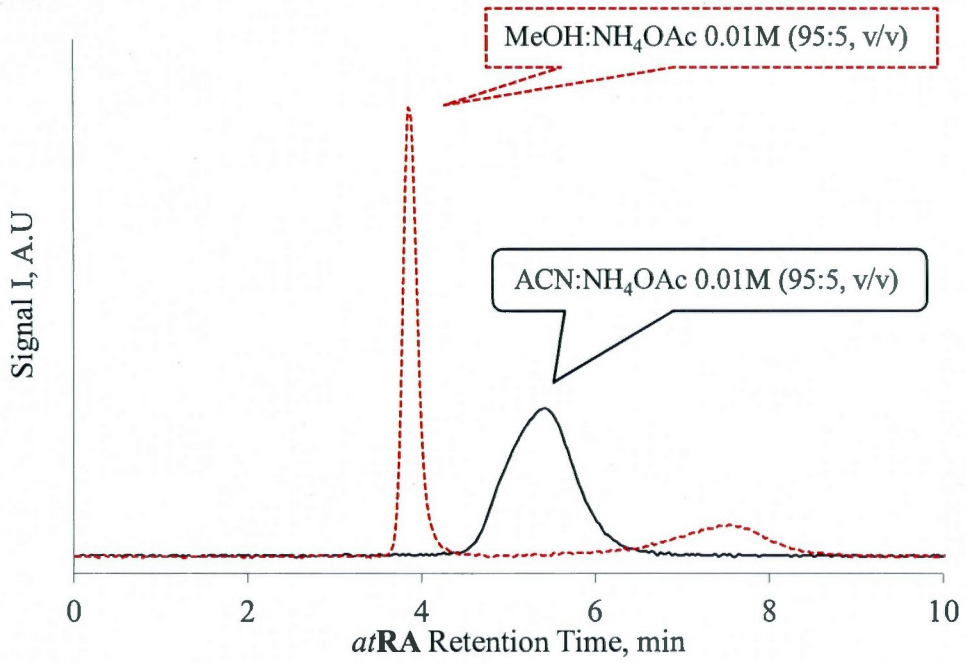


Figure 4.25: Chromatograms for Comparison of 1 µg/mL *at***RA** Solution Eluted with Mobile Phase ACN:NH_4OAc 0.01M (95:5, v/v) *vs* MeOH:NH_4OAc 0.01M (95:5, v/v). HPLC Pump: Waters 590, UV Detector: Waters 2487, Separation Column: Waters X-Terra MS C18 (2.5 µm, 50 mm × 4.60 mm ID) and flow Rate: 0.2 mL/min.

Final Mobile Phase Selection

The concentration of *at***RA** used for the sample was 1 μg/mL while the retention times, peak areas and peak heights of two different mobile phases were compared. From the data shown in Table 4.14 and the chromatograms are shown in Figure 4.26, the following observations were made:

- The signal observed for MeOH:NH_4OAc 0.1M (95:5, v/v) is more intense than that found for ACN:NH_4OAc 0.1M (95:5, v/v).
- The retention time for MeOH:NH_4OAc 0.1M (95:5, v/v) was less than that for ACN:NH_4OAc0.1M (95:5, v/v).
- The concentration of buffer had a small effect on signal intensity and retention time.

Table 4.14: Chromatographic Data for 4 Different Compositions of Mobile Phase.

	Mobile phase	Peak Area	Peak Height	RT (min)
A	MeOH:NH_4OAc 0.1M (95:5, v/v)	6383	160	8
B	MeOH:NH_4OAc 0.15M (95:5, v/v)	6959	173	8
C	ACN:NH_4OAc 0.1M (95:5, v/v)	6591	98	16
D	ACN:NH_4OAc 0.15M (95:5, v/v)	5650	92	19

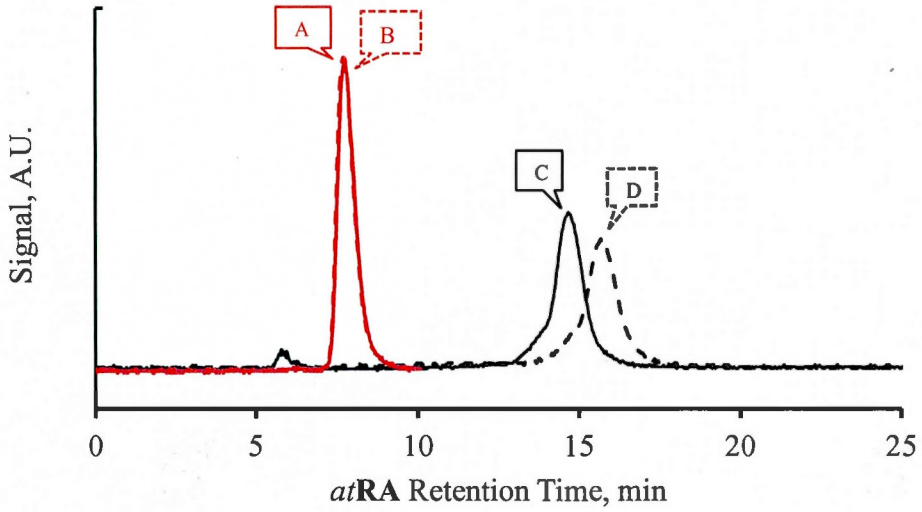


Figure 4.26: *at***RA** Chromatograms Obtained for the 4 Mobile Phases Listed in Table 4.14. HPLC Pump: Waters 590, UV Detector: Waters 2487, Separation Column: Phenomenex Kinetex C18 (2.6 μm, 150 mm × 4.60 mm ID) and Flow Rate: 0.2 mL/min.

Although the mobile phase containing MeOH is more sensitive for the elution of *at***RA** than is that containing ACN, its retention time in the MeOH mobile phase is less than that for the mobile phase made up of ACN. A longer elution time (16 min), and thus the mobile phase with ACN, is preferred in order to avoid undesired interferences from more polar compounds present in physiological media; which are anticipated to elute earlier. Thus ACN:NH_4OAc 0.1M (95:5, v/v) better meets the method development objectives and so was the chosen mobile phase.

✓ Stationary Phase Characterization

Stationary phase characterization began using five different columns: the Thermo Hypersil, the Waters X-Terra, Phenomenex Luna and kinetex columns. The Luna had a P/D of 5μm and the another of 3μm. The effect of length and, more particularly the diameter, on sensitivity and retention time was studied for the quantification of *at***RA** at pharmacological concentrations. Two main factors were considered for the trace level analysis of *at***RA:** resolution and sensitivity which both play critical roles in stationary phase performance. Resolution is the result of three factors, which include selectivity, retention and efficiency. Among these parameters, the column selectivity has the most impact and thus should be focus of method development.

Column selectivity depends on the following factors: hydrophobicity, steric interaction and the ability to form and break hydrogen bonds.

Among these factors, steric interaction is the most important for product analysis since the retinoids undergo a *cis-trans* isomerization. Sensitivity, that is, the signal-to-noise ratio, is related to concentration and depends upon parameters such as efficiency, injection volume, column length, column internal diameter and capacity factor (Phenomenex, 2013). Table 4.15 shows the stationary phase portion of the method development.

Table 4.15: Stationary Phases Studied for Method Development.

Brand Name	Manufacturer	Model	P/D (μm)	Dimension (mm)
Phenomenex	Luna	C18 (2)	5	150×4.6
Thermo Scientific	Hypersil	Gold	3	50×2.5
Waters	X-Terra	MS C18	2.5	50×4.6
Phenomenex	Kinetex	C18	2.6	100×4.6
Phenomenex	Luna	C18 (2)	3	100×4.6

P/D: Particular Diameter

❖ Effect of Column Length on Sensitivity, Signal Shape and Retention Time of *at***RA**.

As shown in Table 4.16 and Figure 4.27, the signal intensities and retention times with two C18 columns (the Phenomenex Kinetex and the Waters X-Terra MS C18) were compared using a solution of *at*RA in MeOH (1 µg /mL) eluted with MeOH:NH_4OAc 0.1 M (95:5, v/v). The solid line represents ***at*RA** eluted from the Phenomenex Kinetex column while the red one represents ***at*RA** eluted from the Waters X-Terra. The main difference between these columns is the column length and ***at*RA** elutes at 7 min from the X-Terra and 14 min from the Kinetex respectively. In addition, a very intense peak which was observed at 6 min on the Kinetex stationary phase was absent from the X-Terra one. The use of the Kinetex column resulted in a better signal shape in comparison to that of the X-Terra one. In addition, no significant difference in the signal sensitivity was found between the two columns.

Table 4.16: Chromatographic Information of Two Different C18 Columns Phenomenex Kinetex and Waters X-Terra MS Using Mobile Phase MeOH: NH_4OAc 0.1M (95:5, v/v).

Column	Peak Area	Peak Height	RT(min)
Phenomenex Kinetex	2320	30	14
Waters X-Terra	2255	37	7

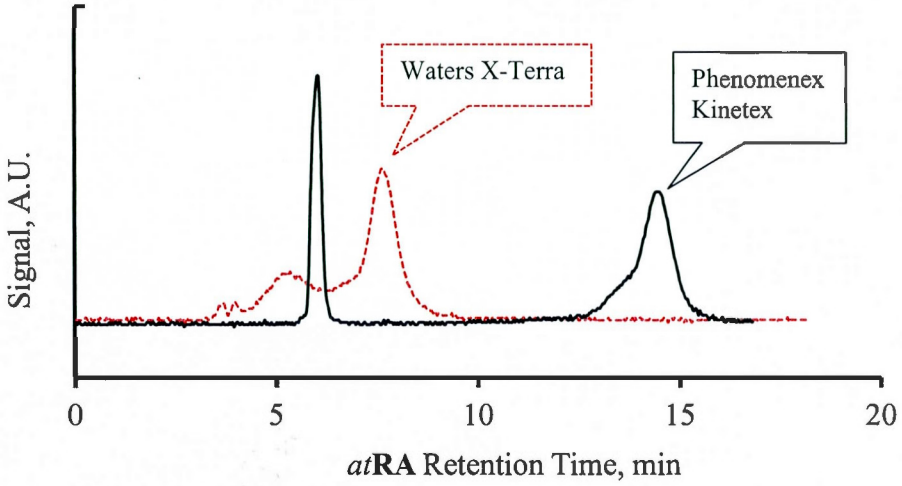


Figure 4.27: Comparison of Signal Shapes and Retention Time Using MeOH: NH_4OAc 0.1 M (95:5, v/v) for a solution of *at***RA** on Two C18 Columns, i.e. the Phenomenex Kinetex and theWaters X-Terra MS.

Another test was performed with ACN: NH_4OAc 0.1 M (95:5, v/v) as the mobile phase and same two columns as above. The *at***RA** eluted at retention time of 11.6 and 18.1 min, respectively. Again, there was no significant difference in the signal intensities.

Final Stationary Phase Selection

Based on the physical characteristics, the Phenomenex Kinetex column, with core shell particles, was chosen as the best stationary phase for *at***RA** method development. The reasons were as follows:

- It is a stationary phase with a high steric interactions and it is the best suited for the separation of conformational isomers (*cis-trans*).
- It offers a high hydrophobicity, which favors stronger retention of these compounds.

- It offers a decreased internal diameter and smaller particle size which improves the sensitivity of the analysis.
- It allows for rapid analysis and for an increased mobile phase flow rate without loss of analytical performance.
- It offers high particle density and an optimal bed structure. This reduces band broadening effects of eddy diffusion allows for faster mass transfer
- It has a porous layer (shell), which decreases the diffusion path length, therefore reducing the time it takes for molecules to diffuse in and out of a particle.

During the last stage of method development, a problem of bacteria precipitation occurred. Therefore, the whole chromatographic system was replaced with new chromatographic material. The Phenomenex Kinetex C18 column (2.6 μm, 100 mm × 4.60 mm ID) separation column was replaced by a Phenomenex Luna C18(2) column (3 μm, 100 mm × 4.6 mm ID). The new column, while not identical, has very similar in properties as compared to the former.

❖ Desorption Solution Selection.

In order to choose a proper desorption solvent, a solution of 1 μg/mL of *at***RA** in MeOH was prepared in four different desorption solutions which are listed in the Table 4.17 and injected to the system using ACN:NH_4OAc (0.1M, 95:5, v/v) as mobile phase. According to the chromatograms shown in Figure 4.28 and data presented in Table 4.17, the desorption solution of ACN:NH_4OAc 0.15M (95:5, v/v) shows the greatest peak surface area and highest peak. Therefore, it was selected as the desorption solution to analyse extracts of *at***RA**.

Table 4.17: *at*RA Desorption Solutions Chromatographic Data.

	Desorption Solutions	Peak Area	Peak Height	RT(min)
A	MeOH:NH_4OAc 0.1M (95:5, v/v)	5680	81	14.6
B	MeOH:NH_4OAc 0.15M (95:5, v/v)	6341	90	14.8
C	ACN:NH_4OAc 0.1M (95:5, v/v)	6591	92	15.9
D	ACN:NH_4OAc 0.15M (95:5 v/v)	8018	121	16.4

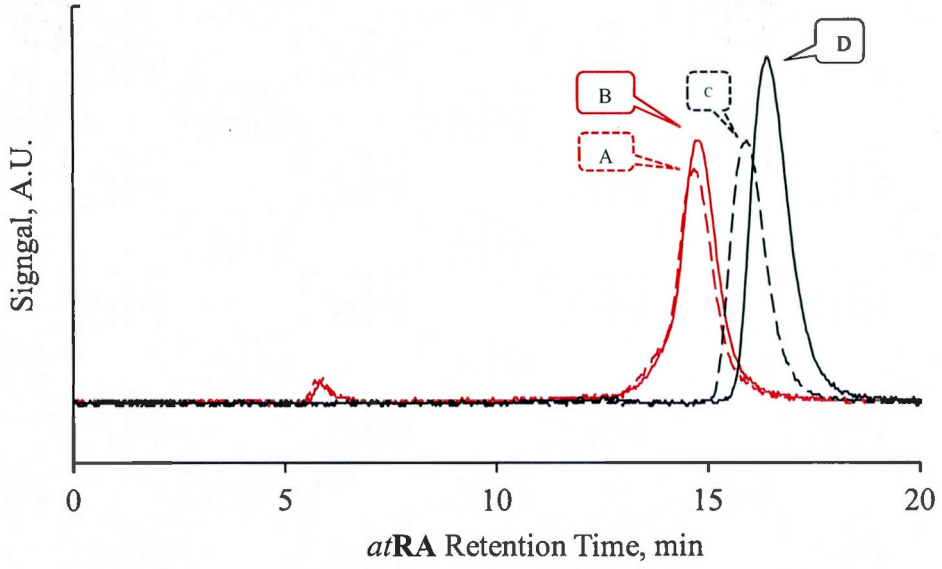


Figure 4.28: *at*RA Chromatograms Obtained for the 4 Different Desorption Solutions listed in Table 4.17 Using Mobile Phase ACN: NH_4OAc 0.1M (95:5, v/v). HPLC Pump: Waters 590, UV Detector: Waters 2487, Separation Column: Phenomenex Kinetex (2.6 µm, 150 mm × 4.60 mm ID) and Flow Rate: 0.2 mL/min.

- Extraction of *at***RA** with EtOAc:Acidified Hexanes and Analysis by HPLC-ESI-MS/MS Using Buffered Mobile Phase

As mentioned previously, HCl (4N, 0.90 %, v/v) was chosen for the acidification of hexanes but some undesired effects were observed with subsequent analysis in non-buffered mobile phase. It was decided to change the acid from HCl (4N, 0.90%, v/v) to glacial HOAc (0.25, v/v). The same extraction protocol was used and the effect of each acid on the recovery rate was evaluated by HPLC-ESI-MS/MS using a buffered mobile phase, as shown in Figure 4.29. The extraction protocol using HCl (4N) proved to be less reproducible than that using glacial HOAc for quantities of *at***RA** from 10 to 80 ng. The recovery rate does not change significantly except for the lowest quantity of *at***RA** (i.e., 10 ng). In this case, extraction with HCl (4N) showed a higher recovery rate.

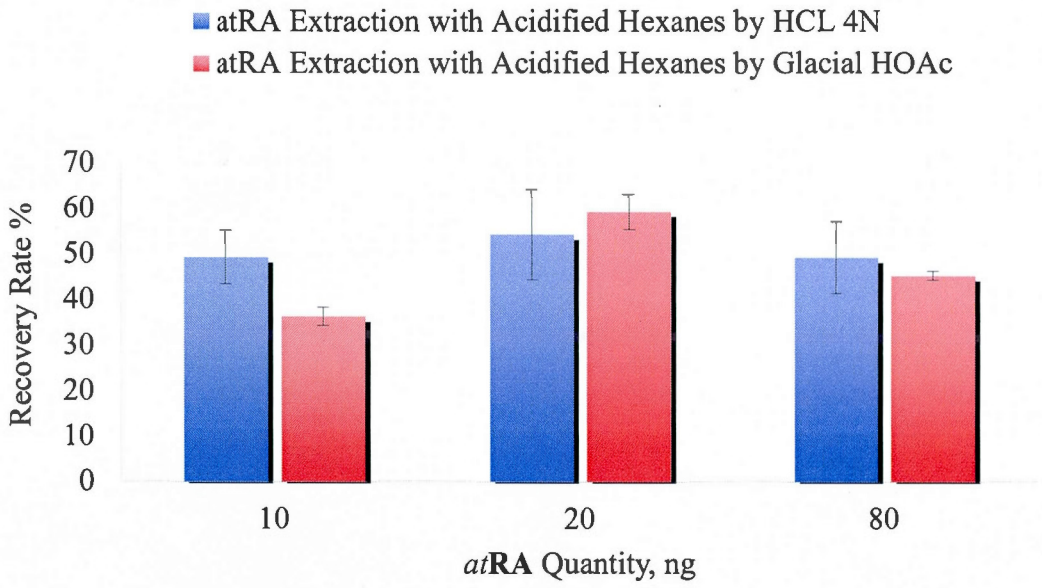


Figure 4.29: Comparison of Recovery Rates Obtained from Extraction of 10, 20 and 80 ng of *at***RA** From a 0.4 mL Aqueous Solution Using EtOAc:Acidified Hexanes

(50:50, v/v) with HCl 4N or Glacial HOAc. HPLC Pump: Waters 2695, Mobile Phase: ACN:NH_4OAc (0.1 M, 95:5, v/v), Separation Column: Phenomenex Luna C18(2) (3 µm, 100 mm × 4.6 mm ID) and Flow Rate: 0.2 mL/min.

❖ Effect of the Quantity of Glacial HOAc in Hexanes on Extraction Profile

As mentioned before, glacial HOAc was chosen for the acidification of the hexanes extraction solvent. As showed in Figure 4.30, the chromatographic profiles for extracts of 20 ng quantity of *at***RA** using EtOAc: acidified hexanes (50:50, v/v) with HOAc (0.25 % v/v, red) and (0.12% v/v, black). The chromatogram of HOAc 0.25% v/v shows a negative peak that appears at 20 min that is at the tail end of *at***RA** peak. This peak is not observed with HOAc of 0.12% v/v. Thus, HOAc (0.12% v/v in hexanes) was chosen in the final extraction protocol.

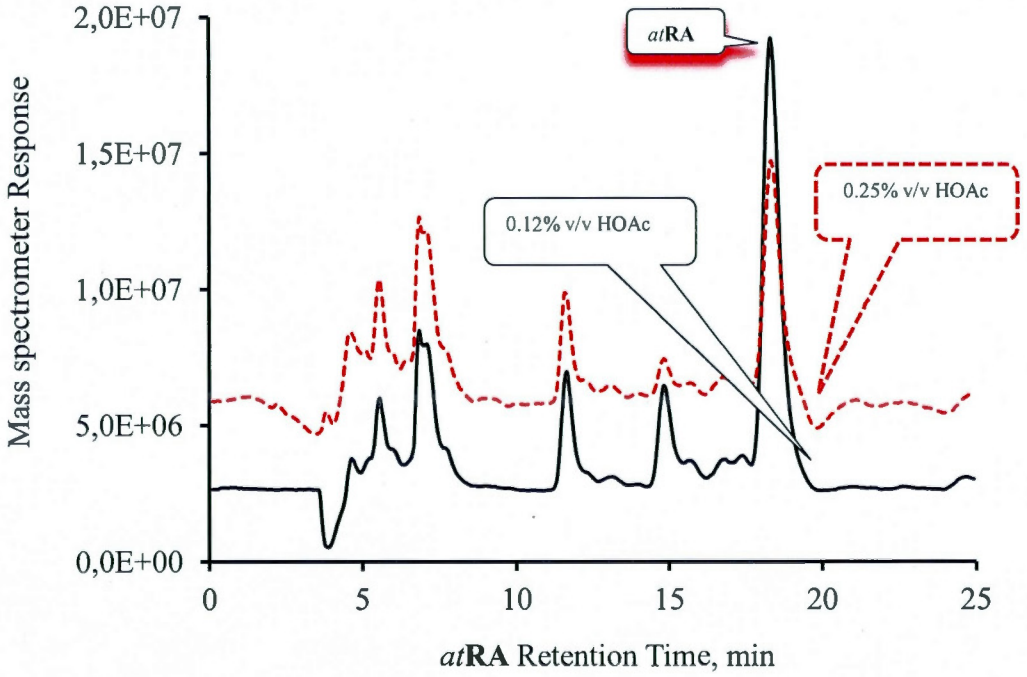


Figure 4.30: Comparison of Chromatograms Obtained for Extractions of 20 ng of *at***RA** Using 0.12% v/v *vs* 0.25% v/v of glacial Acetic Acid in Hex:EtOAc (50:50, v/v) as Organic Extraction Solvents. HPLC Pump: Waters 2695, Mobile Phase: ACN:NH_4OAc 0.1M (95:5, v/v). Separation Column: Phenomenex Luna C18(2) (3 µm, 100 mm × 4.6 mm ID) and Flow Rate: 0.2 mL/min.

- Comparsion of *at***RA** Extracts Analysed by HPLC-ESI-MS/MS Using Buffered and Non-Buffered Mobile Phase

The extraction recovery rates for *at***RA** determined by HPLC-ESI-MS/MS using non-buffered and buffered mobile phases are compared in Figure 4.31. The results show no significant variations in the extraction recovery rates but there was significant differences in reproductibility between using the non-buffered and buffered mobile phases. The buffered mobile phase offers better reproductibility for both standard and extraction solutions of *at***RA**.

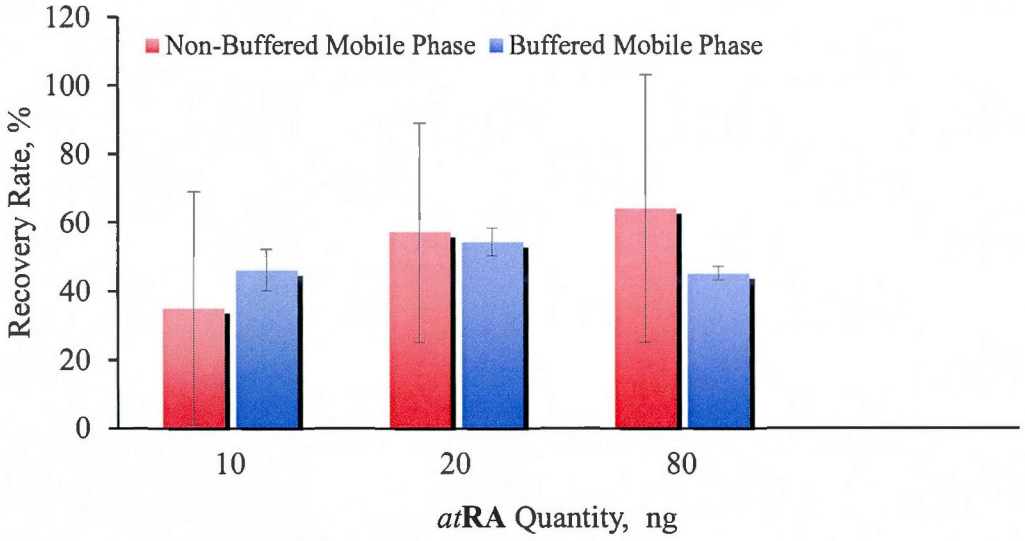


Figure 4.31: Comparison of Extraction Recovery Rates of *at***RA** Determined by

HPLC-ESI-MS/MS Using Buffered and Non-Buffered Mobile Phases. HPLC Pump: Waters 2695, Separation Column: Phenomenex Luna C18 (2) (3 μm, 100mm × 4.6 mm ID) and Flow Rate: 0.2 mL/min.

➢ Optimized Extraction Conditions for *at***RA** analysis

After each parameter had been optimized and the final conditions established, they were used for all subsequent analyses, as presented in chapter 5.

1- The hexanes was acidified with glacial HOAc (0.12% v/v).
2- The mixed extraction solvent was EtOAc: Acidified Hexanes (1.3 mL, 50:50, v/v).
3- The desorption solution was ACN: NH_4OAc 0.15 M (95:5, v/v).
4- The buffered mobile phase used was ACN: NH_4OAc 0.1 M (95:5, v/v).
5- The mobile phase flow rate was 0.2 mL/min.

❖ Optimized Extraction Protocol

1- A *at***RA** mother solution (10 μg/mL in MeOH) was freshly prepared on the day of the analyses by diluting a stock solution warmed to room temperature before use.
2- Aqueous solutions containing *at***RA** were prepared from the mother solution and 0.4 mL of each solution was transferred to a glass tube.
3- HPLC MeOH (0.4 mL) was then added to each tube.
4- Organic extraction solvents: EtOAc:Acidified Hexanes (1.3 mL, 50:50 v/v) contaning glacial HOAc (0.12 v/v) was added to the solution.
5- The mixture was stirred for 5 min at the speed of 5000 RPM.
6- The upper organic phase was transfered to a glass test tube and evaporated almost to dryness under a gentle stream of nitrogen gas.

7- The extraction was performed three times, with the third extraction including acidified hexanes (1.3 mL).

8- The three extracts were combined and evaporated to dryness at room temperature under a gentle stream of nitrogen gas.

9- The residue was dissolved in 200 µL of desorption solution ACN:NH_4OAc 0.15M (95:5,v/v).

10- The final extracted solution was filtered on a 0.45 µm filter before injection.

11- 20 µL was injected into the reverse phase HPLC system.

- Summary of Method Development Optimization

It was found that the MS/MS signal for a non-buffered mobile phase was more sensitive and the specific ion was more abundant than that for the buffered one. Moreover, the non-buffered mobile phase can prevent proliferation and precipitation of in the whole chromatographic system since bacterial growth is encouraged by the presence of ammonium acetate in the buffered mobile phase ACN:NH_4OAc 0.1M (95:5, v/v). Quantitative MS/MS assays were performed with a non-buffered mobile phase. Optimization was investigated using different volumetric amount of acid, that is, ACN:H_2O:HOAc in the following proportions: 1) 95:4.80:0.20; 2) 95:4.90:0.10, v/v and 3) 95:4.99:0.01, v/v. The non-buffered mobile phase was chosen to be ACN:H_2O:HOAc (95:4.99:0.01, v/v) because it increases the signal intensity for both the standard and extraction solutions of *at***RA**. The first analysis and extraction optimization assay using the above mobile phase were performed with HCl (4N) in different proportions ranging from 0.3 to 0.9 % v/v. Finally HCl (4N, 0.9 % v/v) was found to give the highest extraction recovery rate. Therefore, this proportion was chosen to acidify the hexanes. Unfortunately, the standard deviation obtained for the MS/MS analyses of the standard and extracted solutions were quite high. This

behaviour was not observed with UV detection indicating that the non-buffered mobile phase could be causing variability in the MS/MS signal.

In order to obtain a reproducible MS/MS signal, a buffered mobile phase is needed. Therefore, method optimization for *at***RA** analysis was continued using the standard solution with ACN:NH_4OAc 0.1 M (95:5, v/v) as the mobile phase and UV detection. The data shows that this system followed by desorption with ACN: NH_4OAc 0.15 M (95:5, v/v) was the best choice for obtaining the greatest signal. For *at***RA** extraction, HOAc (0.12%, v/v) was chosen to acidify the organic solvents. Thus the method described offers the optimum conditions for high sensitivity.

CHAPTER V

RESULTS AND DISCUSSION

The results and discussion section is divided into two major parts:

The first one covers the quantification and comparison of analytical parameters for different techniques (HPLC-UV, HPLC-FLU and HPLC-ESI-MS/MS) used during the early and final stages of method development. It also includes determination of the limit of detection (LOD), and quantification (LOQ), the linearity range and the coefficient of variation (R^2).

The second part focuses on the study of extracted *at***RA** analysis by HPLC-UV and HPLC-ESI-MS/MS. It includes determination of: the range of recovery rates, the standard deviation, the minimum detectable amount and any matrix effects on *at***RA** analysis (that is, aqueous *vs* serum-supplemented cell culture media αMEM: FBS (90:10, v/v) and their influences on final method development.

5.1 *at***RA** Analytical Parameters of HPLC-UV, HPLC-FLU and HPLC-ESI-MS/MS.

As shown in the early method development section in Chapter IV, *at***RA** analysis was starts with aqueous media using a buffered mobile phase MeOH:NaOAc, 0.01M (85:15, v/v) at a high flow rate (1.5 mL/min). Figure 5.1 presents the variation in *at***RA** peak area obtained using a UV or a FLU detector for concentrations ranging from 75 to 1000 ng/mL. Calibration curves were generated by analysing 4 concentrations from which best fit line were determined by least squares regression. Peaks were identified on the bases of their retention times. *at***RA** standards for each analysis were freshly prepared on the day of the run by dilution of the mother solution. Linear calibration curves were obtained, giving a slope of 0.1016 and a

coefficient of variation (R^2) of 0.9127 for HPLC-FLU and a slope of 0.5483 and a R^2 of 0.9974 for HPLC-UV.

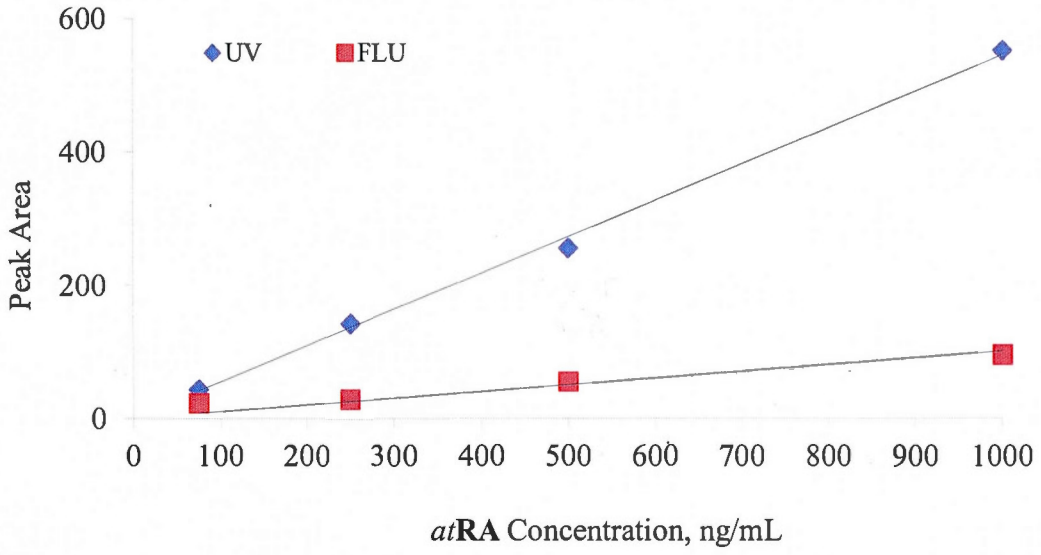


Figure 5.1: Comparison of *at***RA** Standard Calibration Curves Analysed by HPLC-UV and HPLC-FLU. HPLC Pump: Spectra Model P4000, Mobile Phase: MeOH:NaOAc 0.01M (85:15, v/v). Separation Column: Phenomenex Luna C18(2) (5 µm, 150 mm × 4.6 mm ID) and a Flow Rate of 1.5 mL/min.

The sensitivity of both methods is best visualized in Figure 5.2, for which both chromatograms were obtained from the analysis of the same solution of *at***RA** (100 ng/mL in mobile phase) using HPLC-UV (black) and HPLC-FLU (red). The greater sensitivity of the HPLC-UV method is clearly visible.

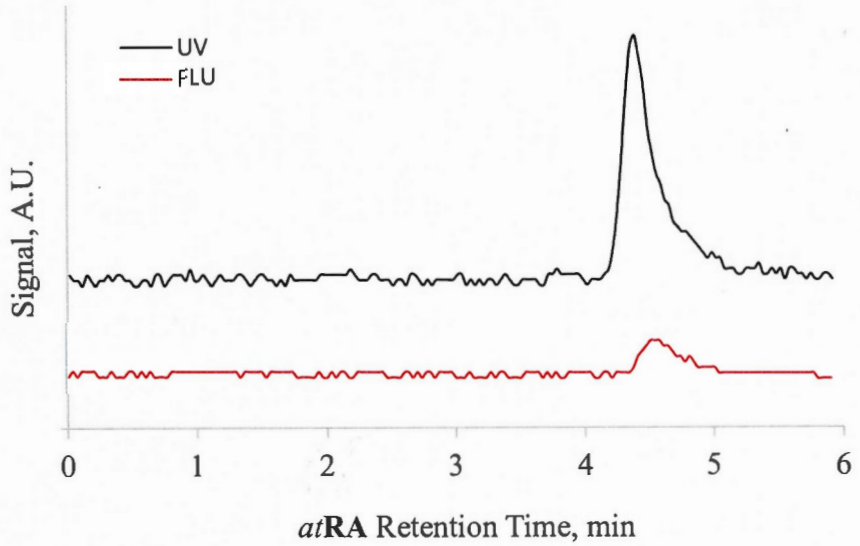


Figure 5.2: Comparative Sensitivities of HPLC-UV *vs* HPLC-FLU for the Analysis of a Solution of ***at*RA** in Mobile Phase (100 ng/mL). HPLC Pump: Spectra Model P4000; Mobile Phase: MeOH:NaOAc 0.01M (85:15, v/v). Separation Column: Phenomenex Luna C18(2) (5 µm, 150 mm× 4.6 mm ID) and a Flow Rate: 1.5 mL/min.

Based on the data shown in Fig. 5.2, the LOD and LOQ for both the HPLC-UV and the HPLC-FLU methods were determined. Their values are shown in Table 5.1. The LOD is defined using S/N=3 while the LOQ is defined using S/N=10 with both being expressed as ng/mL (ppb). The LOD were 7 for HPLC-UV and 24 for HPLC-FLU, respectively. Thus, HPLC-UV detection is three times more sensitive in aqueous media. From this point onwards, HPLC-UV was the detector used for method development.

Table 5.1: Comparison of Analytical Parameters for HPLC-UV and HPLC-FLU.

Analytical Parameters	S/N	HPLC-UV	HPLC-FLU
LOD (ng/mL)	3	7	24
LOQ (ng/mL)	10	23	80

The final step of method development for the analysis of *at***RA** was carried out using a HPLC-ESI-MS/MS at a low flow rate (0.2 mL/min). The composition of the mobile phase was changed from MeOH:NaOAc 0.01M, (80:15, v/v) to ACN:NH_4OAc 0.1M, (95:5 v/v) and a HPLC-UV was used to monitor the behaviour of the HPLC-ESI-MS/MS. Standard calibration curves of *at***RA** were generated by analyzing separately, 5 concentrations of standard solution ranging from 75 to 550 ng/mL. Figures 5.3 and 5.4 show the standard calibration curves obtained by HPLC-UV and by HPLC-ESI-MS/MS of fresh *at***RA.** Their coefficent of variation was found to be greater than 0.999. The sensitivities of the methods were compared at a reduced eluent flow rate using a volatile buffer in the mobile phase.

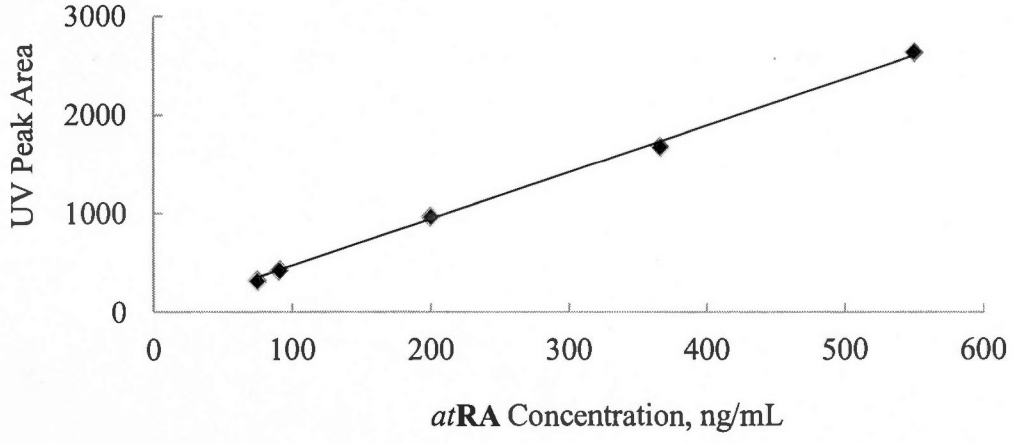


Figure 5.3: *at***RA** Standard Calibration Curve Analysed by HPLC-UV

HPLC Pump: Waters 590, Mobile Phase: ACN:NH_4OAc 0.1M (95:5, v/v), Separation Column Phenomenex Kinetex C18, and Flow Rate: 0.2 mL/min.

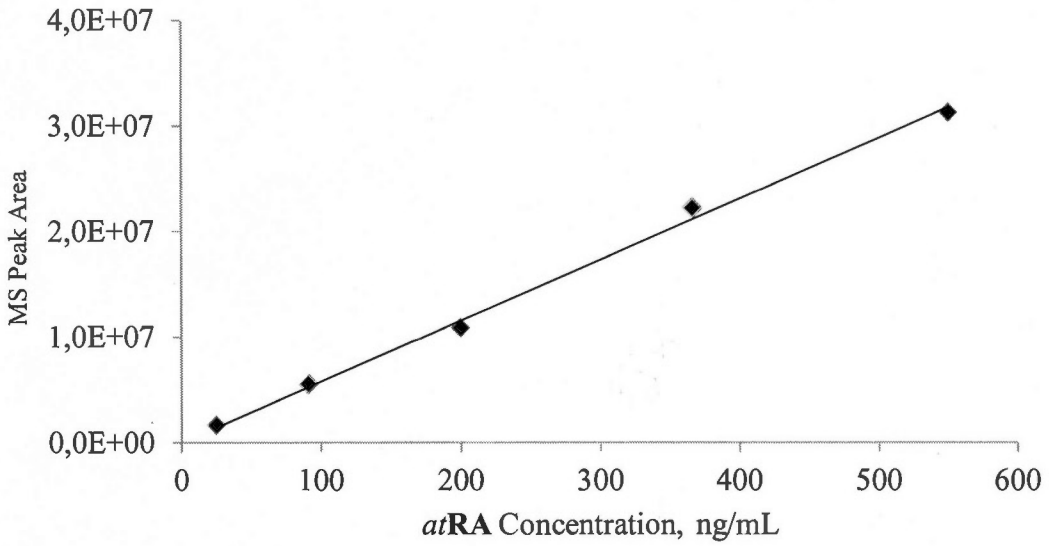


Figure 5.4: *at***RA** Standard Calibration Curve Analysed by HPLC-ESI-MS/MS. HPLC Pump: Waters 590, Mobile Phase: ACN:NH_4OAc 0.1M (95:5 v/v), Separation Column Phenomenex Kinetex C18 and Flow Rate: 0.2 mL/min.

Figure 5.5 shows two chromatograms for the analysis of a same solution of *at***RA** (75 ng/mL) by HPLC-UV (left) and by HPLC-ESI-MS-MS (right). It can clearly be seen that, while both traces have a similar signal intensity, the latter chromatogram has less noise.

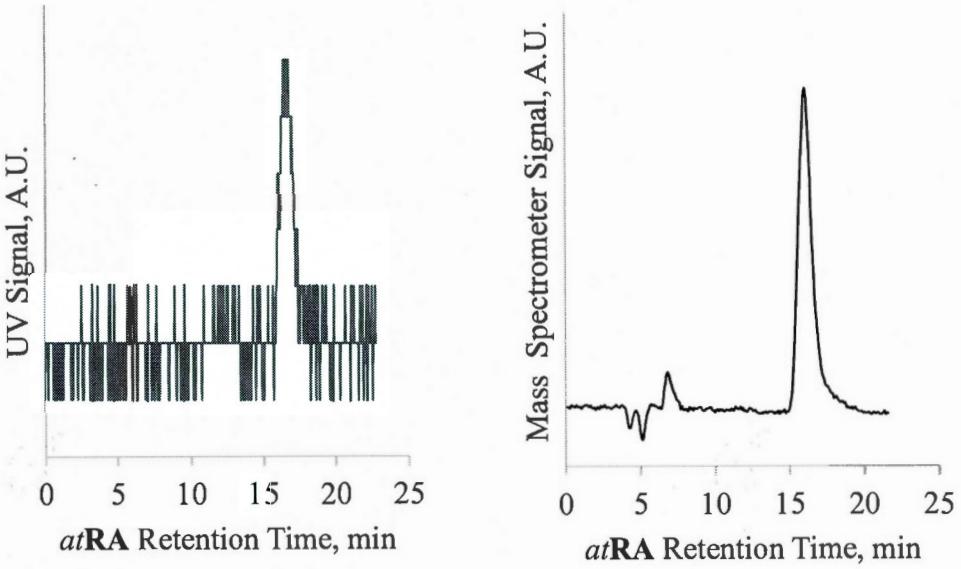


Figure 5.5: Sensitivity of HPLC-UV *vs* HPLC-ESI-MS/MS Analysis of an *at***RA** Standard Solution (75 ng/mL). HPLC Pump: Waters 590, Mobile Phase: ACN:NH_4OAc 0.1 M (95:5, v/v). Separation Column: Phenomenex Luna and Flow Rate: 0.2 mL/min.

The analytical parameters are summerized in Table 5.2 and show that detection using a HPLC-ESI-MS/MS is ten times more sensitive than that using a HPLC-UV.

Table 5.2: Comparison of Analytical Parameters for HPLC-UV and HPLC-ESI-MS/MS.

Analytical Parameters	LOD (ng/mL)	R^2	Linearity Range (ng/mL)
HPLC-UV	10	0.9988	75-550
HPLC-ESI-MS/MS	1	0.9973	5-550

The LOD of *at***RA** obtained by HPLC-UV using MeOH:NaOAc (0.01 M) as the mobile phase at 1.5 mL/min (100 ng/mL) as shown in Table 5.1, was comparable to that obtained from ACN:NH_4OAc 0.1 M (95:5, v/v) at 0.2 mL/min; which indicates that the LOD is independent of the nature of the mobile phase and flow rate.

5.2 Recovery Rate of *at***RA** Extracted in Aqueous and Serum-Supplemented Cell Culture Media

The extraction of *at***RA** was performed in triplicate for each experiment and the recovery rates presented in Tables and Figures in this section are averages.

5.2.1 Analysis of *at***RA** Extracts in Aqueous Media

The extraction of *at***RA** was performed for quantities ranging from 20 to 120 ng in aqueous medium using a HPLC-UV. The average recovery rates are shown in Table 5.3 and Figure 5.6. The results show an average recovery rate of 72±4 % which demonstrates the reliability of the extraction method.

Table 5.3: *at***RA** Extraction Recovery Rate in Aqueous Media Obtained Using HPLC-UV Analysis.

Quantity (ng)	MRR [a] (%)
20	72±7
30	73±5
50	58±2
60	86±6
120	64±3

[a]: Mean Recovery Rate ± SD for three analysis.

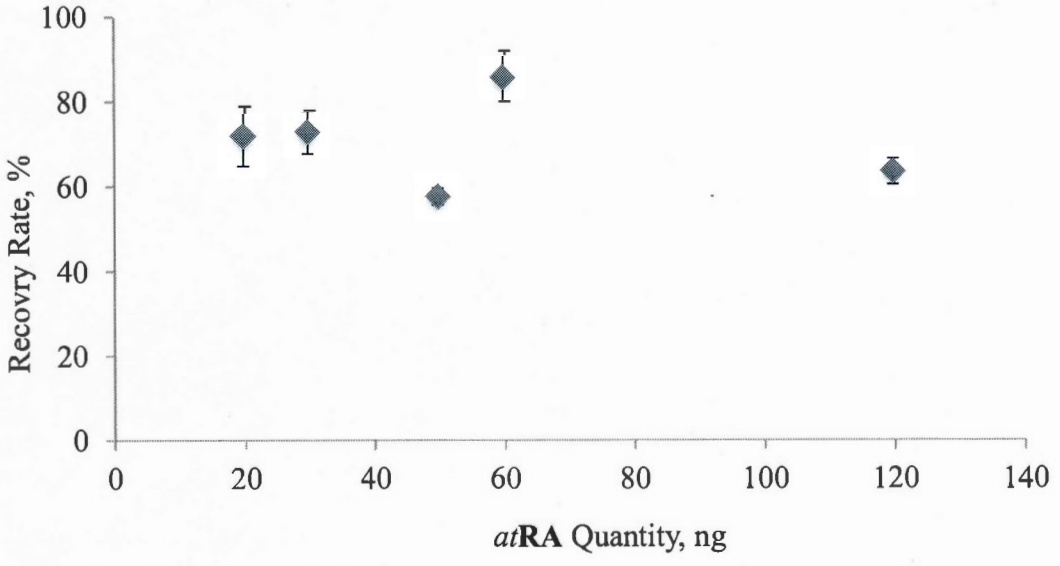


Figure 5.6: Analysis of Extracted in Aqueous Media by HPLC-UV at a Low Flow Rate. HPLC Pump: Waters 590, UV Detector: Waters 2487, Mobile Phase: ACN:NH_4OAc 0.1 M (95:5, v/v), Separation Column: Phenomenex Luna C18(2) (3 μm, 100 mm ×4.6 mm ID) and Flow Rate: 0.2 mL/min.

For HPLC-ESI-MS/MS, *at***RA** extractions were performed for quantities ranging from 5 to 80 ng and the recovery rate results are presented in Table 5.4 and Figure 5.7. The results show a constant recovery rate of 35% over the range from 5 to 10 ng which increases to 54% for quantities from 20 to 80 ng. The low standard deviation observed indicates the reliability of the extraction protocol using a HPLC-ESI-MS/MS. Similar deviations were found using a HPLC-UV. The standard deviation of ±10 % for 40 ng may be attributed to manipulation errors during the extractions.

Table 5.4: *at***RA** Extraction Recovery Rate in Aqueous Media Obtained Using HPLC-ESI-MS/MS Analysis.

Quantity (ng)	MRR %
5	34±3
10	36±1
20	59±4
40	59±10
80	45±2

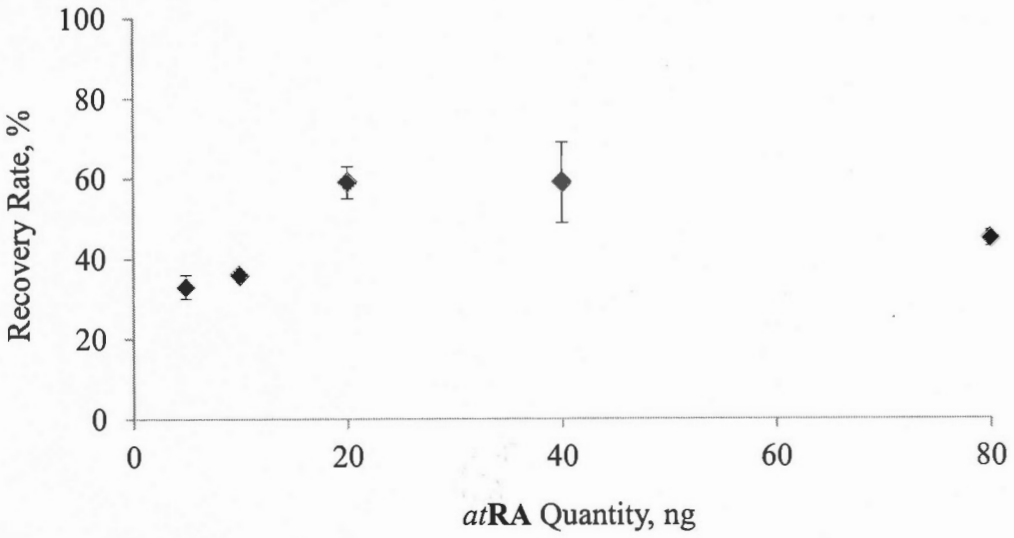


Figure 5.7: *at***RA** Recovery Rate in Aqueous Media Analysed by HPLC-ESI-MS/MS. HPLC Pump: Waters 2695, Mobile Phase: ACN:NH_4OAc 0.1 M (95:5, v/v), Separation Column: Phenomenex Luna C18(2) (3 µm, 100 mm x 4.6 mm ID) and Flow Rate: 0.2 mL/min.

- Comparsion the Recovery Rates of *at***RA** Extracted from Aqueous Medium Analysed by HPLC-UV and HPLC-ESI-MS/MS

Table 5.5 summerizes the extraction recovery rates obtained using HPLC-UV and HPLC-ESI-MS/MS for *at***RA** quantities ranging from 5 to 120 ng. For the HPLC-UV, the extraction started at 20 ng (for an analyte concentration of 100 ng/mL) due to the limitation of detection for this instrument. The low recovery rates for 5 and 10 ng observed using the HPLC-ESI-MS/MS can be explained by a loss of some *at***RA** during sample preparation. Therefore these values were discarded. As shown in Table 5.5, a difference in mean recovery rates (MRR) for the two methods was observed; the MRR of HPLC-UV was 30% higher than that of HPLC-ESI-MS/MS which

reflects real behavior. It is possible that this is due to the presence of acetic acid, used during the extraction process, since this acid is known to suppress the MS/MS signal. Depending on how the extracted product is evaporated, a small residual amount of acetic acid may be present in the extracted *at***RA** (Table 5.5). Hence during HPLC-ESI-MS/MS analysis, the presence of acetic acid could cause *at***RA** ion suppression resulting in a lower signal (Antignac *et al.*, 2005; Lee and Volmer, 2006). This behavior was not observed when HPLC-UV was used. To confirm whether the presence of acetic acid is causing a lower signal. The extracted *at***RA** should be dried for a longer time so as to more completely remove any remaining acetic acid in the sample. A study of the effect of presence of acetic acid, used to acidify the hexanes on *at***RA** recovery rate might be worthwhile.

Table 5.5: Comparsion of *at***RA** Recovery Rates from Aqueous Media Obtained Using HPLC-UV and HPLC-ESI-MS/MS.

	*at***RA** Detection Method	
Quantity (ng)	HPLC-UV	HPLC-ESI-MS/MS
5	-	34±3
10	-	36±1
20	72±7	59±4
30	73±5	-
40	-	59±10
50	58±2	-
60	86±6	-
80	-	45±2
120	64±3	-
MRR	72±5	54±5

5.2.2 Analysis of *at***RA** Extracted From Serum-Supplemented Cell Culture Media αMEME:FBS (90:10, v/v)

Table 5.6 shows the experimental recovery rate of *at***RA** extracted from serum-supplemented cell culture media. Because of the limited availability of this medium, the volume of serum was not as consistent as for the aqueous extractions. Therefore its volume was reduced from 0.4 to 0.3 mL. Consequently, the pre-concentration factors were 1.5 and 2 for the extractions carried out with 0.3 mL and 0.4 mL, respectively.

Table 5.6: *at***RA** Recovery Rate from Serum-Supplemented Cell Culture Media αMEM:FBS (90:10, v/v) Obtained by HPLC-UV Analysis.

Quantity (ng)	ARR (%)
50	60±3
60	72±1
90	75±10
100	85±4

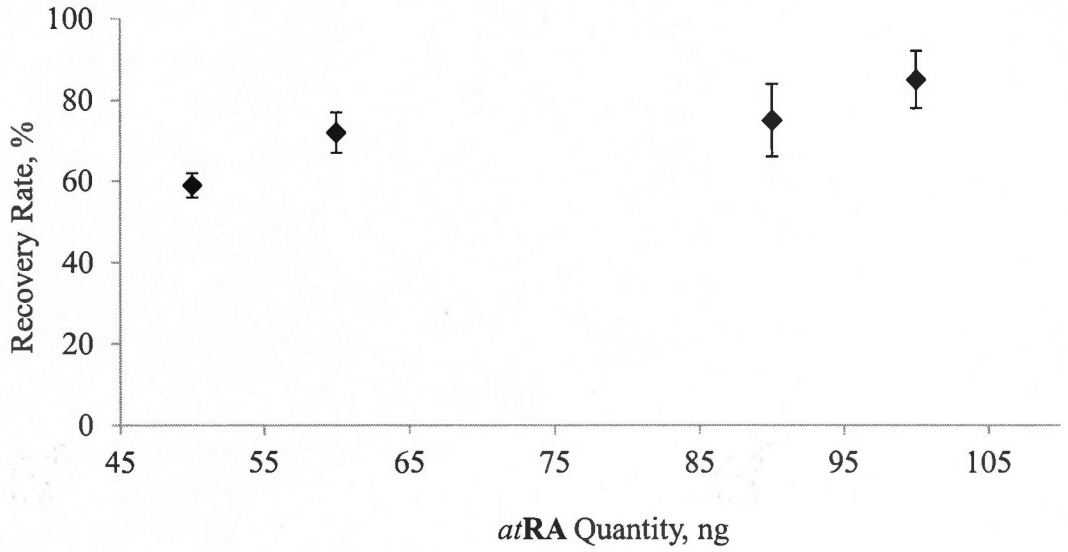


Figure 5.8: *at***RA** Analysis of Extract in Serum-Supplemented Cell Culture Media αMEM:FBS (90:10, v/v); HPLC Pump: Waters 2695, Mobile Phase: ACN: NH_4OAc (0.1 M, 95:5, v/v) and Desorption Solution: ACN:NH_4OAc 0.15 M (95:5, v/v) Separation Column: Phenomenex Luna C18 (2) and Flow Rate: 0.2 mL/min.

- Comparison of *at***RA** Extracted From Aqueous and Serum-Supplemented Cell Culture Media Analysed by HPLC-UV

The data from the analysis of *at***RA** extracts, as shown in Figures 5.6 and 5.8, were compared in quantities ranging from 20 to 120 ng and from 50 to 100 ng in aqueous and serum-supplemented media, respectively. Because of the limited availibility of the serum, *at***RA** quantities below 50 ng were not analysed. The results demonstrate that there was no matrix effect nor was there any co-eluting interference observed for the analysis of extracted *at***RA**.

Table 5.7: Comparsion of *at***RA** Extract Recovery Rates from Aqueous and Serum-Supplemented Culture Media αMEM:FBS (90:10, v/v) Obtained by HPLC-UV. Analysis.

Quantity (ng)	Recovery Rate From	
	Aqueous Medium (%)	Serum-Supplemented Cell Culture Medium (%)
20	64±7	-
30	64±5	-
50	-	60±3
60	86±2	72±5
80	73±6	-
90	-	75±10
100	-	85±4
120	72±3	-
MRR	72±5	73±6

5.3 Comparison of Our Methods *vs* Other Validated Assays

It is difficult to compare the results of this method with the others because some of the analytical parameters, such as: the type of instrument, the chromatographic conditions, the separation modes, the extraction protocol, the injection volume and the type of media analysed were quite different from other, reported assays. Table 5.9 summarizes a comparison of various parameters derived from our methods (UQAM) with those from other validated methods. These parameters includes: the detection method, the limit of detection (LOD), the detected quantity limit and the extraction recovery rates (ERR) for analysis of *at***RA** from different matrices.

Table 5.8: Comparison of Ours *vs.* Other Validated Analytical Methods.

Methods Author	Detection Method	LOD	Tested Quantity (ng)	MRR %	Matrices Analysed
(Bérube *et al.*, 2010)	HPLC-UV	10 (ng/mL)	20	72±5	Nano pure Water (Aqueous)
(Bérube *et al.*, 2010)	HPLC-UV	10 (ng/mL)	50	73±6	Serum Supplemented Cell Culture Media
(Bérube *et al.*, 2010)	HPLC-ESI-MS/MS	1 (ng/mL) 133 (fmol)	5	54±5	Nano pure water (Aqueous)
(Wu *et al.*, 2010)	UPLC-ESI-MS/MS	20 (ng/mL)	Not Reported	59	Influent, Effluent, River and Sea Water
(Kane *et al.*, 2008)	HPLC-APCI -MS/MS	0.625 (fmol)	Not Reported	80±11 to 95±4	Mouse Serum and Tissue
(Ruhl, 2006)	HPLC-APCI-MS/MS	0.2 (ng/mL)	Not Reported	89	Serum and Cell
(Kane *et al.*, 2005)	HPLC-APCI-MS/MS	0.15 (ng/mL)	0.3	80±4	Serum, Tissue

Note: The extraction recovery rate for our method was calculated by comparing of the peak areas of *at***RA** obtained for an extracted *vs.* a standard solutions (external method) while the other validated methods used an internal standard in the extract solutions.

5.4 Advantages of Our Methods

- These methods allow for extracted *at***RA** in aqueous or serum-supplemented cell media to be analysed in a single run by HPLC-UV or HPLC-ESI-MS/MS.
- These methods are applicable to the quantification of *at***RA** at both pharmacological and physiological levels.

- These methods offer a desirable flat baseline in both aqueous and cell culture media.
- The analysis can be carried out using different buffered mobile phase (ACN- or MeOH) at a high or low flow rate.
- An aqueous, buffered mobile phase was chosen to maximize ***at*RA** ion generation when ESI-MS/MS detection is used since this leads to good reproducibility of signal intensity for the standard and extracted solutions.
- LLE is an inexpensive, simple and time efficient way to extract ***at*RA** from the desired media (aqueous or serum) and offers protocol efficiency over a specific range.
- The extraction protocol was optimized for interference suppression of the ***at*RA** peak area as well as address minimization of ion suppression in ESI-MS/MS.
- These methods offer a reasonable standard deviation for the extracted ***at*RA** solution.

5.5 Disadvantages of Analytical Methods

- The extraction recovery rate was determined by the comparison of the area and height of the ***at*RA** peak *vs* that of a standard solution which offers less accuracy than an internal standard in the ***at*RA** to be extracted.

CHAPTER VI
CONCLUSION

This study focused on the development of analytical methods for the quantification of *at***RA** in pharmacological level (μg) and physiological levels (ng) by HPLC-UV and HPLC-ESI-MS/MS in aqueous and serum-supplemented cell culture media αMEM:FBS (90:10, v/v). *at***RA** is a light sensitive compound which easily isomerizes upon exposure to light. We demonstrated that the highly sensitive and selective HPLC-ESI-MS/MS was an efficient technique for the quantification of *at***RA** at very low concentrations. Since *at***RA** is ionisable, it is important to control mobile phase pH so as to obtain a high signal intensity. In this study, non-buffered and buffered mobile phases were studied. The use of a buffered mobile phase allowed for the generation of a parent ion at *m/z* 299 for $[at\mathbf{RA}\text{-H}]^-$ with relatively good signal stability and reproducibility. The use of a non-buffered mobile phase resulted in the formation of an abundant fragment ion at *m/z* 255 for $[at\mathbf{RA}\text{-}CO_2]^-$, but the signals were not sufficiently reproducible for either the standard or extracted solutions. A serious limitation associated with the use of a buffered mobile phase is bacterial growth in the chromatographic system. Thus, a protocol was developed to avoid this problem. Tandem mass spectroscopy in ion filtering mode offered a better signal-to-noise ratio and, therefore, a better detection limit for *at***RA**. The LODs for the HPLC-UV and HPLC-FLU at a high flow rate (1.5mL/min) with a mobile a phase of MeOH:NaOAc 0.01M (85:15, v/v) were 7 and 24 ng/mL, respectively, for aqueous solutions. The HPLC-UV was found to be three times more sensitive than HPLC-FLU in aqueous media. However, at lower flow rate (0.2 mL/min) and with the mobile phase ACN:NH_4OAc 0.1M (95:5, v/v) as the mobile phase, the LOD for the HPLC-UV was found to be 10 ng/mL; while HPLC-ESI-MS/MS method was 1 ng/mL. That is, this method is ten times more sensitive than is the HPLC-UV. The

type and proportion of acid, used for acidification of organic extraction solvent (hexanes) was an important parameter in the extraction protocol affecting both the recovery rate and chromatogram profile. Liquid-Liquid extraction and analysis by HPLC-ESI-MS/MS permitted the quantification of 5 ng of *at***RA** in aqueous media with a 34% recovery rate, while at 50 ng, the sample extracted from the serum-supplemented cell culture media could be analysed by HPLC-UV with a with 60% recovery rate.

CHAPTER VII
PERSPECTIVES

As mentioned in the introduction, the realisation of this project was planned to have in two phases. The first phase was the analysis of ***at*RA** *in vitro* aqueous, serum-supplemented cell culture media αMEM:FBS (90:10, v/v) and the second phase was its analysis *in vivo* (P19 embryonic stem cell cultures). Due to time limitations, ***at*RA** analysis focused on the *in vitro* and remain to be validated *in vivo*. Because of severe sample volume limitations of the P19 embryonic stem cell culture medium, the following should also be investigated:

- A study of the scope and limitations of our method for the analysis of ***at*RA** metabolites.
- An optimization of instrumental parameters (ion source and analyser) to have sufficiently abundant specific ions derived from ***at*RA** and the other metabolites in stem cell extracts and culture media.
- An optimization of the extraction protocol to achieve the highest recovery rate at very low possible quantity of analytes.
- A study of the effect of the mobile phase buffer ionic strength on ion suppression and signal intensity.
- A study of the effects of ion-pairing contaminants on the electrospray signal. Suppression of ***at*RA** and metabolites present in cell culture media based on literature for other analytes (Holcapek *et al.*, 2004).
- A study of the scope and limitations of our method for the analysis of other available synthetic retinoids (such as E19, E23) which are not light sensitive and can be used for application in modeling cell growth.
- A study of ***at*RA** and other metabolites stabilities in stem cell culture media. (Sharow, Temkin and Asson-Batres, 2012)

APPENDIX A

Developed Protocol for Quantification of *at***RA** using a HPLC Waters 2695 Separation Module.

Solvent Management for the Analysis of *at*RA by HPLC-2695 Separation Module

1. Remove old mobile phase from reservoirs A and B (if B was used with buffer) and replace them with Pure Water.
2. Select 100% A and then, direct function then wet prime for at least 2 minutes at 7.5 mL/ min if the pressure is not stable abort wet prime and perform dry prime.
3. Draw the solvent from the bottle and close the valve by pressing cancel.
4. Repeat the wet prime from reservoir A until the pressure is stable (2-3% fluctuations).
5. Purge the injector with mobile phase ACN:NH_4OAc: 0.1M (95:5, v/v).
6. Make sure that reservoirs C and D are filled with solvents (H_2O, MeOH or ACN).
7. Select the appropriate mobile phase composition and perform direct function –pure injector.
8. Make sure the seal wash inlet is in the water-enriched solution, go to menu screen (initial screen) and select diag seal wash. Check that the liquid flows properly in the tubing around the pump heads and abort.
9. Make sure that the needle wash inlet is in the appropriate solution ACN:H_2O (80:20, v/v).
10. Go to menu screen (initial screen) select diag-needle wash. Let it finish on its own. Needle wash needs to be repeated if the solvent composition is

changed.

11. Switch the composition to the method's composition and start flowing it throw the column.
12. Watch the pressure and make sure it has stabilized (2-3% fluctuations)
13. Increase the flow at 1mL with mobile phase.
14. Condition the separation column at flow rate of 1mL for 30 min with mobile phase.
15. Select the appropriate instrument method and monitor the baseline until it stabilized the system is ready for injections.

At the end of Analysis

16. Perform needle, injector and seal wash with (MeOH 100%).
17. Recondition the separation column at 1mL/min flow rate for 30 min with ACN:H_2O (50:50, v/v)
18. Press stop and turn off HPLC module.

Important note :

- Algae and bacteria can grow in aqueous mobile phase, especially in the pH 4-8 range, replace buffers on the regular basis (every 2 days is a good practice).
- Always use amber clean bottles for storing buffers and aqueous mobile phases.
- Depending on their buffering capacity, buffers are very likely to absorb CO_2 and induce a pH change. Protection of the mobile phase against pH change can be done by flushing an inert gas in it and the using an ascarite trap.
- Ammonium acetate is particularly hydroscopic thus it should be used freshly prepared and stored immediately in a desiccator.

APPENDIX B

Mass and Composition of Cell Culture Media (Catalog No. 310-011 1X Liquid αMEM).

Amino Acids	Con (mg/L)	CAS	M.W ($g\ mol^{-1}$)	Formula
L-Alanine	25.00	56-41-7	89.09	$C_3H_7NO_2$
L-Arginine-HCl	126.50	1119-34-2	210.66	$C_6H_{14}N_4O_2$
L-Asparagine-H_2O	50.00	70-47-3	132.11	$C_4H_8N_2O_3$
L-Aspartic Acid	30.00	617-45-8	133.10	$C_4H_4NO_4$
L-Cysteine HCl-H_2O	100.00	7048-04-6	175.6	$C_3H_7NO_2S.HCl.H_2O$
L-Cystine-2HCl	31.20	30925-07-6	313.22	$C_6H_{12}N_2O_4S_2.2HCl$
L-Glutamic acid	75.00	56-86-0	147.13	$HO_2CCH_2CH_2CH(NH_2)CO_2H$
L-Glutamine	292.00	56-85-9	146.14	$H_2NCOCH_2CH_2CH(NH_2)CO_2H$
Glycine	50.00	56-40-6	75.07	NH_2CH_2COOH
L-Histidine-HCl-H_2O	41.90	71-00-1	155.15	$C_6H_9N_3O_2$
L-Isoleucine	52.50	73-32-5	131.17	$C_2H_5CH(CH_3)CH(NH_2)CO_2H$
L-Leucine	52.50	61-90-5	131.17	$(CH_3)_2CHCH_2CH(NH_2)CO_2H$
L-Lysine-HCl	72.50	10098-89-2	182.65	$C_6H_{14}N_2O_2.HCl$
L-Methionine	15.00	63-68-3	149.21	$CH_3SCH_2CH_2CH(NH_2)CO_2H$
L-Phenylalanine	32.50	63-91-2	165.19	$C_6H_5CH_2CH(NH_2)CO_2H$
L-Proline	40.00	147-85-13	115.13	$C_5H_9NO_2$
L-Serine	25.00	56-45-1	105.09	$HOCH_2CH(NH_2)CO_2H$
L-Threonine	47.60	72-19-5	119.12	$CH_3CH(OH)CH(NH_2)CO_2H$
L-Tyrosine.2Na-2H_2O	51.90	-	-	-
L-Tryptophan	10.00	73-22-3	204.23	$C_{11}H_{12}N_2O_2$
L-Valine	46.80	72-18-4	117.15	$(CH_3)_2CHCH(NH_2)CO_2H$

Vitamins	Con (mg/mL)	CAS	M.W (g mol^{-1})	Formula
Ascorbic Acid	50.00	50-81-7	176.2	$C_6H_8O_6$
D-Calsium pantothenate	1.00	-	-	-
Biotin	0.10	58-85-5	244.31	$C_{10}H_{16}N_2O_3S$
Choline chloride	1.00	67-48-1	139.62	$(CH_3)_3NCH_2CH_2OH$
Folic acid	1.00	59-30-3	441.40	$C_{19}H_{19}N_7O_6$
Nicotinamide	1.00	98-92-0	122.12	$C_6H_6N_2O$
Pyridoxine-HCl	1.00	58-56-0	205.64	$C_8H_{11}NO_3.HCl$
Riboflamin	0.10	83-88-5	376.36	$C_{17}H_{20}N_4O_6$
T-Diamine HCl	1.00	67-03-8	337.27	$C_{12}H_{17}ClN_4OS.HCl$
Vitamin B12	1.36	68-19-9	1355.3	$C_{63}H_{88}CON_{14}O_{14}P$
D-Glucose	1000.0	50-99-7	180.16	$C_6H_{12}O_6$
Lipoid acid	0.20	1200-22-2	206.33	$C_8H_{14}O_2S_2$
Sodium pyruvate	110.00	113-24-6	110.4	$CH_3COCOONa$

Nucleosides	Con (mg/mL)	CAS	M.W (g mol^{-1})	Formula
Thymidine	10.00	50-89-5	242.23	$C_{10}H_{14}N_2O_5$
Adenosine	10.00	58-61-7	267.24	$C_{10}H_{13}N_5O_4$
Cytidine	10.00	65-46-3	243.22	$C_9H_{13}N_3O_5$
Guanosine	10.00	118-00-3	283.24	$C_{10}H_{13}N_5O_5$
2-Deoxycytidine-HCl	11.00	3992-45-5	263.68	$C_9H_{13}N_3O_4.HCl$
2-Deoxyguanosine	10.00	312693-74-4	285.26	$C_{10}H_{13}N_5O_4.H_2O$

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