University of South Bohemia in České Budějovice, Faculty of Science,
Biology Centre, Czech Academy of Sciences

Oximation in LC-MS analysis of ecdysteroids

Bachelor Thesis

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Biological Chemistry

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Annotation:
Investigation of hydroxylamine hydrochloride as an oximation reagent for increased sensitivity in LC-MS analysis for ecdysteroids.

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In České Budějovice, 06.05.2019

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Acknowledgement

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I want to thank Stanislav Opekar Ph.D for his guidance during the practical work and the writing process. Further I want to thank him for his remarks which greatly increased the quality of this thesis and learned me a lot about scientific writing.

Additionally, I want to thank Anna Rázková for her help with proofreading of the thesis drafts.

Abbreviations:

20E – 20-hydroxyecdysone

7dHC – 7-dehydro-cholesterol

CID – collision induced dissociation

ESI – electrospray ionisation

ISTD – internal standard

LC – liquid chromatography

LPE – liquid phase extraction

MeOH – methanol

MS – mass spectrometry

MS/MS – tandem mass spectrometry

MTBE – methyl *tert*-butyl ether

NMR – nuclear magnetic resonance

R_f – retention factor

RP-HPLC – reversed phase high pressure liquid chromatography

SPE - solid phase extraction

TLC – thin layer chromatography

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1 Introduction

Ecdysteroids are a group of natural and synthetic, biologically active compounds. Their role in the endocrinal system of insects and crustaceans makes them a biomarker for development and stress response. Biosynthesis in various plants and invertebrates, as well as their profile in arthropods upon dietary changes, are not fully illuminated.[1-3] They exhibit positive effects on mammalian organisms and are therefore used as dietary supplements. Ecdysteroids can introduce heterogeneous gene expression and are candidates for so called “gene switches” used in gene therapy. [4] The low abundance and structural diversity challenge analytical and clinical chemistry. Increase in ionization efficiency using electrospray ionization is lowering the detection limits of ecdysteroid profiling methods and might allow deeper investigation of unresolved questions.

1.1 Aims of the thesis

The Laboratory of Analytical Biochemistry & Metabolomics, Biology Centre CAS is doing comprehensive work in the field of metabolite analytics. This thesis is a part of their efforts for improvement in the quantitative and qualitative analysis of ecdysteroids using LC-MS methods and has the following aims:

- To gain basic knowledge about the biological and physiological importance of ecdysteroids as well as existing derivatization methods.
- To test the 6-keto group common to ecdysteroids for reactivity towards hydroxylamine hydrochloride and determine qualitatively the oxime products formed on an analytical scale.
- To evaluate the process efficiency of hydroxylamine hydrochloride derivatization on ecdysteroid standards.
- To determine the response improvement of derivatized ecdysteroids using ESI in LC-MS/MS detection and identification of the major fragmentation ions.

2 Theoretical background

2.1 Ecdysteroids

2.1.1 Structure

Ecdysteroids contain, similarly to all steroidal compounds, the cyclopentano-perhydrophenanthrene polycycle. All ecdysteroids bear a 7-ene-6-one chromophore, a cis (5β-H or OH) confluence of ring A and B as well as a trans (14α-OH or -H) confluence of ring C and D (Figure 1). Further common functionalities are a 3β-OH group and an alkyl side chain on C17, both already present on their sterol precursor. The multitude of ecdysteroids structures arises from different degree and positions of hydroxylation, as well as varying length and functionality of the side chain. Free ecdysteroids are polar and water-soluble molecules. Names of ecdysteroids are generally derived from the trivial name of their parent molecule e.g. 20-hydroxyecdysone. [5-6] Ecdysteroids are found either free or conjugated *via* hydroxy groups. Common conjugates are sugars (via α- and β-glycosidic bonds), ethers (acetonide and methyl ether), esters with carboxylic acids (acetate, benzoate, cinnamate and others) as well as sulphate and phosphate esters which are all dominantly found on the side chain and the vicinal C3,2 diol group on the steroid backbone. Chemical and enzymatic hydrolysis of ester bonds is possible. Esterification and the formation of ether bonds render the solubility as well as their activity of ecdysteroids in biological systems. Reversible defunctionalisation by conjugation for temporary deposition in organisms or offspring is described in insects. [7] The formation of conjugates is proposed to direct the active compounds and intermediates between the cellular compartments. [5]

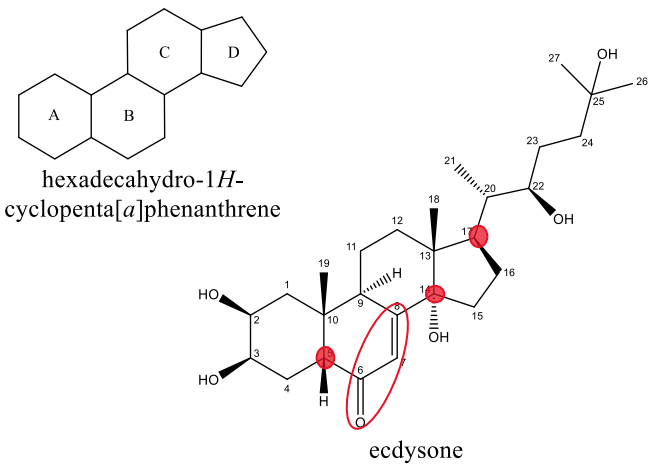


Figure 1: Carbon backbone of all ecdysteroids (upper left) and ecdysone bearing the characteristics of ecdysteroids (lower right).

2.1.2 Zoo-ecdysteroids

Arthropods possess an exoskeleton made of rigid chitin for attachment of muscles, protection and prevention of water loss. Due to inelasticity, the cuticle restrains growth and is shed (moulted) in order to enable growth. Ecdysteroids are a part of the endocrinal system of arthropods and regulate moulting, growth, reproduction and stress response. Concentrations of ecdysteroids in the haemolymph rise and reach climax before moulting and are low in-between the cycles.

The biosynthesis of ecdysteroids starts from a sterol precursor and is mediated by a group of reductase and hydroxylase enzymes. Mutations on the genome of *Drosophila melanogaster*, *Bombyx mori* and other model organisms led to elucidation of genes involved in the final steps of the biosynthesis in insects. Conversion of sterol to 7-dehydro-cholesterol (7dHC) in the endoplasmic reticulum is the first step followed by the introduction of a 7-ene-6-one chromophore to the steroid backbone. The multi-step conversion of 7dHC to the Δ^4-diketol is called the "Black Box" due to insufficient knowledge about the intermediates. [3,8] The reductions of Δ^4-diketol on the 5β and/or 3β position occur in the cytosol and microsomes and yield either the 5β-ketodiol or the 5β-diketol. Starting from one of the earlier compounds the final hydroxylation producing ecdysone and the active compound 20-hydroxy-ecdysone are catalysed by cytochrome P_{450} mono-oxygenases (Figure 2). The genes coding for the different reductases and hydroxylases are named the Halloween family and consist out of phantom (Cyp306a1), disembodied (Cyp302a1), shadow (Cyp315a1), shade (Cyp314a1) and spook (Cyp307a1). While phantom, disembodied, shadow and shade regulate the final steps of hydroxylation, spook is regulating an early step in the "Black Box". [9] Functional homologues have been found in crustaceans which imply the conservation of these genes in the arthropods. [10]

All arthropods are sterol auxotroph and must intake it through their diet in order to synthesise ecdysteroids. [11] Most animals depend on cholesterol which is a C27 sterol, while higher plants utilize different C28 and C29 sterols such as sitosterol, campesterol and stigmasterol for their physiological needs. Carnivore species directly use ingested cholesterol while many phytophagous species can dealkylate plant sterols to cholesterol. Those able to excess cholesterol mostly produce ecdysone while those unable to do so are producing a C28 or C29 analogue such as makisterone A or makisterone C. [12] Some species are able to switch between C27 and C28 ecdysteroids depending on their intake of sterols. [13]

The moult cycle in insects is controlled by a cascade of neuropeptides which trigger the production of ecdysteroids. Secretion of ecdysteroids is differentiated into the release of an inactive prohormone into the haemolymph by steroidogenic cells (in most cases ecdysone), followed by hydroxylation through a 20-hydroxylase in the mitochondria of the peripheral cells, yielding the active hormone (in most cases 20-hydroxyecdysone). Proliferation and cell growth in the target tissues is trigger by binding to a heterodimer receptor, which is stabilized by the active ecdysteroid.

The ecdysteroid induced activities in insects and crustaceans vary due to differing life cycles. Ecdysteroids are reported in non-arthropod invertebrate species like molluscs, nematodes, porifera and anthozoans but an indigenous origin or function in their endocrinal systems is not proven. [14-15] The use of insects and crustaceans as model organisms and their succession into virtually all habitats on this planet, make ecdysteroids a valuable biomarker. [16-17]

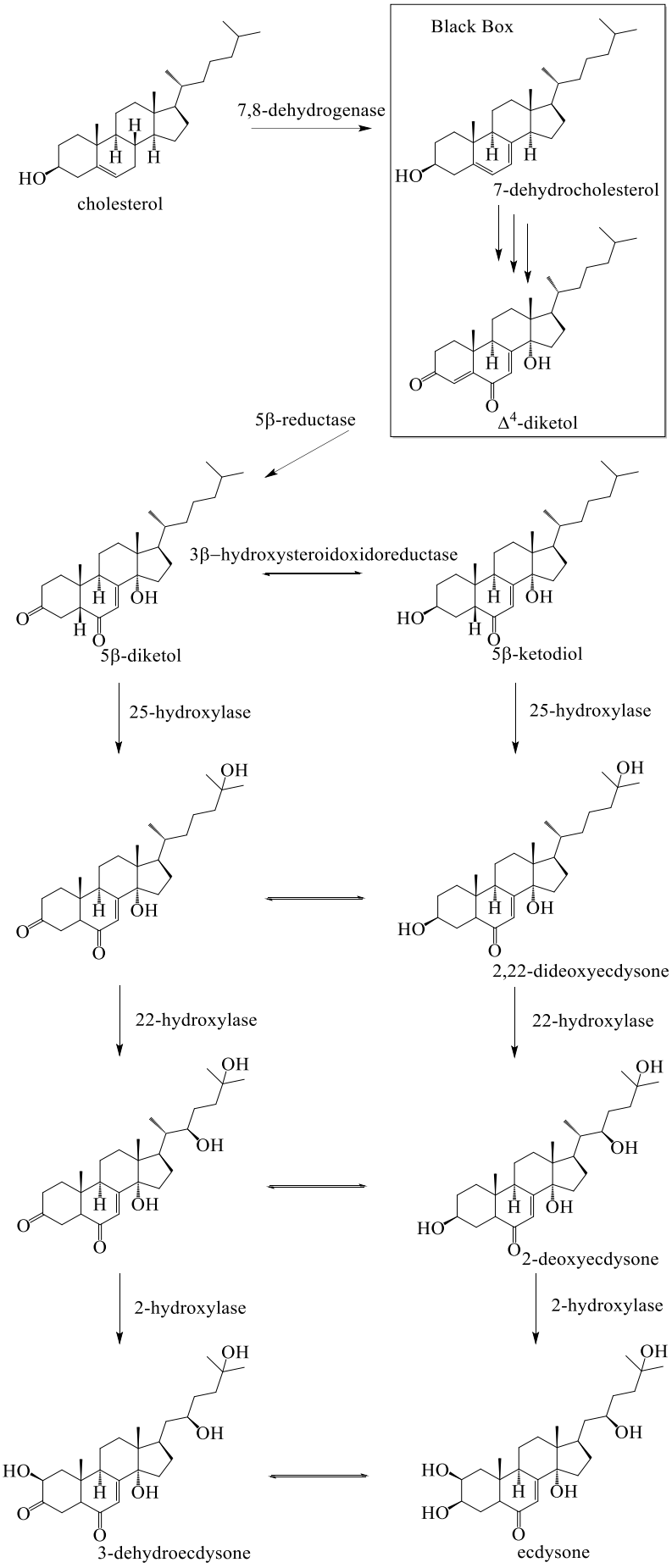


Figure 2: Biosynthesis of ecdysone in *Drosophila melanogaster*.

2.1.3 Phyto-ecdysteroids

Ecdysteroids can be found in many species belonging to the super group of Archaeplastida. Surveys showed significant amounts in over 6 % of the vascular plants. [6] The exact cell type or tissue responsible for synthesis and accumulation is not identified, but indices for increased accumulation in tissues vital for endurance and reproduction exist. [18] Likewise, the biosynthetic pathway for phyto-ecdysteroids remains unidentified. Differing precursors like sterol, sitosterol, campesterol and stigmasterol as well as different conjugates indicate the existence of varying pathways in the plant taxa. Surveys suggest the ability to produce ecdysteroids in most plants, but their accumulation is common just in some [6,19] Nevertheless, it is not completely understood if these accumulations are caused by environmental or genetic factors. Genome sequencing of ecdysteroid producing plants revealed genes coding for enzymes of the cytochrome P_{450} family and therefore an endogenous origin of phyto-ecdysteroids is probable. [2] Varying occurrence and high concentrations in some species, as well as the identification of brassinosteroids as the major plant steroid hormones make an endocrinal role in physiological plant processes unlikely. Negative effects observed on invertebrates, microbes and seedlings, in combination with their seasonal variation and location in plants point towards allelochemical functions. [20-21] Most occurring ecdysteroids in plants are 20-hydroxyecdysone and polypodine B. Usually plants contain few major ecdysteroids making up a fundamental amount of total ecdysteroids and a small but complex fraction of structural derivatives. Plants containing very high concentrations of ecdysteroids (2-3 % of dry weight) are the major source for commercially available ecdysteroids. [19]

2.1.4 Physiological effects in mammalian organisms

Ecdysteroids are not produced in the mammalian biosynthetic pathways but can be found in tissues and fluids due to dietary intake or parasites. Structural similarities to human androgenic steroids like the cyclopentanoperhydrophenanthrene polycycle, a *trans* convolution of ring C and D and a keto group conjugated with a double bond underline steroid character. Differentiation in the form of polyhydroxylation, the β-position and length of the alkyl side chain as well as the position of the chromophore prohibit interaction with androgenic steroid receptors. Nevertheless, they possess biological activity in humans and other mammals. [22] Observed effects include increased protein metabolism and increase in muscle mass, stimulation of liver secretion as well as promotion in keratinocyte differentiation and psoriasis inhibition. [23-25] A variety of plant extracts containing ecdysteroids in different purities meant for performance enhancement in sport is currently on the market. [26] If these positive effects are caused by ecdysteroids, their metabolites or cross activity with other compounds is not yet illuminated. Ecdysteroids and derivatives are investigated as promoting molecules for heterogeneous gene expression in mammalian systems. Insertion of genes coding for receptors can promote the *in situ* synthesis of pharmaceutically active peptides. The synthesis can be controlled over activation of the ligand binding receptor which regulates the engineered gene expression system. The regulation of expression using the ecdysteroid receptor EcR is proven in cell models. [4,27] Such systems could provide treatment for cancer, diabetes, cardiovascular disease and many others. Ecdysteroids offer great properties for these systems due to low interference with the endocrinal system of mammals and low toxicity.

2.2 Derivatization of ecdysteroids

The endocrinal function of ecdysteroids combined with the demand for small and cheap model organisms request highly sensitive quantification techniques to determine ecdysteroids in biological samples. Immunoassays are the traditional tool of choice but are not entirely selective and do not allow the detection of novel compounds. [28-29] Ecdysteroids are polar, non-volatile as well as thermally labile and therefore can't be separated by GC. Their low abundance in biological samples and low polarizability are a major drawback for the sensitivity of LC-MS using atmospheric pressure ionization techniques. Derivatizations can be used to render the physical properties of ecdysteroids in order to allow chromatographic separation in combination with mass spectrometric detection. Derivatization targets common to all ecdysteroids are the hydroxy groups and the 6-keto group.

2.2.1 Silyl ether

The conversion of hydroxy groups to silyl ethers reduces dipole-dipole interactions and allows the separation by GC due to the lower boiling points of the derivatives. Trimethylsilyl reagents are most commonly used, since steric hindrance of bulkier substituents causes decreased product formation. Derivatization proceeds through a nucleophilic attack (SN_2) of the hydroxy group and therefore must be carried out in water free conditions. [30] From the variety of silylation reagents known, N-trimethylsilylimidazole is suitable for ecdysteroids due to its ability to selectively donate the silyl group to hydroxy groups while not promoting the formation silyl-enol ethers (Figure 3). [31] The derivatization can lead to different degrees of silylation due to varying rates of reaction of the hydroxy groups (primary > secondary > tertiary), which can be controlled by reaction temperature and time. For conjugated ecdysteroids, enzymatic or chemical hydrolyses must be utilized in order to obtain the free hydroxy group and therefore limits the detection of ecdysteroid-conjugate complexes. Typical column temperatures used for the separation of silyl ether derivatives of ecdysteroids are above 300°C. [32] Coupling to MS using vacuum ionization techniques like electron impact ionization provides a sensitive detection method although less sensitive than immunoassays. [33]

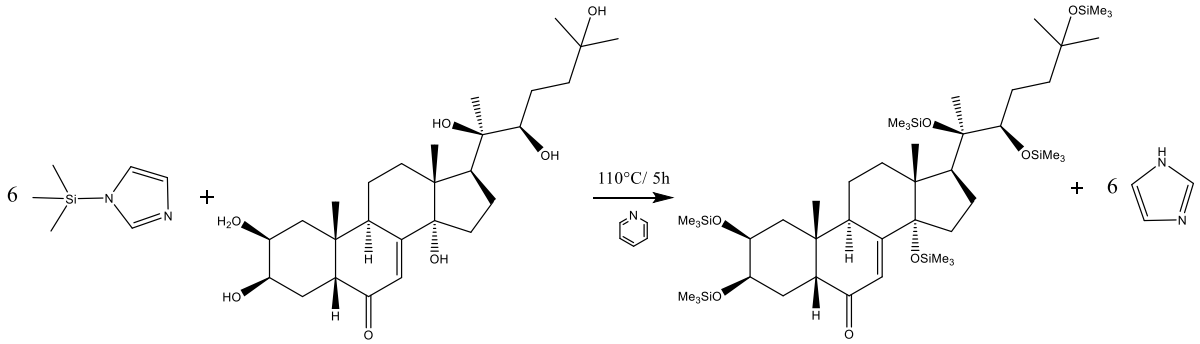


Figure 3: Reaction scheme for the complete silylation of 20E with N-trimethylsilylimidazole. [32]

2.2.2 Boronate esters

Boronic acids selectively form cyclic esters with 1,2- and 1,3-diols as well as α-hydroxy acids. The reaction with phenylboronic acid can be used as a recognition moiety for the detection of ecdysteroids possessing a 20,22-diol (Figure 4). This is possible since phenylboronic acid exclusively reacts with the diol system but does not form cyclic esters with the axial C2 and equatorial C3 hydroxy group under mild conditions. The reaction is quantitative and the formed ecdysteroid derivatives are in comparison to other boronic acid esters stable under protic conditions. The derivatization directs the fragmentation to the C17/20 bond and allows structure elucidation of the sterol skeleton and side chain. [34]

This selectivity can be exploited to determine the presence of a 20,22-diol system and directs the fragmentation by chanced chromatographic behaviour or mass spectrometry. [35] This method is suitable for HPLC methods and can further be used for the separation affinity chromatography with immobilized phenylboronic acid. [36]

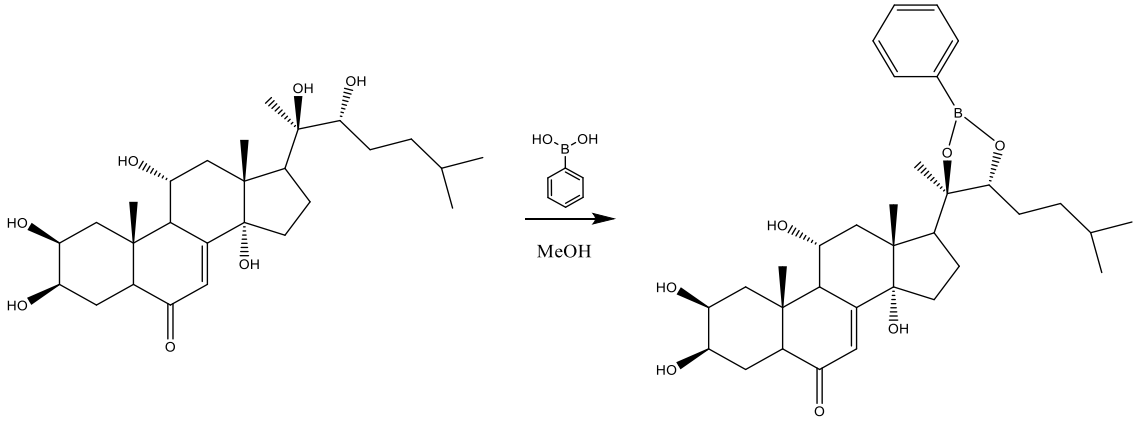


Figure 4: Formation of a cyclic boronic acid ester with the 20,22-diol moiety on ajugasterone C using phenylboronic acid. [34]

2.2.3 Hydrazones

Carbonyl compounds can be converted to hydrazones by different hydrazine and hydrazide reagents (Figure 5). [37-38] Quaternary ammonium hydrazides (Girard reagents) which "classically" possess quaternary ammonium or pyridinium moiety have been used to derivatize the 3-keto group of oxysterols. [39] This reaction can be utilized for the 6-keto group of ecdysteroids to enhance their solubility in polar solvents and the sensitivity using ESI-MS detection. [40] The increased sensitivity results from a permanent charge retained on the derivatized compound (charge derivatization). [41] The derivatization yields *E* and *Z* isomers which can be resolved using RP-HPLC. Depending on the used Girard reagent the detection in positive or negative ion mode is possible. Increased sensitivity can also be achieved by monitoring the specific fragment ions (pyridine, trimethylamine) upon fragmentation at MS/MS experiments. [40]

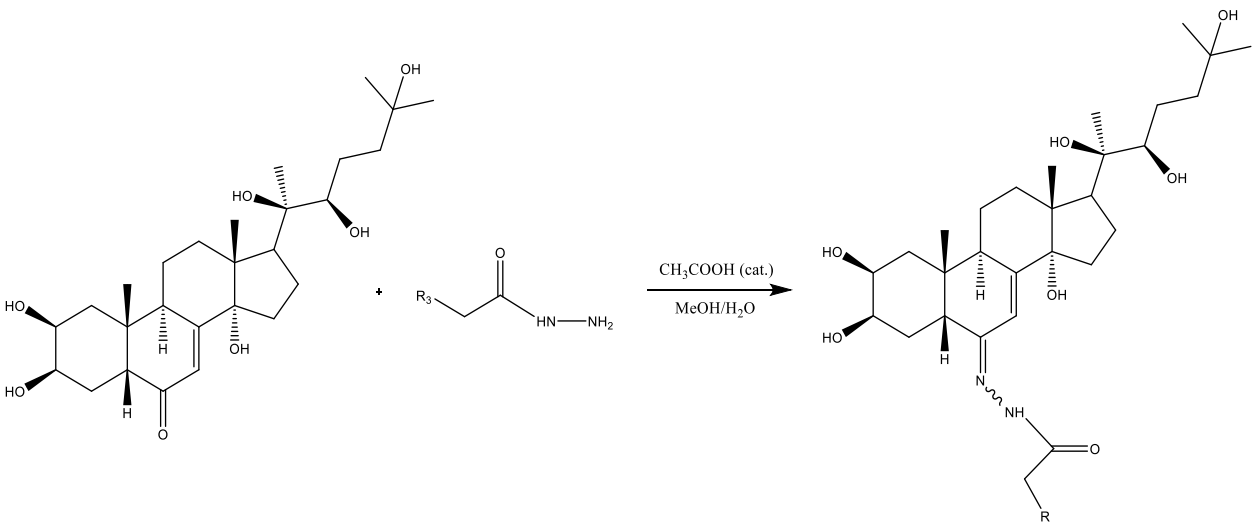


Figure 5: Scheme for the formation of a hydrazone derivative of 20E using a Girard reagent. Girard P: R = pyridine; Girard T: R = trimethylamine; Girard C = COOH. [40]

2.2.4 Oximes

To increase the ionization capacity of steroidal compounds the introduction of a basic functional group containing nitrogen can be used. A possibility is the conversion of a carbonyl group to an oxime. This method was previously used for keto groups on oxosteroids and proved to be depending on the position of the keto group valuable for the ion abundance upon ESI. It was also possible to obtain fragments of use for the structural analysis and assignment of positions to the hydroxy and keto groups of the target compounds.[42-44] This method was previously examined for the quantification of ecdysteroids in biological samples with similar sensitivity enhancement. [45]

2.3 Oxime formation

Organic oximes can be formed by reduction of nitroalkanes and reduction-nitrosation of olefines, but the most general method remains condensation of hydroxylamine with carbonyl compounds. The condensation of hydroxylamine yields an oxime compound at acidic and basic reaction conditions and can be carried out in a variety of solvents such as water, ethanol and pyridine. [46] The formation at neutral pH is initiated by the nucleophilic attack of the nitrogen lone pair and proceeds via an intermediate as shown in Figure 6. [47] Depending on the starting material being an aldehyde or a ketone an aldoxime or ketoxime is formed. The restricted rotation around the C=N double bond leads to the formation of two isomers, which are labelled *syn* (*Z*) and *anti* (*E*). In contrast to the isomers of imines which rapidly interconvert, are isomers of oximes stable and can be separated by crystallization and chromatography. Due to low differences in internal energy and Gibbs-Energy for the isomers, the interactions with the surrounding medium can determine which stereoisomer is formed. [48] Isomerisation can be induced either by an acid-base catalysed mechanism or photochemically. [49-50]

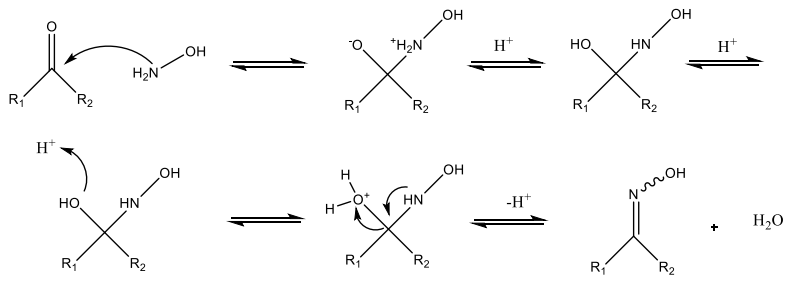


Figure 6: Condensation mechanism for the oxime formation using hydroxylamine (R_1=R_2= alkane, aryl, H).

3 Materials and Methods

3.1 Equipment

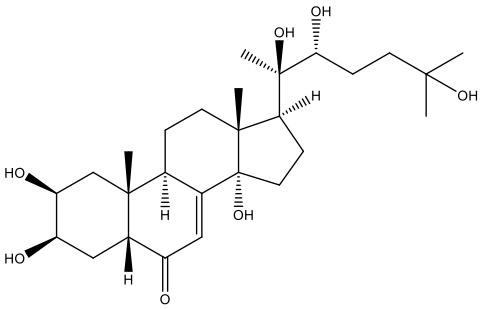
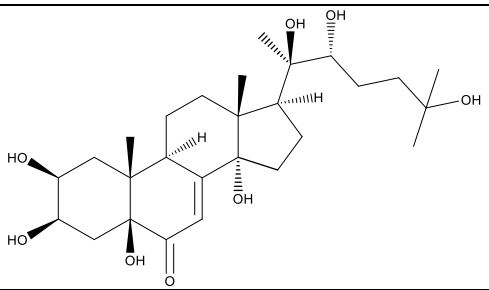
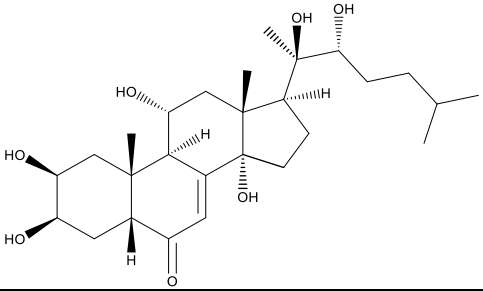
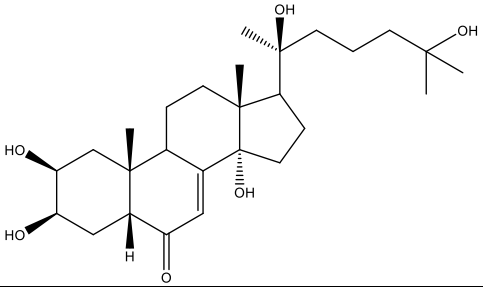
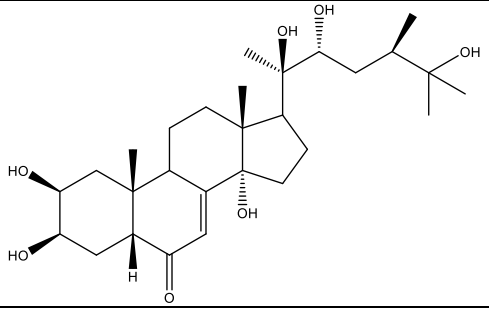
LC-MS analysis was performed using a combination of an Accela 600 pump coupled to an LTQ-XL mass spectrometer built by Thermo Scientific. Stationary phases for the LC setup were columns supplied by Phenomenex (Kinetex C18, Synergi Polar RP).

The NMR spectra were recorded on a Bruker Avance III 400. TLC plates coated with silica 60 (layer thickness 0.2 mm) purchased from Merck and a heat gun were used for quick analysis. Solid phase extraction cartridges from Waters (HLB Oasis 10 mg) and Agilent (Bond Elute Plexa 10 mg) were utilized for sample work up.

3.2 Chemicals

The 20-hydroxyecdysone was isolated by Helena Zahradníčková, Anna Heydová and Marie Texlová in the Laboratory of Analytical Biochemistry & Metabolomics, Biology Centre CAS. 20E with a purity of ≥ 85 % was used for preparative and ≥ 95 % for analytical purposes. Other ecdysteroids (Table 1) obtained from *Leuzea carthamoides* extract in the Institute of Organic Chemistry, Prague CAS were gifted by Juraj Harmatha and had an unknown purity. [51] Hydroxylamine hydrochloride (≥ 98.0 %) was purchased from Merck. Ethanol, methanol, chloroform, methyl-*tert*-butyl ether, ethyl acetate and pyridine have been obtained from various sources. Pyridine for synthetic purposes was purified by distillation from potassium hydroxide. Methanol (LC-MS Grade, Merck) and water (purified by Thermo Scientific™ Barnstead™ Nanopure™ System) were used as mobile phases. A vanillin staining solution (60 g L^{-1} in ethanol) was used for quick analysis of TLC plates.

Table 1: Used ecdysteroids.

Name	Structure	Molecular weight [g mol^{-1}]
20-hydroxyecdysone		480.64
Polypodine B		496.64
Ajugasterone C		480.64
Taxisterone		464.64
Makisterone A		494.67

3.3 Analytical methods

3.3.1 Liquid chromatography

All separations were performed using reversed phase columns with fully porous ether linked phenyl particles (Synergi Polar RP) or core shell particles with C18 chains (Kinetex C18) as stationary phases. All separations were carried out at 35°C unless stated otherwise. An injection volume of 5 µL in 30 % methanol/water dissolved sample was loaded onto the columns, except for low concentration (Table 3) where 20 µL was loaded.

3.3.1.1 Mobile Phase

Different gradient programs of methanol and water containing 5 mmol L^{-1} ammonium formate with flow rates between 400 µL min^{-1} or 300 µL min^{-1} have been utilized.

Program A

A gradient starting from 30 % methanol/water linearly increasing to 70 % methanol/water in 14 minutes a subsequent linear increase to 90 % methanol/water in 2 minutes followed by 30 % methanol/water for 4 minutes was used. The mobile phase flow rate was 400 µL min^{-1}.

Program B

A gradient starting from 30 % methanol/water linearly increasing to 65 % methanol/water in 8 minutes a subsequent linear increase to 100 % methanol in 4 minutes followed by 30 % methanol/water for 4 minutes. The mobile phase flow rate was 300 µL min^{-1}.

Program C

A gradient starting from 30 % methanol/water linearly increasing to 100 % methanol in 8 minutes, continued 100 % methanol for 1.6 minutes and followed by 30 % methanol/water for 3.4 minutes. The mobile phase flow rate was 400 µL min^{-1}.

3.3.2 Mass spectrometry

All signals were obtained using an LTQ-XL mass spectrometer using positive ion mode. Ionization was achieved with ESI using a capillary temperature of 300°C, and a capillary voltage 40 V for all measurements.

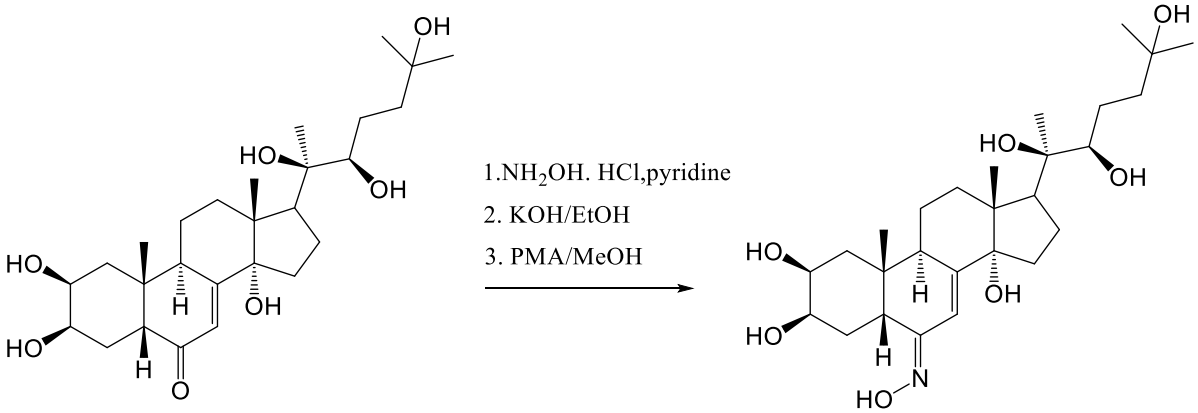
The collision cell was operated using an isolation width of 3.0 m z^{-1}, collision energy of 30 eV and a wide band activation mode for recording of MS/MS spectra (unless stated otherwise).

3.3.3 NMR-spectrometry

The NMR spectra were recorded on a Bruker Avance III 400 with the help of Vladimír Pejchal in Pardubice. Spectra for ^{1}H at 400.13 MHz have been recorded in deuterated pyridine at ambient temperatures.

3.4 Synthesis of 14,15-anhydro-20-hydroxyecdysone-6-oxime

A literature procedure for the derivatization of 20-hydroxyecdysone with hydroxylamine hydrochloride was used. [52] 20-hydroxyecdysone (145 nmol; 70 mg; 1 equiv.) was dissolved in freshly distilled pyridine (1.5 mL). Hydroxylamine hydrochloride (0.875 mmol; 60 mg; 6 equiv.) was added and the mixture heated and stirred for 72 hours in a closed vessel at 70°C (Figure 7). After cooling to 0°C the mixture was neutralized with ethanolic potassium hydroxide solution (0.5 mL, 100 mg L^{-1}). Subsequently, water (5 mL) was added and extracted three times with ethyl acetate (8 mL). The organic phase was collected, dried with Na_2SO_4 and the ethyl acetate was evaporated. The obtained residue was dissolved in anhydrous methanol (1.5 mL) and treated with 0.2 mg of phosphomolybdic acid. The mixture was neutralized with saturated $NaHCO_3$ solution (1 mL) and again extracted with ethyl acetate (5 mL), dried with Na_2SO_4 and concentrated using a rotary evaporator. The crude product obtained was 60 mg (126 nmol) of yellowish crystalline 14,15-anhydro-20-hydroxyecdysone-6-oxime which is corresponding to a yield of 87 %.



20E (6*Z*)-14,15-Anhydro-20E-6-oxime

Figure 7: Reaction scheme for the preparation of 14,15-anhydro-20-hydroxyecdysone-6-oxime.

3.5 Preparation of solutions

Solid ecdysteroids were weighted and dissolved in appropriate amounts of methanol. Concentrations for reactivity and signal experiments are listed in Table 2. Solutions of 20E, reference and internal standard used for recovery experiments were produced by dissolving and subsequent 100 × dilutions of the corresponding solid materials (Table 3).

Table 2: Summary of used amounts of ecdysteroid, solvents and solution concentrations.

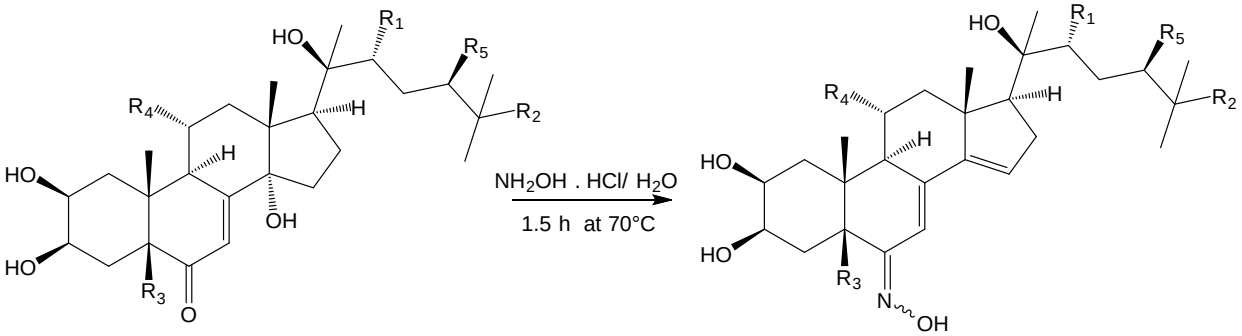
Compound	**Amount [mg]**	**Volume MeOH [µL]**	**Concentration [nmol µL^{-1}]**
20-hydroxyecdysone	24µL Table 3)	276	0.5
Makisterone A	0.29	1172	0.5
Polypodine B	0.32	1280	0.5
Taxisterone	0.14	602	0.5
Ajugasterone C	0.52	2168	0.5
Mix (All above mentioned)	50 µL (of all above mentioned final solutions)	0	0.1 (each)

Table 3: Solutions for process efficiency experiments (20-hydroxyecdysone, reference and ISTD solution).

High concentrations			
Compound	**Amount [mg]**	**Volume MeOH [µL]**	**Concentration [nmol µL^{-1}]**
20-hydroxyecdysone	4.88	1626	6.25
14,15-anhydro-20-hydroxyecdysone-6-oxime (reference)	2.38	798	6.25
Makisterone A (ISTD)	0.21	2100	0.2
Low concentrations			
Compound	**Amount [µL]**	**Volume MeOH [µL]**	**Concentration [pmol µL^{-1}]**
20-hydroxyecdysone	10	990	62.5
14,15-anhydro-20-hydroxyecdysone-6-oxime (reference)	10	990	62.5
Makisterone A (ISTD)	10	90	20

3.6 Analytical scale ecdysteroid derivatization

Solutions from section 3.5 (10 µL of each) were transferred into a vial, dried under nitrogen flow and mixed with 500 µL of aqueous hydroxylamine hydrochloride solution (100 mg mL^{-1}) using a vortex mixer. [45] The mixture was heated to 70°C for 90 min (Figure 8). Work-up according to section 3.6.1 or section 3.6.2 was carried out subsequently.



Ecdysteroid

Dehydro-ecdysteroid-6-oxim

Figure 8: Scheme for the analytical scale derivatization of ecdysteroids to their dehydro-ecdysteroid-6-oximes. 20-hydroxyecydysone: $R_1 = R_2 = OH$, $R_3 = R_4 = R_5 = H$; polypodine B: $R_1 = R_2 = R_3 = OH$, $R_4 = R_5 = H$; ajugasterone C: $R_1 = R_4 = OH$, $R_2 = R_3 = R_5 = H$; taxisterone: $R_2 = OH$, $R_1 = R_3 = R_4 = R_5 = H$; makisterone A: $R_1 = R_2 = OH$, $R_3 = R_4 = H$, $R_5 = CH_3$.

3.6.1 Liquid phase extraction work-up

The mixtures were cooled to room temperature and extracted twice with 500 µL of solvent. After each addition of solvent, the vial was vigorously mixed on a vortex mixer for 5 seconds. The organic layer was collected using a syringe, transferred into a vial and dried under nitrogen flow. The organic solvents used were ethyl acetate, chloroform and methyl-*tert*-butyl ether.

3.6.2 Solid phase extraction work-up

Mixtures were cooled to room temperature and extracted using solid phase extraction cartridges. Each cartridge was conditioned and equilibrated by hoovering 1 mL methanol and 1 mL water over it. The sample solution was applied to the column and sucked through the absorbent with the lowest continuous rate possible. Afterwards washing solution (5 % methanol in water, 1 mL) was inserted and vacuum applied untill the column was dry. Three millilitres of eluent solution (5 % water in methanol) were used to release the ecdysteroid derivatives from the cartridge. Liquids were concentrated using a Speedvac Jouan RC 10.10.

4 Results

4.1 Reactivity of C6-carbonyl-group of ecdysteroids

The affinity of the 6-keto-group for nucleophilic attack of hydroxylamine was investigated by comparison of signal intensities from residual ecdysteroids after derivatization according to section 3.6.2 and signals from same amounts of untreated ecdysteroids. Low ecdysteroid signals in reaction mixtures from ajugasterone C, taxisterone, makisterone A and 20-hydroxyecdysone emphasize high affinity of their 6-keto groups for nucleophilic attack of hydroxylamine hydrochloride. Polypodine B the only tested ecdysteroid with a 5-hydroxy- next to the 6-keto-group proved mainly unreactive under the reaction conditions. The obtained signals are visualized in Figure 9 and can be seen in Appendix A. An estimation of the reaction turnovers is summarized in Table 4. Derivatives and ecdysteroids were separated according to gradient programme C on a Kinetex C18 column.

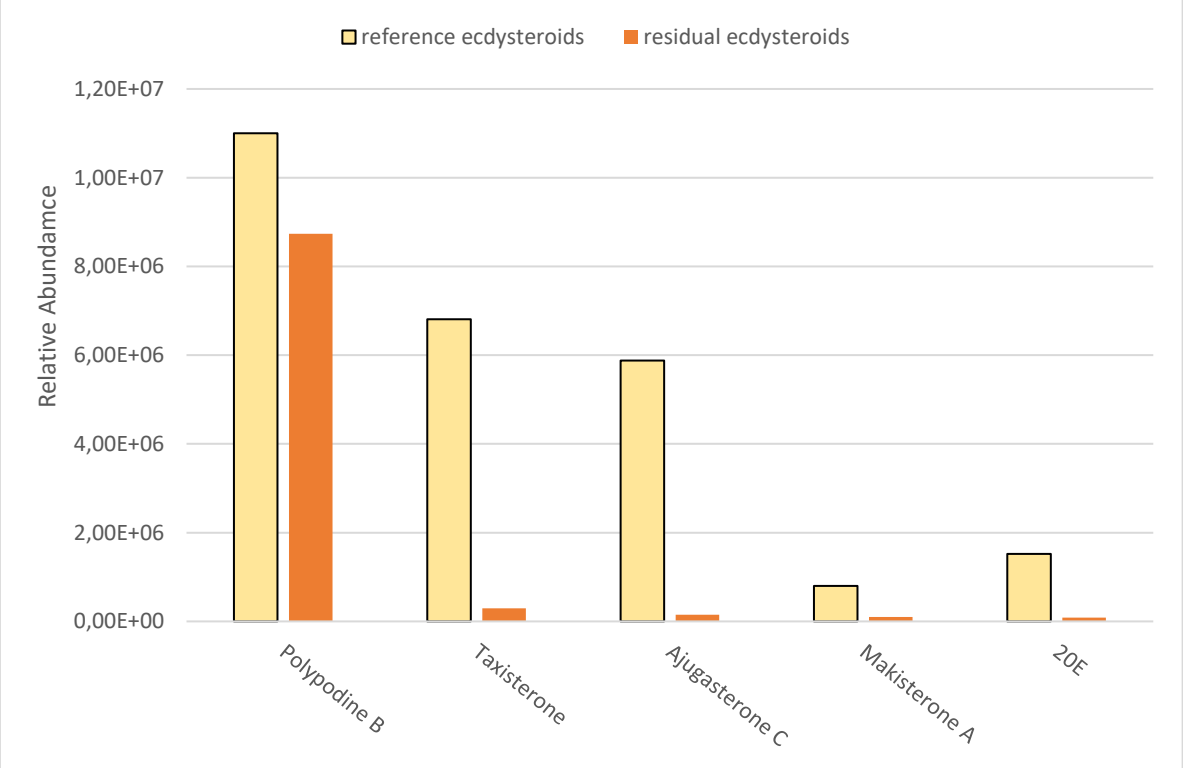


Figure 9: Peak areas of unreacted ecdysteroids after derivatization (section 3.6.2) (orange colour) and blanks spiked with untreated ecdysteroids (Table 3) (yellow colour).

4.2 Ionization efficiency

Ionization efficiency was determined by comparing the signals from ecdysteroid-6-oximes obtained by derivatization according to section 3.6.2 and blanks spiked with corresponding untreated ecdysteroid (Table 2). The obtained data proved increased ionization efficiency upon ESI for the anhydro-ecdysteroid-oximes in comparison to the precursors. 20-hydroxyecdysone and makisterone A both forming an anhydro-6-oxime derivative as the major product, showed significantly increased signals. The signals for the bisanhydro-derivatives are minor compared to anhydro-derivatives, except for taxisterone. A signal improvement factor for each ecdysteroid was calculated as the ratio of the ESI-MS signals (peak areas) from anhydro-ecdysteroid-6-oximes to their corresponding ecdysteroid. The obtained signals are visualized in Figure 10 and can be seen in Appendix A. The response improvement factors are listed in Table 4.

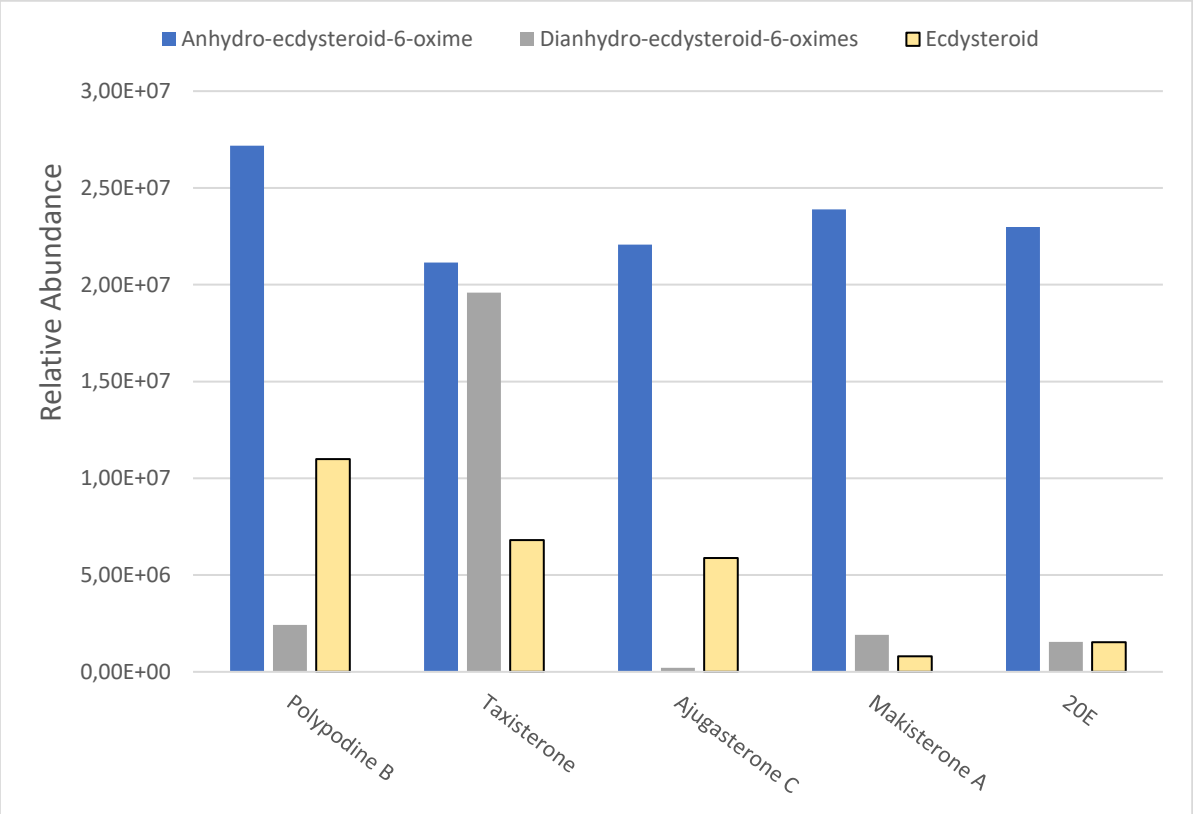


Figure 10: Signals of blanks spiked with ecdysteroids by post extraction addition (yellow colour) and ecdysteroid-oxime derivatives (blue and grey colours) after reaction and work-up according to section 3.6.2 and in detection with ESI-MS.

Table 4: Turnover and response increase of ecdysteroids derivatized according to 3.6.2.

Compound	**Unreacted Material (reaction mixture) [%]**	**Signal improvement factor**
Polypodine B	79	2.5
Taxisterone	4	3.1
Ajugasterone C	3	3.8
Makisterone A	12	29.8
20-hydroxyecdysone	6	15.1

4.3 Relative recovery of oximation procedure

The recovery of the oximation protocol was determined with 20E as a representative compound. The efficiency of the oximation and extraction processes (derivatization recovery) were determined by comparison of two separated experiments. Signals of 14,15-anhydro-20-hydroxyecdysone-6-oxime either obtained by derivatization and LPE (section 3.6.1) or SPE (section 3.6.2) work-up of 20E, or blank samples containing ISTD spiked post extraction with 10 µL of the reference solution (Table 4) were compared. Derivatization followed by SPE work-up with HLB Oasis columns showed the highest efficiency for high and low sample concentrations. Obtained recoveries can be seen in Figure 11 and demonstrate the process efficiency of the oximation for 20E. Chloroform and ethyl acetate also used in LPE work-up showed inferior recoveries compared to MTBE (data not displayed). Signals of reference and sample were compared by taking the ISTD into account.

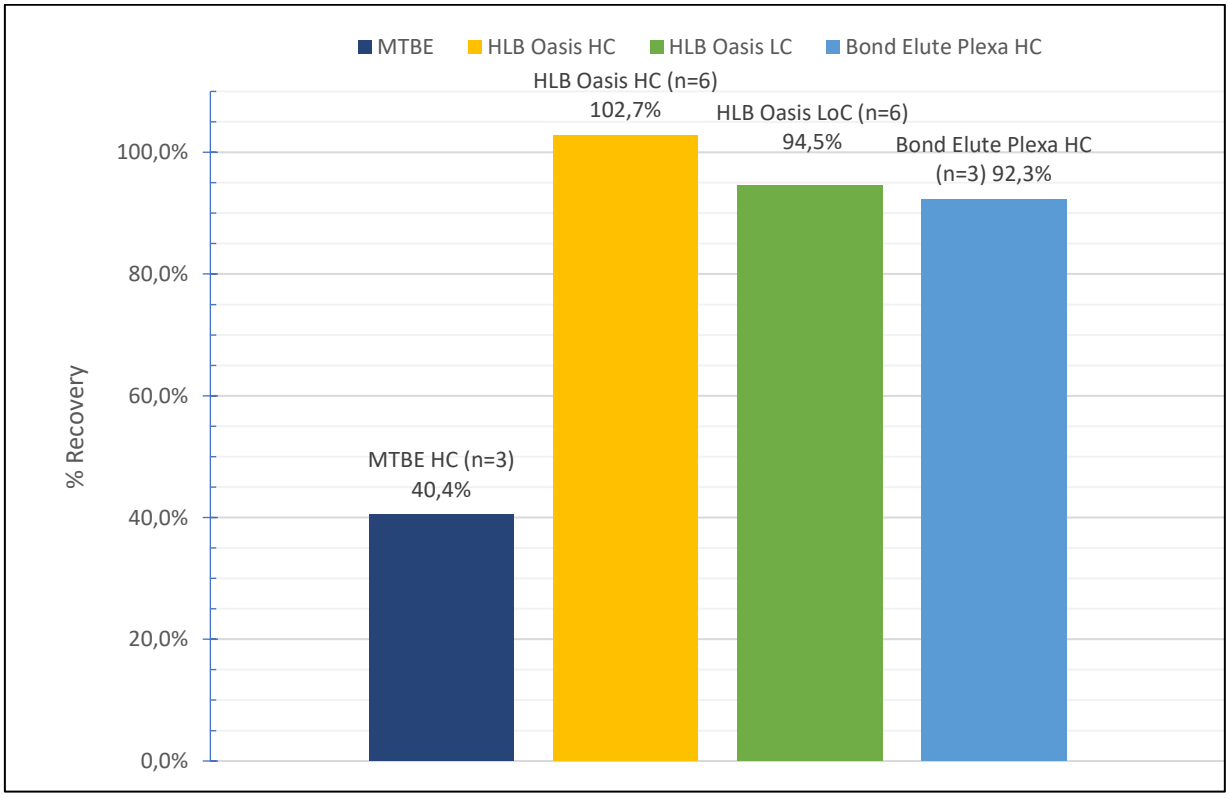


Figure 11: Relative recovery of the oximation method (Section 3.6) for 20E, using SPE and LPE work-up with sample amounts from 62.5 nmol (HC) to 625 pmol (LoC). n = number of experiment repetitions.

4.4 Fragmentation patterns and structural information

All tested ecdysteroid-oximes fragmented upon collision cell settings. A summary of the produced ions can be found in Table 5. Loss of up to four water molecules is common to all tested ecdysteroid derivatives. The observed product ion patterns suggest stability of the vicinal hydroxy groups on C2 and C3 upon CID of the C17-C20 bond. The fragment ions originating from the cleavage of the C17-C20 bond (Figure 12) differ in mass depending on the position of hydroxylation. Ecdysteroids with a hydroxy group at C5 or C11 produce ions with mass 314.2 m z^{-1} while ecdysteroids with no additional hydroxy group to the C2,3 diol on the steroid backbone produce an ion with mass 316.2 m z^{-1}.

Table 5: Parent ions, fragmentation patterns and collision cell settings for all tested ecdysteroids.

Compound	Parent Ion [m z^{-1}]	Product Ion [m z^{-1}]	Collision Energy [eV]
Anhydro-polypodine B-6-oxime	494.3		0
		458.3	30
		422.3	30
		314.2	30
Anhydro-taxisterone-6-oxime	462.4		0
		426.3	30
		316.2	30
Anhydro-ajugasterone-6-oxime	478.3		0
		442.3	30
		332.1	30
		314.1	30
Anhydro-makisterone-6-oxime	492.4		0
		438.2	30
		316.2	30
14,15-anhydro-20-hydroxyecdysone-6-oxime	478.3		0
		424.2	30
		316.2	30
Bisanhydro-taxisterone-6-oxime	444,3		0
		316.2	30

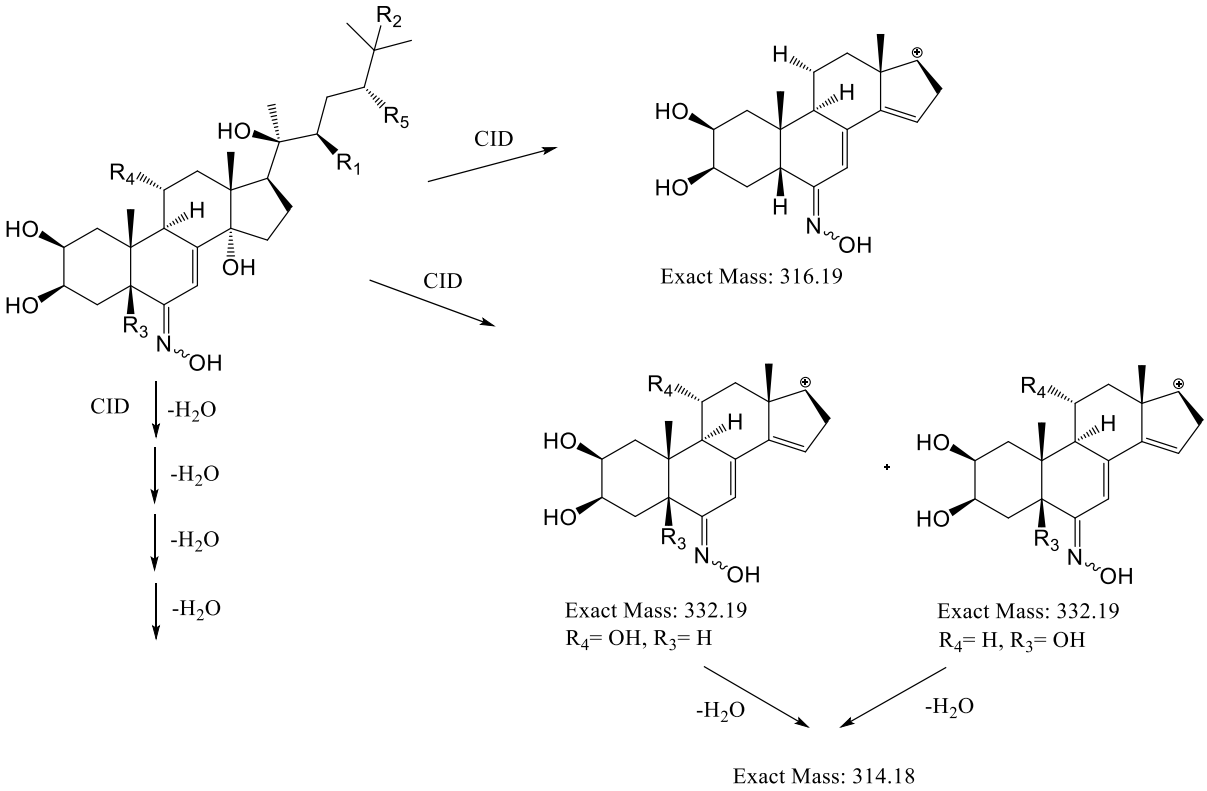


Figure 12: Dehydro-ecdysteroid-6-oxime fragments obtained by CID with N_2. 20-hydroxyecydysone: $R_1 = R_2 = OH$, $R_3 = R_4 = R_5 = H$; polypodine B: $R_1 = R_2 = R_3 = OH$, $R_4 = R_5 = H$; ajugasterone C: $R_1 = R_4 = OH$, $R_2 = R_3 = R_5 = H$; taxisterone: $R_2 = OH$, $R_1 = R_3 = R_4 = R_5 = H$; makisterone A: $R_1 = R_2 = OH$, $R_3 = R_4 = H$, $R_5 = CH_3$.

4.5 Separation of (6*E*) and (6*Z*) isomers

LC-MS analysis of the crude 14,15-anhydro-20-hydroxyecdysone-6-oxime obtained according to section 3.4, showed a split peak with 478.3 m z^{-1} in the chromatogram (Appendix B). Purification by silica gel column chromatography using a chloroform/MeOH (7:1) mobile phase gave two fractions. The earlier fraction (A) was a colourless solid with a R_f (TLC) of 0.33. LC-MS analysis showed a shoulder peak with 478.3 m z^{-1} in the chromatogram. The later fraction (B) was also a colourless solid with R_f (TLC) of 0.30 and 0.33. LC-MS analysis showed a split peak with 478.3 m z^{-1} (Appendix C) in the chromatogram The ^{1}H-NMR spectrum of B (Appendix D) showed signals with chemical shifts of 5.86 and 5.92 ppm, which were assigned to the 7H on (6*Z*)-14,15-anhydro-20-hydroxyecdysone-6-oxime and (6*E*)-14,15-anhydro-20-hydroxyecdysone-6-oxime by comparison with data from literature. [52] The relative abundance of the isomers was determined through the ratio of the 7H peak integrals and found to be 46:54 (*E*:*Z*). Comparison of signal ratios from LC-MS and NMR analysis enabled the conclusion of the retention times and elution sequence for the isomer (Table 6). This indicated that the crude product was a nearly equimolar mixture of (6*E*)-and (6Z)-14,15-anhydro-20-hydroxyecdysone-6-oxime. A complete separation of *E* and *Z* isomers could neither be achieved on a preparative nor on an analytical scale.

Table 6: Relative isomeric composition of different fractions of 14,15-anhydro-20E-6-oxime and elution sequence.

Fraction	**Gradient Program**	**Column**	**RT_1 [min]**	**RT_2 [min]**	**Ratio (*E*:*Z*)**
Crude Product	A	Kinetex C18 (22°C)	10.37 (*E*)	10.48 (*Z*)	46:54
A	A	Kinetex C18 (22°C)	10.63 (*E*)	10.76 (*Z*)	75:25
B	A	Kinetex C18 (22°C)	10.62 (*E*)	10.76 (*Z*)	40:60
B	B	Synergi Polar RP	7.93 (*Z*)	8.16 (*E*)	41:59
B	NMR				43:57

Separation of the 6-oxime ecdysteroid stereoisomers obtained by derivatisation according to section 3.6 proved to be difficult using RP-HPLC. Best results were obtained using gradient program B and a Synergi Polar RP, summarized in Table 7. Complete peak separation was not achieved. Assignment of obtained peaks was just accomplished for (6*E*)-14,15-anhydro-20-hydroxyecdysone-6-oxime and (6*Z*)-14,15-anhydro-20-hydroxyecdysone-6-oxime from the retention times obtained above (Table 6). Differentiation in fragmentation upon used CID parameters for 6*E* and 6*Z* isomers were not observed. Corresponding chromatograms and MS/MS spectra can be seen in Appendix E-I.

Table 7: Ratios and retention times of anhydro-ecdysteroid-6-oxime using gradient B and a Synergi Polar RP column.

Compound	Ratio	RT_1 [min]	RT_2 [min]
14,15-anhydro-20-hydroxyecdysone-6-oxime	40:60 (*E*:*Z*)	8.00 (*Z*)	8.19 (*E*)
Anhydro-makisterone-6-oxime	56:44	8.97	9.18
Anhydro-polypodine B-6-oxime	70:30	8.23	8.89
Anhydro-taxisterone-6-oxime	51:49	9.80	10.00
Anhydro-ajugasterone-6-oxime	71:29	9.41	9.78

5 Discussion

Hydroxylamine hydrochloride is showing a high affinity for the 6-keto group on 20-hydroxyecdysone, makisterone A, taxisterone and ajugasterone C. The effectiveness of this derivatization protocol in biological matrixes as previously shown for some ecdysteroids looks promising for the earlier mentioned compounds, but has to be determined by further experiments with biological material. [45] The reduced affinity of hydroxylamine for the 6-keto group of polypodine B can be explained by increased steric constraints and an increased keto-enol tautomerism due to intramolecular hydrogen binding by the C5 hydroxy group. [34,53] A general trend for the decreased affinity of hydroxylamine for the 6-keto group in connection to the degree of hydroxylation on the sterol backbone or the side chain was not observed.

Previously described increased ionization efficiency of steroidal oxime derivatives compared to the natural compounds was observed for all anhydro-6-oxime derivatives upon the used ESI parameters. [42,44-45] The experiments strongly emphasised the use of solid-phase extraction cartridges over liquid extraction due to their convenience and efficiency. Using the solid phase extraction cartridges for the derivatization procedure of 20E, a relative recovery of 95 % could be achieved at the concentration level of 625 pmol.

The dehydration occurring during the derivatization process in all tested ecdysteroids seems to be a result of the elevated temperatures and the acidic derivatization conditions. The observed fragment ions speak for the formation of the double bond between C14 and C15 at all tested ecdysteroids, although the additional hydroxy group on the cyclopentanoperhydrophenanthrene backbone of polypodine B and ajugasterone C would allow different locations. [45,52] The fragmentation patterns also allowed assigning the second dehydration occurring on taxisterone to the side chain, but the complete structural assessment was due to the limited amounts of substance not carried out.

The collision induced neutral loss of up to four water molecules, previously observed for derivatized and natural ecdysteroids was also observed for the anhydro-6-oxime derivatives. [40,53] The major product ions observed resulted from cleavage of the C17-20 bond. The previously reported stability of the vicinal C2,3 hydroxy groups upon CID was also observed and allows the assignment of hydroxy group elimination either to the sterol backbone or the side chain and is therefore valuable for identification of ecdysteroids using MS/MS. [40,53-54] Other characteristic fragment ions which would contribute to structural elucidation were not observed in positive ion mode.

Assignment of *E* and *Z* isomers was successful for the 14,15-anhydro-20E-6-oxime although complete chromatographic separation could not be achieved. The by I. Galyautdinov *et. al.* as stereoselective described synthesis procedure for 14,15-anhydro-20-hydroxyecdysone-6-oxime yielded in above described synthesis a mixture of *E* and *Z* isomer. [52] Evaluation of the chromatograms for all compounds revealed an approximately 50:50 distribution of *E* and *Z* isomer for ecdysteroids bearing no additional hydroxy group than the vicinal diol at C2,3 on the sterol backbone, while the existence of a further hydroxy group either at C5 or C11 leads to increased stability of one isomer.

6 Conclusion and perspectives

The conversion of ecdysteroids to their oxime derivatives provides a method for an increase in ionization efficiency using ESI and is therefore, a promising method for enhanced sensitivity of LC-MS analysis for this hormone class. The observed total recovery of the applied method meets basic criteria for further research in the direction of implementation to an analytical method for picogram quantities. The observed fragmentation patterns in combination with the different turnover to the corresponding oxime derivatives provide means to differentiate between hydroxylation on the steroidal backbone and the side chain as well as the hydroxylation on C5 or C11. The non-invasive derivatization with respect to conjugates, in combination with the increased sensitivity and possibility for structural elucidation, might allow the identification of novel ecdysteroids in biological mixtures using this derivatization method.

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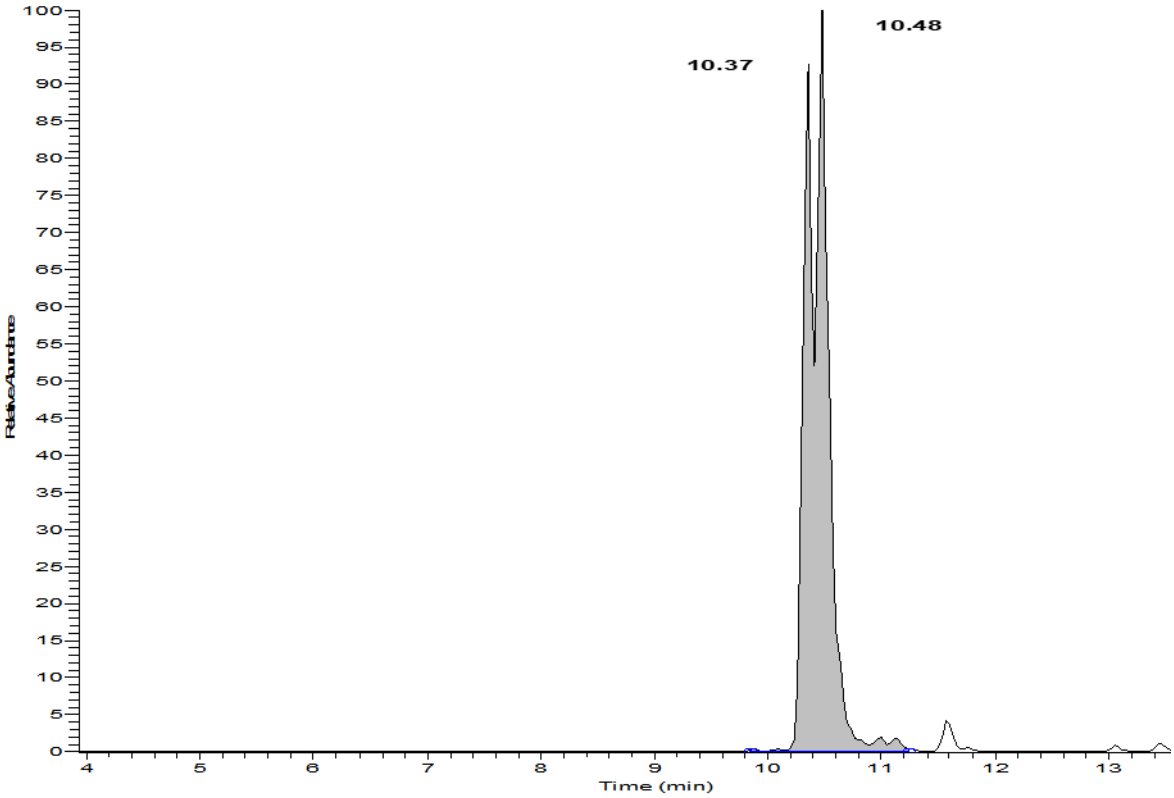
Appendix

Appendix A: Signal intensities after derivatisation according to section 3.6.2 of anhydro-ecdysteroid-6-oximes and residual ecdysteroids (Derivatized Ecdysteroids) as well as signal intensities of same amounts of unreacted ecdysteroids (Untreated Ecdysteroids).

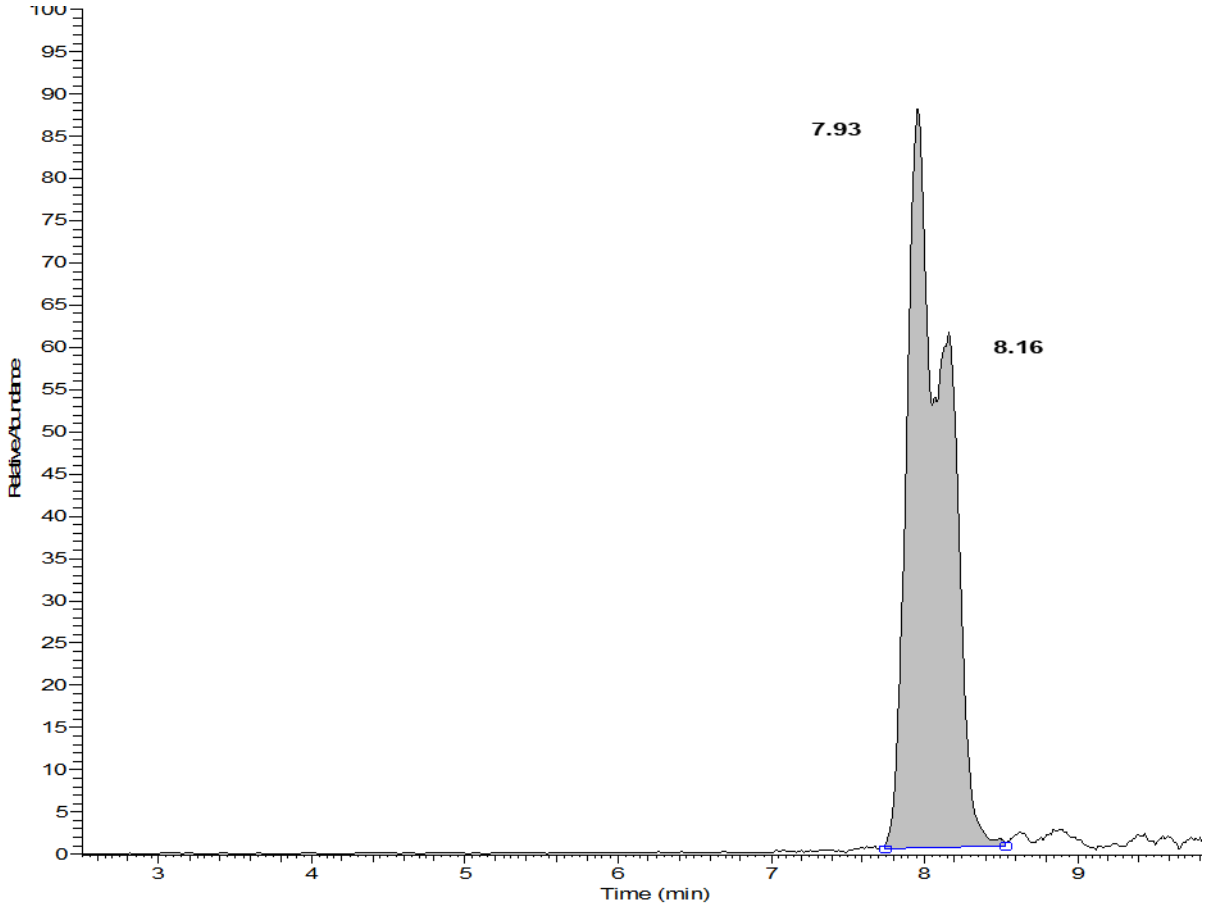
Derivatized Ecdysteroids			
Compound	**Peak Areas [arbitrary units]**	**Residual Compound**	**Signal Intensity [arbitrary units]**
14,15-anhydro-20-hydroxyecdysone-6-oxime	21062007	20-hydroxyecdysone	75619
	24901894		97424
Anhydro-makisterone A-6-oxime	26518819	Makisterone A	116580
	21273914		81421
Anhydro-polypodine B-6-oxime	22890902	Polypodine B	8231939
	31487120		9245329
Anhydro-taxisterone-6-oxime	20665726	Taxisterone	266504
	21630359		326236
Anhydro-ajugasterone-6-oxime	20980492	Ajugasterone	143226
	23168507		160720

Untreated Ecdysteroids	
Compound	**Peak Areas [arbitrary units]**
20-hydroxyecdysone	1524013
Makisterone A	802938
Polypodine B	10998599
Taxisterone	6810840
Ajugasterone	5877865

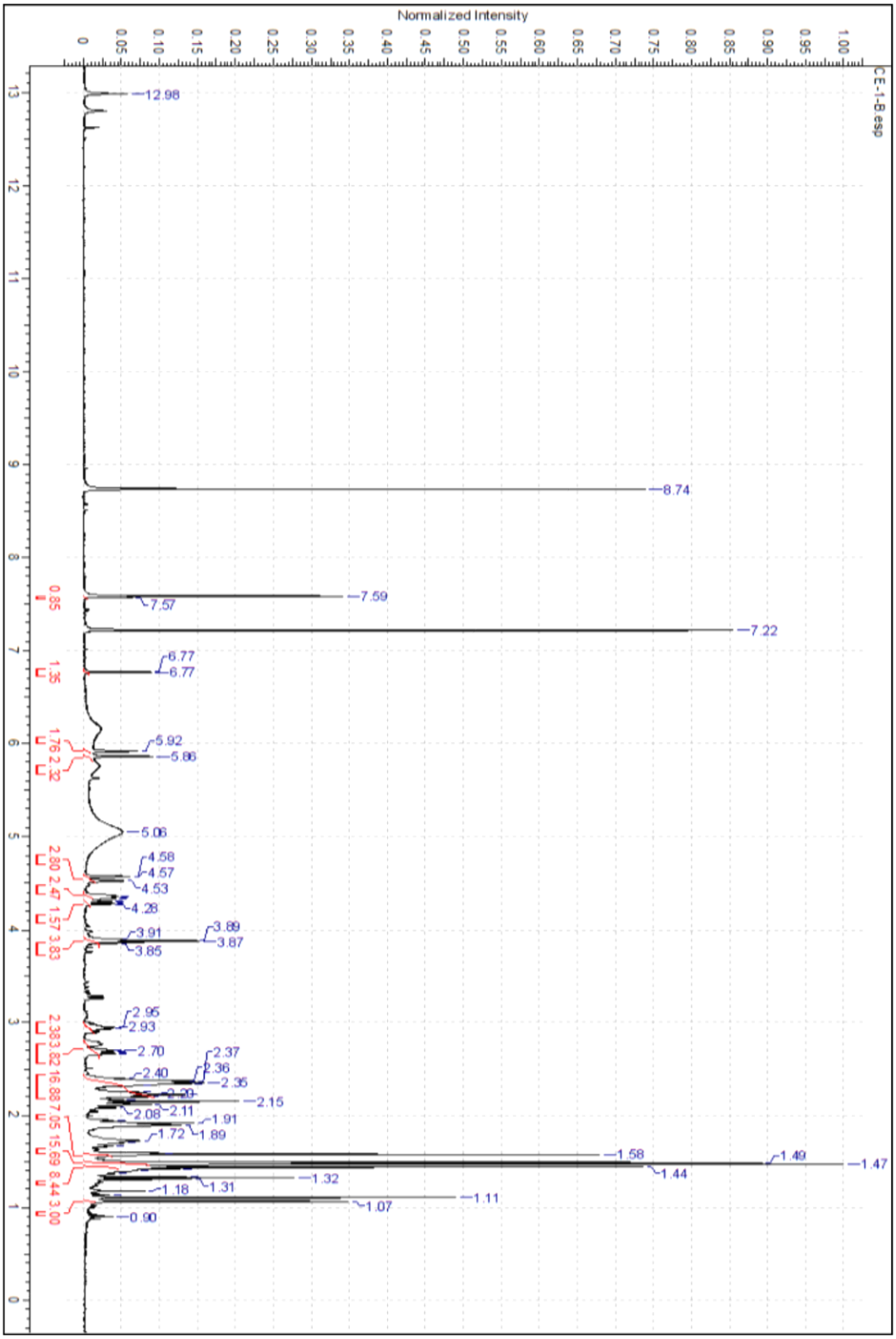
Appendix B: Chromatogram of the crude product (14,15-anhydro-20E-6-oxime) using gradient A on a Kinetex C18 column.



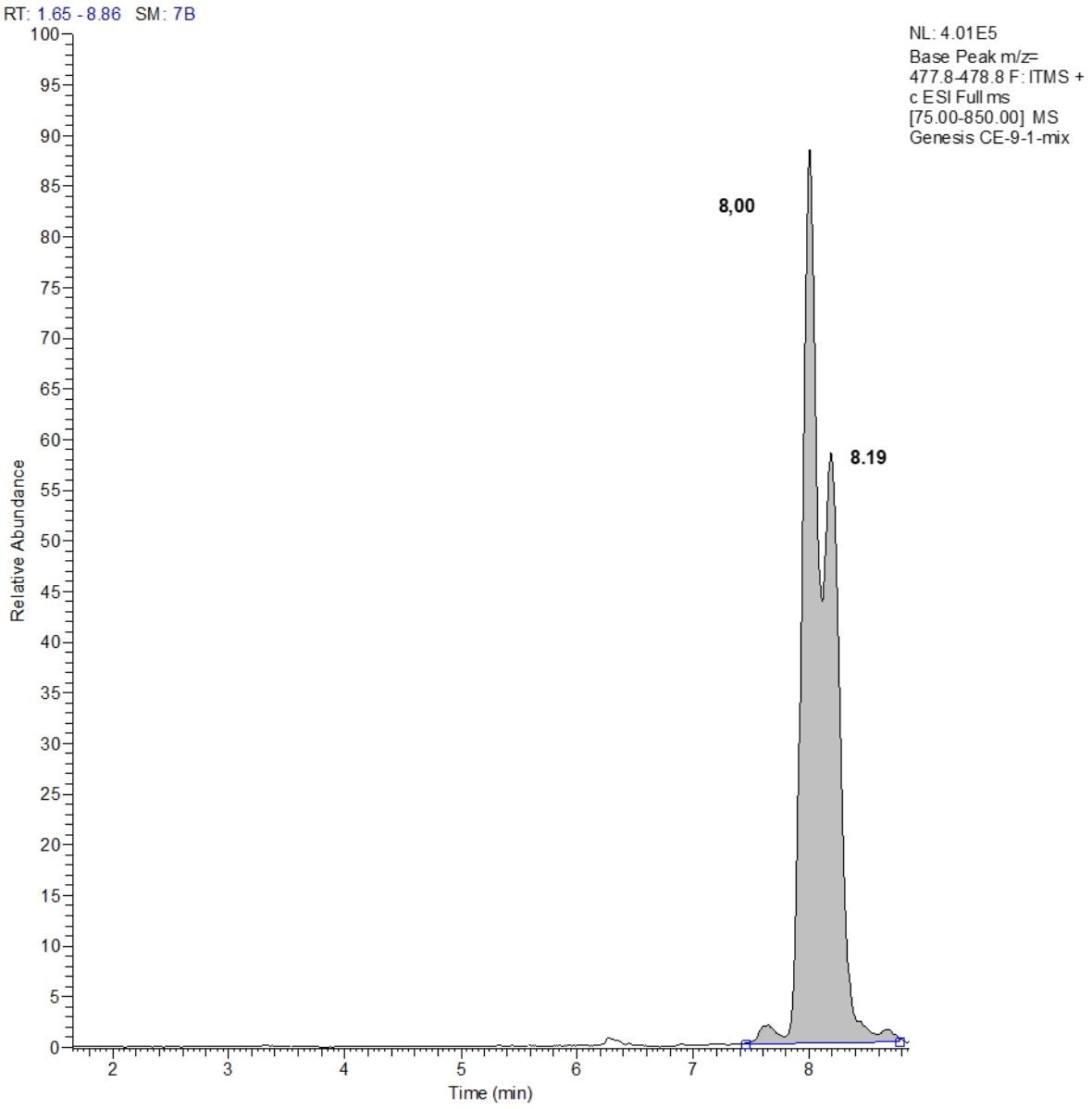
Appendix C: Chromatogram of fraction B (14,15-anhydro-20E-6-oxime) using gradient B on a Synergi Polar RP column.



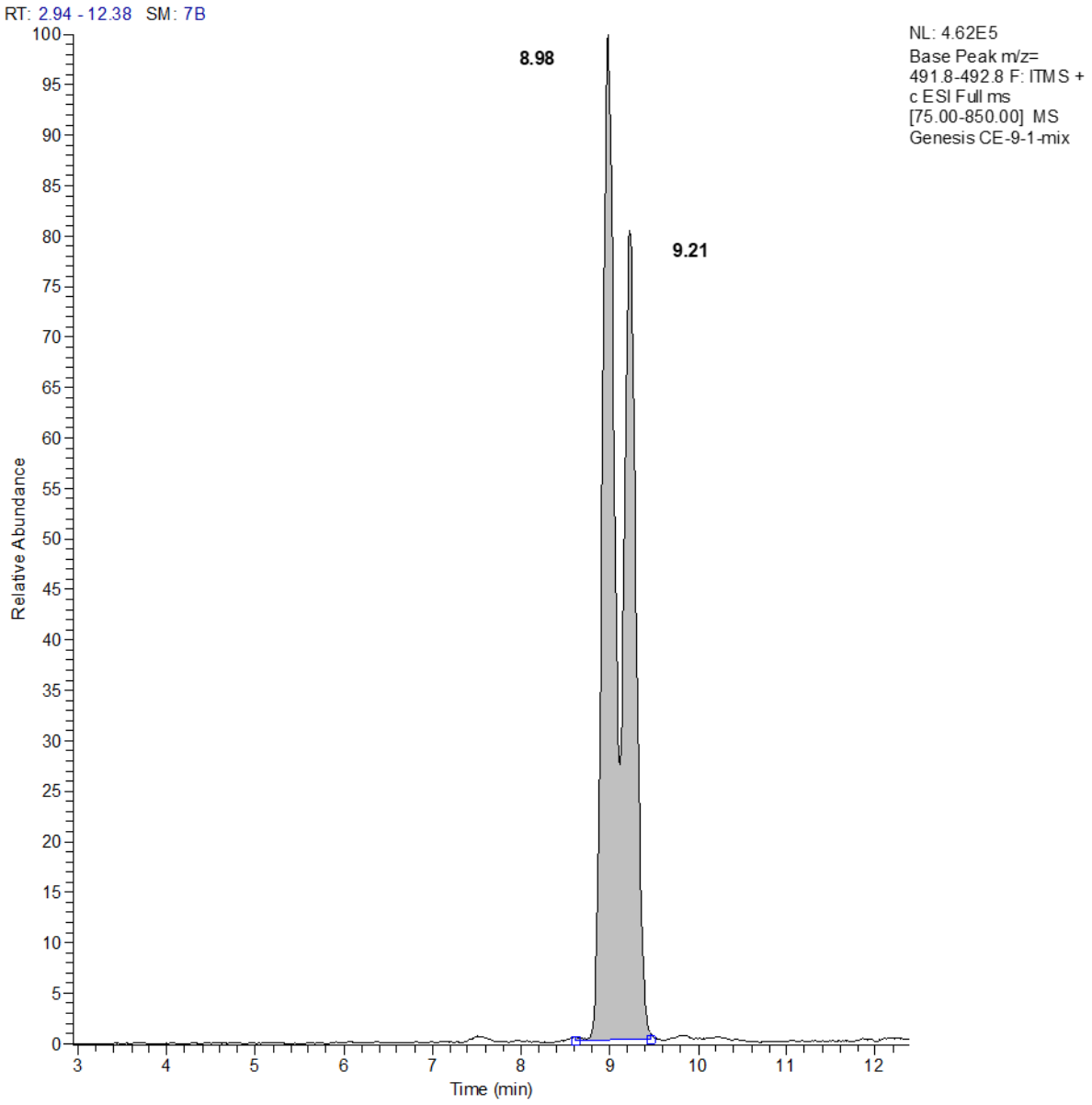
Appendix D: ^{1}H-NMR spectrum of 14,15-anhydro-20E-6-oxime (fraction B) (shifts in ppm).



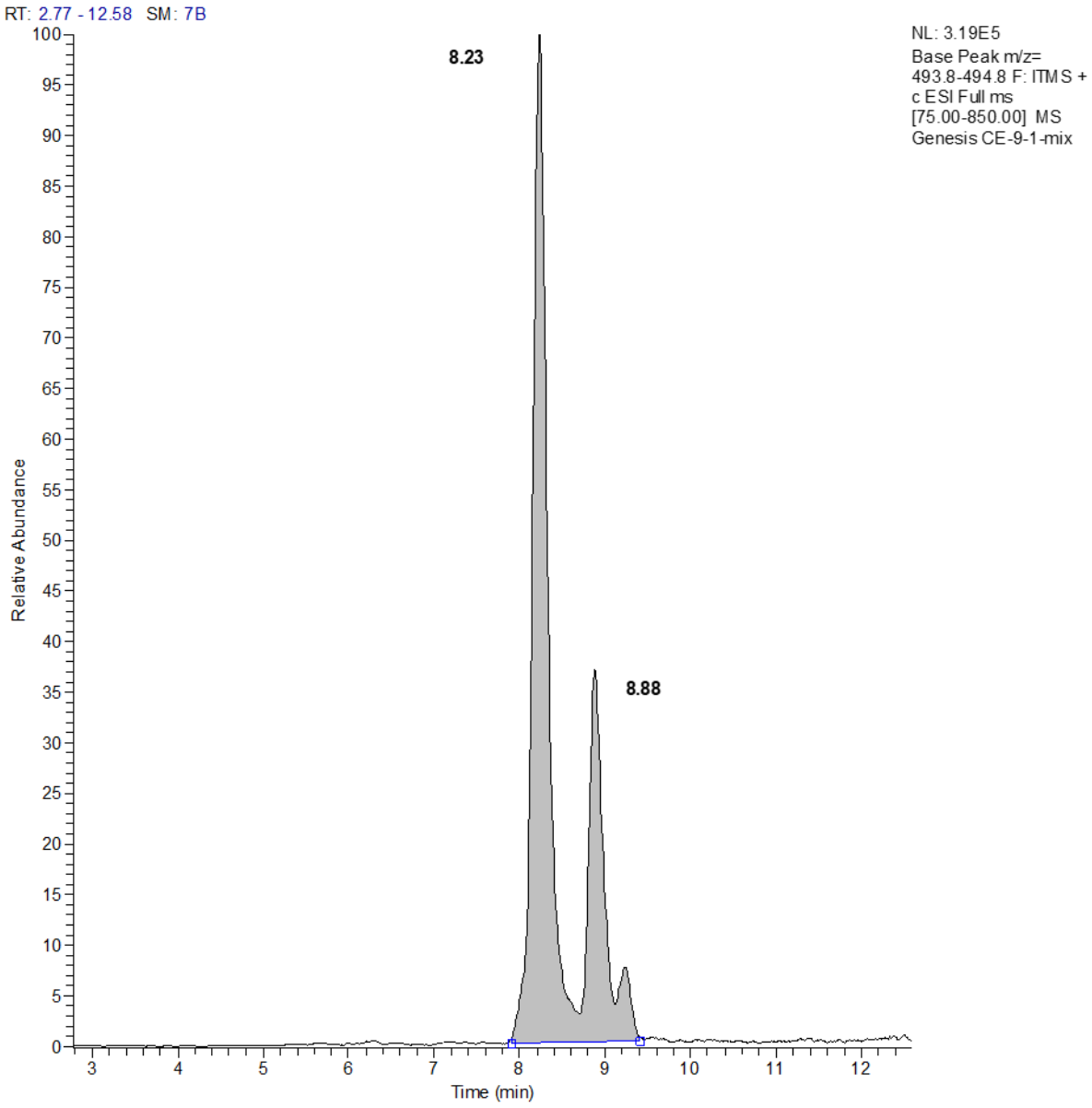
Appendix E: Chromatogram of *E*- and *Z*- 14,15-anhydro-20E-6-oxime (*Z* at 8.00 min, *E* at 8.19 min) separated on a Synergi Polar RP using gradient B.



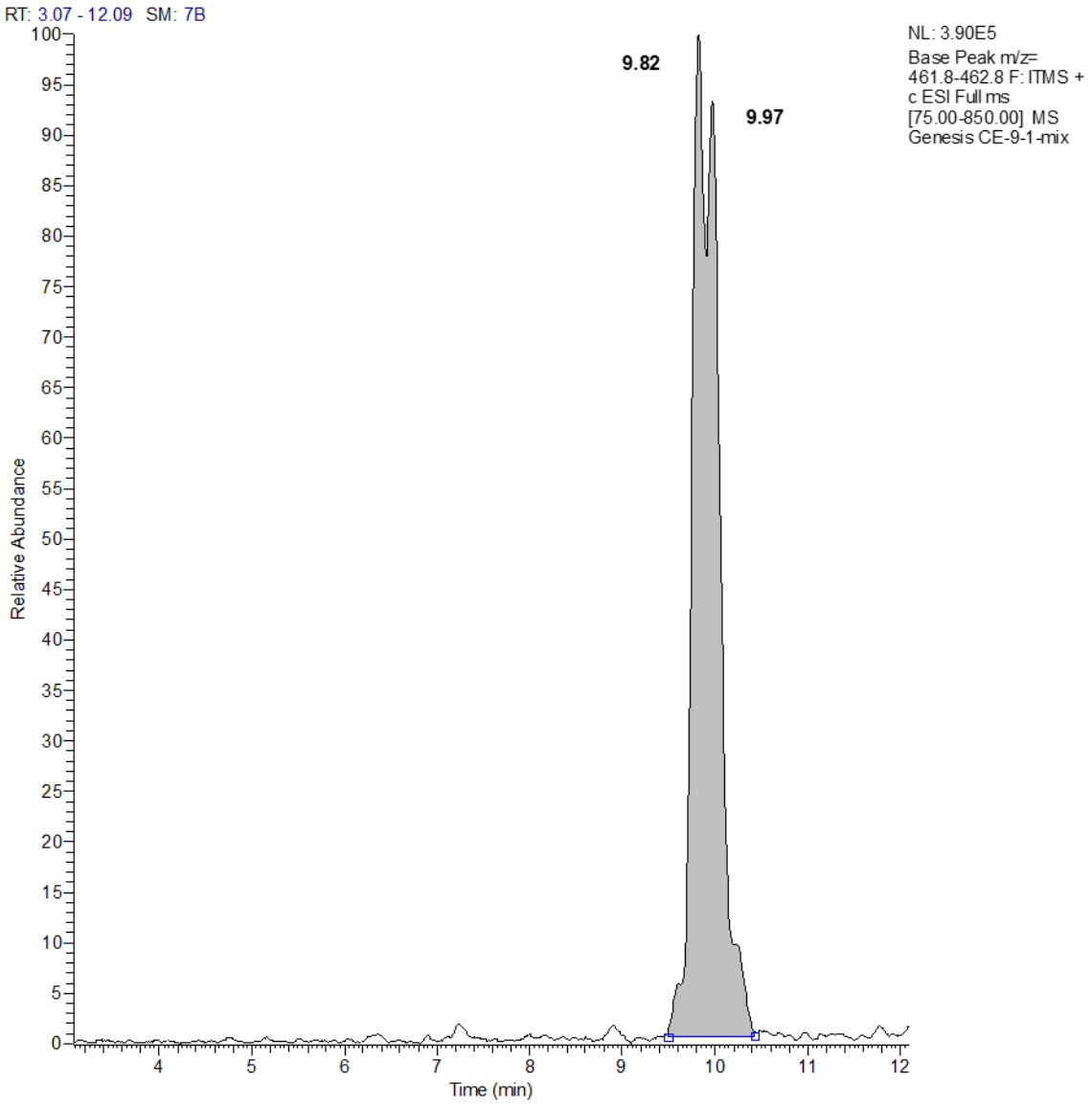
Appendix F: Chromatogram of *E*- and *Z*- anhydro-makisterone-6-oxime separated on a Synergi Polar RP using gradient B.



Appendix G: Chromatogram of *E*- and *Z*- anhydro-polypodine B-6-oxime separated on a Synergi Polar RP using gradient B.



Appendix H: Chromatogram of *E*- and *Z*- anhydro-taxisterone-6-oxime separated on a Synergi Polar RP using gradient B.



Appendix I: Chromatogram of *E*- and *Z*- anhydro-ajugasterone C-6-oxime separated on a Synergi Polar RP using gradient B.