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Quantitative Analysis of Steroids by ^{13}C NMR Spectroscopy

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QUANTITATIVE ANALYSIS OF STEROIDS BY ^{13}C NMR SPECTROSCOPY

by

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Steroids are usually analyzed quantitatively by using HPLC with ultraviolet detection and are confirmed by FTIR, GC-MS. Quantitative ^{13}C NMR method provide an alternative means to analyze steroids. Chromatographic separation and subsequent identification by match with a spectroscopic data base spectrum is not required for quantitative analysis of mixture components. Instead, equivalent information is obtained by creating subspectra from resonances with equivalent peak areas within a quantitative NMR spectrum. Techniques for eliminating Nuclear Overhauser Enhancement (NOE) effect is described.

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CHAPTER I

INTRODUCTION

Synthetic anabolic steroids were developed in 1930s to help rebuild body tissue and to prevent the breakdown of tissue during debilitating disease. Clinically, these agents are prescribed as replacement therapy in men with central or peripheral hypogonadism and in some adolescents with marked delay of pubertal development.

Anabolic steroids increase protein synthesis in skeletal muscles mass and reverse catabolic processes [1]. Because of these properties some athletes use anabolic steroids in an attempt to improve their strength, athletic performance and appearance.

Estrogenic hormones are often prescribed to hasten sexual maturation in females [2]. The widest use of estrogens is in the treatment of menopause, in which they supplement of natural estrogens. Estrogens are also used in the control of cancer of the prostate in the male. Female hormones include progesterone, estrogen and progestins, i.e., synthetic progesterone-like compounds which have no natural counterpart in the body. Their use includes a variety of conditions: functional uterine bleeding, absence of menstruation (amenorrhea) used at times with estrogens, painful menstruation (dysmenorrhea), infertility, habitual abortion in order to maintain pregnancy, and in fact, to suppress ovulation hence their use as antifertility drugs. Certain progestins such as norethindrone combined with an estrogen, are the principal components of birth control pills which suppress ovulation. Since there is no egg to fertilize, conception does not take place. Progesterones have been used as hormone

substitutes after hysterectomy and to treat dysmenorrhea, endometriosis, functional uterine bleeding, amenorrhea and habitual abortions [3].

However, these agents can cause serious side effects such as: symptoms of intolerance; disturbance of the excretory function of the liver; virilization in children and women; acceleration of skeletal maturation; antigonadotropic, antiestrogenic, or gestagenic properties of anabolic steroids in men or women; disturbances of water and electrolyte metabolism. Because of these serious side effects and questionable usefulness of products introduced in the 1960s, most of them are controlled and have been withdrawn from the U.S. market. The few remaining anabolic steroids are approved only for specific uses in treating serious illnesses, such as aplastic anemia or breast cancer, while some products are intended only for veterinary use.

In place of the limited availability of approved products, we have seen the appearance of a black market in anabolic steroids. This black market is sustained by the increasing demand for these products, which are used by athletes and body builders.

Law enforcement agencies have been attempting to stop the illegal distribution of anabolic steroids, many of which are mislabeled or improperly formulated. Investigations and legal actions require that the products be analyzed to determine their identity and potency.

Steroids

Steroids are the generic name for one of a group of substances which include the sex hormones, bile acids, saponins, vitamins, some cardiac drugs, certain constituents of the body, etc. In common usage, the steroids also include sterols, which are related substances containing the -OH group. Steroids and sterols are often found in association with fats and oils. Some of the more familiar steroids include the

following: aldosterone (a hormone of the adrenal cortex), androsterone (a male hormone), cholesterol (found in animal fats but not in vegetable fats), cortisone (used in shock and allergic conditions), ergosterol (a precursor of vitamin D), estriol (an estrogenic hormone), progesterone (a hormone produced in the ovary), and testosterone (a male sex hormone produced in the testicles) [2].

The anabolic-androgenic steroids are derivatives of testosterone, which is responsible for the anabolic and androgenic effects noted during male adolescence and adulthood. Androgenic effects are those that relate to the growth of the male reproductive tract or to the development of secondary sexual characteristics in men.

Anabolic effects are the changes that occur in the somatic or nonreproductive tract tissues and include an acceleration of linear growth that appears before bony epiphyseal closure, enlargement of the larynx and thickening of the vocal cords, the development of libido and sexual potentia, and finally an increase in muscle bulk and strength as well as a decrease in body fat. This androgen is also probably responsible for the increase in aggressive and sexual behavior, although its role in the psychological and behavioral aspects is controversial [2].

Steroids are compounds containing the cyclopentanoperhydrophenanthrene ring system, Figure 1. The three six-sided rings (A, B, C) constitute the phenanthrene nucleus to which is attached a five-sided ring (D), cyclopentane. The prefix "perhydro" refers to the fact that all the necessary hydrogen atoms have been added to the compound to make it fully saturated. This class of compounds includes such natural products as sterols (e.g., cholesterol), bile acids (e.g., cholanic acid), sex hormones (e.g., estrogens, androgens), and some alkaloids (e.g., solasodine). Cholesterol will be used to illustrate the numbering system for a steroid shown in Figure 2 and Figure 3 [4]. The most descriptive name for cholesterol is Δ^5-cholestene-3β-ol. The symbol Δ^5 indicates the presence and position of a double bond

between carbons 5 and 6, and the term 3β-ol indicates the presence and direction of orientation of the hydroxyl group. Identification of the positions of substitution in the

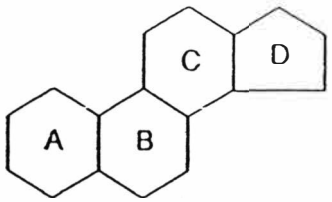


Figure 1. The Cyclopentanoperhydrophenanthrene Ring System.

sterol molecule is by means of the numbering system shown in Figure 2 for the structure of cholesterol. Figure 3 shows the conformational representation of cholesterol. In a sterol molecule, the substituents around a ring may lie above or below the plane of the ring. In the drawings, solid lines linking substituents to a ring refer to bonds above the ring plane, and dotted lines indicate substituents below the plane. In cholesterol, for example, the -OH group is above the plane; in this condition we speak of a β-oriented group (i.e., perpendicular to the plane of the rings). If the

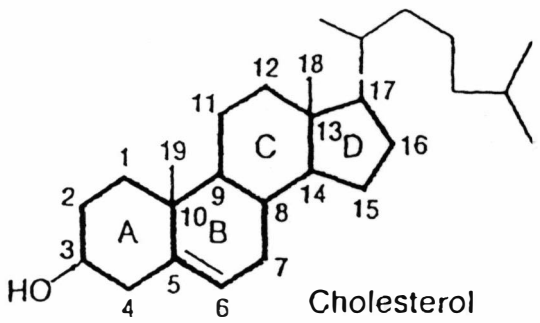


Figure 2. Ring Numbering System for Steroids.

group lies below the ring plane, it is termed α-oriented. In androgen, an unsaturated bond is at position 4 and 5, a ketone group at C-3, and a hydroxyl group (or -COOR)

in the β position at C-17. Functional groups on the β side of the molecule are denoted by solid lines; those on the α side are designated by dotted lines. Side

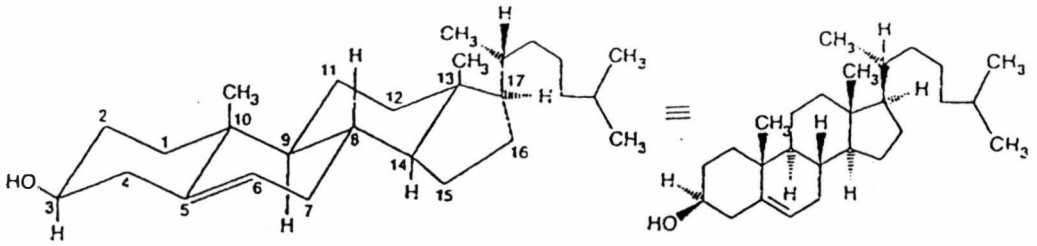


Figure 3. Conformation of Cholesterol

chains at position 17 are always β unless indicated by dotted lines or in the nomenclature of the steroid (e.g., 17α)).

Current Analytical Method

Established analytical methods are available for the legitimate products, but are not always applicable to the wide variety of different steroids, mixtures, and dosage forms found on the illegal market. Anabolic steroid products found in the illegal market are primarily oil-based injectables or tablets and often do not contain the ingredients declared on the label.

Qualitative and quantitative analyses of these steroids in their mixtures have been carried out with both chemical method and with physical methods. (In general, analysis of organic mixtures is a two-step procedure requiring isolation of mixture components followed by identification of the pure materials. Computer-assisted methods employing chromatographic separation, followed by detection using infrared or mass spectrometers, which provide information-rich spectra for library searches of

spectral data bases, are common. Currently, high performance liquid chromatography (HPLC) with an ultraviolet (UV) or diode array detector is the most popular method for the analysis of steroids and is usually confirmed with Fourier transform infrared (FTIR) or gas chromatography (GC)/ mass spectroscopy (MS) [5], [6].

For most of anabolic steroids, this method works well. A few of the steroids, for example, oxandrolone and mesterolone, have very low UV absorptivity because of lack of conjugation in their structures and poor solubility in the 90% methanol eluant used. They can be easily overlooked by HPLC screening. Some steroids slowly react with methanol in solution, like oxymetholone and it is the reaction product rather than the free oxymetholone that is seen as a symmetrical peak in liquid chromatography (LC). The oxymetholone molecule has a polar hydroxyl group that is attracted to the silanol sites on the LC column, resulting in poor chromatography.

Identity of the steroids is usually confirmed by direct insertion probe MS or KBr microdisk FTIR. Because the extracts obtained in the sample preparation procedures often contain more than one steroid, traces of oil, preservatives, and other excipients, they are usually not suitable for direct IR or MS analysis. Before identity can be confirmed, the components of interest must be isolated and purified by a semi-prep LC cleanup that consists of injecting large portions of the sample extract and collecting eluate fractions corresponding to the peaks of interest. Usually a single LC fraction collection run is sufficient for MS, but the process may need to be repeated numerous times for enough pure component to be collected for KBr microdisk IR analysis.

Qualitative and Quantitative Analysis by ^{13}C NMR

^{13}C nuclear magnetic resonance (NMR) spectroscopy could be an alternative

method for analyzing the anabolic steroids. Qualitative analysis of mixtures may be effected via pattern recognition techniques [7], [8]. As a tool for quantitative chemical analysis, NMR spectroscopy offers several important advantages, such as not requiring a chromophoric group, ease with which multicomponent mixtures can be analyzed directly, and its nondestructive nature.

Because of the importance of carbon in chemistry, ^{13}C NMR has been developed to a level where it now is possible to obtain ^{13}C spectra from micromoles of material. ^{13}C spectra are usually measured with broad band ^{1}H decoupling, resulting in a series of singlets. Often a well resolved singlet for each type of carbon in the molecule is observed. One advantage of the low natural abundance of ^{13}C is that ^{13}C-^{13}C pairs are rare and their couplings are not observed with unenriched samples. The probability that two ^{13}C nuclei exist in the same ^{13}C(unenriched) molecule is low, e.g. about 1 in 10^4 for any specified pair of positions in the molecule. These spin interactions between ^{13}C nuclei are not observed. The ^{13}C spectra obtained under heteronuclear decoupling conditions (in this case ^{13}C-^{1}H) consist of singlets. For example, a proton-decoupled 25.1 MHz ^{13}C spectrum of cholestane (which contains 27 carbon atoms) exhibits 26 resolvable resonances; the chemical shift range for the resonances of these saturated carbons present in the molecule is 45 ppm. The same molecule when examined at the corresponding proton frequency (100 MHz) yields only five decipherable signals in the proton spectrum, and all proton resonances are compressed within the chemical shift range of 2 ppm. These results clearly indicate that ^{13}C NMR spectroscopy is a much more sensitive probe for studying chemical shifts. Furthermore the mixtures of steroid injectables often can be analyzed without separation simultaneously.

Principles of NMR Spectroscopy

The nuclei of certain isotopes have an intrinsic spinning motion around their axes. The spinning of these charged particles, or their circulation, generates a magnetic moment along the axis of spin, Figure 4a. If the nuclei are placed in an external magnetic field, their magnetic moment can align with or against the field. The individual nucleus spins around its axis and precesses about the force line of the applied magnetic field. These precessions are actually circular movements with respect to the force line and are restricted to a distinct number of angles between the field line and axis, Figure 4b. The field aligns the spinning nuclei against the disordering tendencies of thermal processes. However, the nuclei do not align perfectly parallel (or antiparallel) to the imposed magnetic field. Instead, their spin

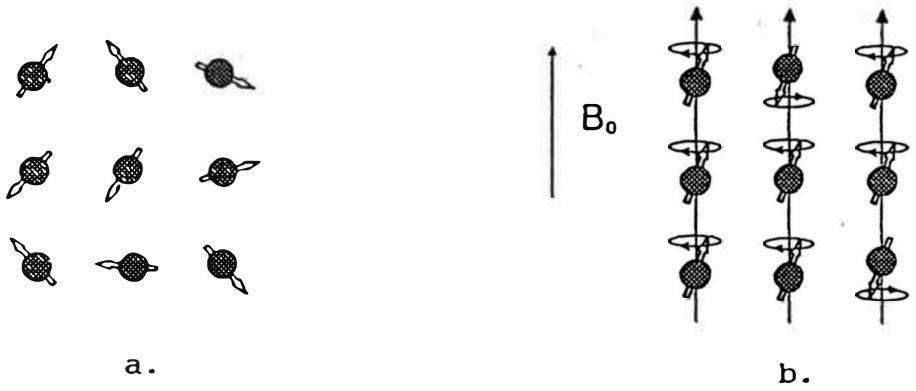


Figure 4. Nuclear Spins: (a) Without an External Magnetic Field, (b) in the Presence of an External Magnetic Field.

axes are inclined to the field and precess about the field direction, behaving like a gyroscope in a gravitational field. Each pole of the nuclear axis sweeps out a circular path in xy-plane. Increasing the strength of the field only makes the nuclei precess faster. The frequency of precession, v_0, is known as the Larmor frequency of the

observed nucleus.

At equilibrium the population of the various nuclear energy levels is predictable by use of a Boltzmann distribution

$$\frac{N_+}{N_-} = e^{-\frac{\Delta E}{kT}} \qquad (1)$$

where N_+ is the number of nuclei populating the high-energy state; N_- is the number of nuclei populating the low-energy state; ΔE is the energy difference between the two states; k is the Boltzmann constant and T is the absolute temperature. For a magnetic nucleus of spin 1/2 in a field of 1.409 Tesla, the distribution predicts a population ratio of 0.9999904 at room temperature. The lower energy level (orientation parallel to the applied magnetic field) is favored to the extent of approximately 9.5 excess nuclei out of every million. Thus, for a sample containing approximately 10^{19} nuclei, the effective participating population will be about 10^{14} nuclei.

A radio frequency (rf) magnetic field, H_1, is applied along the y-axis. As the frequency approaches that of the nuclear resonance frequency, there is increasing of rf field H_1 and the precessing magnetic moment. When the frequencies are identical, resonance absorption occurs and the nuclei "flip" from the lower energy level to an upper energy level, that is the spins originally precessing with H_0 flip over, and now precess against H_0. If the frequency of the rf field is swept through the region of the resonance frequency, peak absorption of energy from the rf oscillating field will be observed at the resonance frequency. Since there is a linear relation between resonance frequency and magnetic field, H_0, spectra may be expressed as intensity of absorption versus resonance frequency at fixed H_0, or against H_0 at fixed resonance frequency if the rf frequency is fixed and the magnetic field swept.

Pulsed FTNMR

The conventional continuous wave (CW) NMR spectrometer scans the spectrum at a slow rate in order to avoid passing over a spectral line too rapidly since the lines are usually narrow. The spectrometer spends most of its time recording background, only occasionally does it record the desired information. Efficiency and consequently the sensitivity of such a system is far from optimum. The time required to observe a NMR spectrum by the CW method is Δ/r, where Δ is the spectral width and r is the resolution desired. For ^{13}C at 25MHz, where delta is typically about 5 kHz and the line widths are about 1Hz, one must scan the 5-kHz region at a rate of 1 Hz/sec, or slower. This requires a minimum time of 5000 sec (or 83 min). To obtain good resolution, 100 scans are required to increase the S/N ratio. Thus, 83 x 100 = 8300 min=138 hr and 20 min. In other words, more than five days. It is obvious that this is not a feasible approach to the problem.

Fortunately, an alternative method has been developed e.g. pulse NMR. If a spectrum is thought of as a large number of small increments in frequency, each increment being just large enough to contain a typical spectral line, these increments can be examined simultaneously. This removes the constraint on scanning rate, since there is no scan.

Naturally the instrumentation must be modified appropriately to accomplish the simultaneous excitation of all the spectral lines and to sort the resulting information into the conventional representation of spectral lines. This is accomplished by applying, e.g. a strong pulse of rf energy (H1) to the sample for a very short time(1-1000 μsec). Under the influence of the pulse of rf energy, the magnetic moment spirals away from the z-axis in the direction of the static field. If the pulse is of the proper strength and duration, the magnetic moment is tipped by 90° and comes to rest

in the xy-plane. After the pulse has terminated, the restoring torque of the static field, HO, causes a precession around the z-axis at the resonance frequency. The free precession of the nucleus under the influence of only the static field induces a decaying sinusoidal voltage in a coil of wire surrounding the sample. The voltage decays partly because the nuclear magnetic moment vector slowly spirals back up to its original position with an exponential time constant, T1, the spin-lattice relaxation time. After several time constants (3T1 to 5T1), the nuclei will have regained equilibrium and a second pulse can be applied to repeat the process. Another reason for the decay of the free induction signal results from magnet inhomogeneity over the entire sample volume. Thus, some otherwise identically precessing nuclei begin to precess at slightly different rates and slowly lose phase coherence. Here we are speaking of the spin-spin relaxation time constant, T2. The free induction decay (impulse response) and the conventional continuous-wave display (steady state equilibrium) of the NMR spectrum form a Fourier transform pair. That is, the time response of the spins can be calculated from their frequency domain spectrum, and vice versa. The response of the entire spin system is picked up in the normal manner, amplified, and detected in the spectrometer. The free induction decay signal following each repetitive pulse is digitized by a fast analog-to-digital (ADC) converter, and the successive digitized transient signals are coherently added in the computer until an adequate signal-to-noise ratio is obtained. Using the Cooley-Tukey algorithm, a computer then performs a fast Fourier transformation to the frequency domain to plot a normal spectral presentation of the NMR absorption versus frequency in a matter of 10-20 sec. Due to chemical shielding, each nucleus may resonate within a range of Larmor frequencies, depending on the chemical environment. In order to rotate all nuclear spins within that range by the same angle, the strength of the rf pulse must meet the following requirement: the pulse width, t_p, must be much shorter than the relaxation times, that is $t_p << T_1, T_2$, so

that relaxation is negligible during the pulse [9].

Nuclear Overhauser Enhancement Effect (NOE)

In an ordinary ^{13}C NMR experiment, broad band decoupling technique is used for removing proton splitting from ^{13}C NMR spectra. Although decoupling increases the sensitivity of NMR experiments because the intensities of all multiplet lines in a coupled spectrum are accumulated in one singlet signal in the decoupled spectrum, the intensity of the ^{13}C signal often increases much more than expected. This effect is called Nuclear Overhauser Enhancement effect (NOE). The NOE effect arises from an intramolecular dipole-dipole relaxation mechanism. In an ^{13}C-^{1}H decoupling experiment, the transitions of ^{1}H are irradiated while the resonance of ^{13}C nuclei are observed. Since the irradiating field is very strong, the homonuclear relaxation processes are not adequate to restore the equilibrium population of 1H nuclei, and these nuclei transfer their energy to the ^{13}C nuclei via internuclear dipole-dipole interaction. The carbon nuclei receiving these transferred amounts of energy, behave as if they had been irradiated themselves and relax. Consequently, the population of the lower energy level of ^{13}C increases, and the intensity of the carbon signal is enhanced [10].

Quantitative Analysis

It is well known that quantitative analysis with ^{13}C NMR is difficult to perform as a result of several factors. The most important of these are differential relaxation and Nuclear Overhauser Enhancement effects. Both of these may effect carbon signal intensity, such that carbon concentrations are not accurately obtained from the spectrum. Apart from this potential problem, ^{13}C NMR spectroscopy is

ideal for quantitative analysis since chemical shifts are well resolved under proton-decoupling conditions and the shift range is large. This usually leads to a favorable situation where there are always some signals well removed from any interference.

Two methods for quantitative analysis have been suggested. The first method involves the addition of 0.1 M $Cr(AcAc)_3$ or $Fe(AcAc)_3$ which is called a "shiftless shift reagent" or "relaxation agent" to the solution, and using a short pulse delay and gated decoupling. The relaxation agent quenches the Nuclear Overhauser Effect for all carbons and shortens all T_1 values enough so that pulses can be repeated at about 1-sec intervals. It should be noted that any method that relies on chemical agents may yield erroneous results due to selective relaxation or shielding of the agent from the center of interest. Paramagnetic relaxation reagents, in addition to shortening the relaxation times of carbons not bonded to hydrogen, will also suppress the NOE for carbons bonded to hydrogen.

The second method involves a long pulse delay ($>5T_1$) and gated decoupling. A gated decoupling sequence can be used to eliminate the NOE while still decoupling the protons; however, this technique requires long delay times between pulses, and the process can be quite time-consuming. If the NOE is completely suppressed, quantitative integration of peaks in the ^{13}C NMR spectra can be performed.

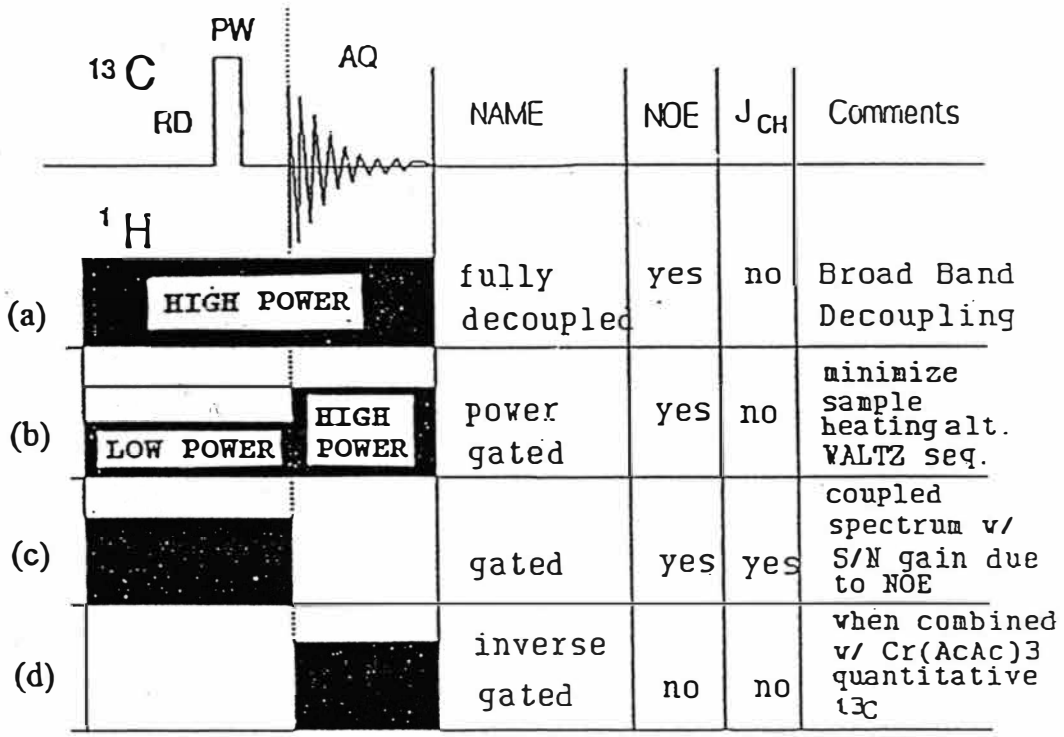
In this project both techniques for eliminating NOE effects were investigated. The use of relaxation reagents can help when one is dealing with compounds having long relaxation times. This reagent shortens relaxation times and allow faster acquisition rates. Paramagnetic (or relaxation) reagent methods employ 0.1 M chromium acetylacetonate $Cr(AcAc)_3$ or $Fe(AcAc)_3$ solution. This technique reduces the NOE factors of all ^{13}C nuclei uniformly toward unity and permits a shorter recycle time by shortening the T_1 values. [9]

Gated-decoupling techniques are also necessary for optimizing ^{13}C

quantitative determinations. To obtain ^{13}C NMR spectra, the following decoupling technique are used for different purposes. The decoupling sequences are shown in Figure 5.

Broad-band ^{1}H-^{13}C Decoupling

The proton decoupler is on during the whole period (Figure 5a). NOE is retained and coupling is eliminated. This technique is used for qualitative analysis.



RD = relaxation delay; PW = pulse width;
AQ = acquisition

Figure 5. NMR Decoupling Techniques.

Power gated-decoupling

The proton decoupler is on with low power during the relaxation delay and pulse period and high power level during the acquisition period (Figure 5b). The NOE is maintained and coupling is eliminated.

Gated-decoupling

In this method, the proton decoupler is on during the first period which is relaxation delay and pulse process, and is gated off during the second period-the acquisition process. Both NOE and ^{13}C-$\{^{1}H\}$ coupling result in the spectra (Figure 5c). Power gated-decoupling and gated-decoupling techniques are used in NOE study for obtaining the structure data and are also used in 2D spectroscopy.

Inverse-gated-decoupling Technique

The proton decoupler is gated off during the relaxation delay and pulse period, and is on during the acquisition period (Figure 5d). By this method, neither NOE nor ^{13}C-$\{^{1}H\}$ coupling are observed in the ^{13}C spectra. This is due to the fact that the multiplets collapse instantaneously and the NOE requires a time of the order of T_1 to build up. Figure 6 shows the pulse sequence employed. When combined with shiftless shift reagent ($Cr(AcAc)_3$, quantitative analysis can be performed [11].

CHAPTER II

EXPERIMENTAL PROCEDURE

The NMR spectrometer used in these studies was purchased in 1987 from IBM Instruments, Inc. as a model NR/200AF. This instrument was actually an AC 200 made by Bruker Instrumenets. In 1988 IBM sold all their NMR instrument interests to Bruker. Thus, the instrument is now considered to be a Bruker AC 200 NMR spectrometer. This instrument was used to obtain all of the ^{13}C spectra at 50.327 MHz. The AC 200 spectrometer employs an internal deuterium lock system. The NMR samples were contained in 5-mm sample tubes. In all experiments, deuteriochloroform ($CDCl_3$) was used for lock stabilization and as solvent.

The ^{1}H-decoupled ^{13}C FT NMR experiments were performed with inverse-gated heteronuclear decoupling program provided by IBM Instruments, Inc. In this mode, the proton decoupler is on only during the acquisition period and is gated off for a much longer time interval. All experiments were completed within 13 hour. Data acquisition time was 3 seconds after each of 2500 pulses (90^{o} pulse width) with a time delay of 3 seconds between the end of data acquisition and the beginning of the next pulse. In all exepriments, the flip angles used were 90^{o} rf pulses. A total of 2500 free induction decays were collected and time-averaged at $21^{o}C$ in each ^{13}C NMR experiment. The sample tubes were sealed by glue to prevent loss of volatile components and cooling air is on to prevent the sample from overheating. The phase correction and integrations were performed with the standard software provided by Bruker Instruments. Normal ^{13}C acquisition parameters were used as shown in Table 1.

A program PRODEC listed in Table 2 was used for inverse-gated decoupling.

Table 1

NMR Spectrometer Parameters

spectrometer frequency:	50.323
synthesizer frequency:	50.323
observe frequency offset:	6534.000
decoupling frequency offset:	3154.530
data size:	32768 K (1024 points per K)
time domain:	32768 K
spectral width:	18518.518
digital resolution:	1.13
pulse width:	8
relaxation time:	3.000
receiver gain:	320

Table 2

NMR Program (PRODEC) for Inverse-Gated Decoupling for Heteronuclear ^{1}H-decoupled without NOE [12]

1 ZE	Zero memory.
2 D1 BB DO	Set decoupler to BB mode, power DP gated off during D1.
3 GO=2 BB	Turn on BB decoupling simultaneously (RD=0) with PW pulse, acquire data, loop to 2 DS+NS times.
4 DO	Decoupling gated off.
5 EXIT	Exit.

Tris(acetylacetonato)chromium(III) ($Cr(acac)_3$) was used as the relaxation reagent. The NMR samples were prepared by carefully weighing out quantities of the sample and $Cr(acac)_3$. The mixture were then dissolved in a known amount of $CDCl_3$ (deuterochloroform).

Reagents

Steroids: (a) 19-Nortestosterone 17-Decanoate (Nandrolone Decanoate), Sigma Chemical Company; (b) Testosterone (17β-hydroxy-4-androsten-3-one 17-cyclopentylpropionate), Sigma Chemical Company; (c) 6α-methyl-17α-hydroxy-progesterone acetate (Depo-provera) 95%, Sigma Chemical Company; (d) Oxymetholone, U.S. Food and Drug Administration, Detroit District; and (e) Methyltestosterone, Sigma Chemical Company.

Excipients: (a) Methyl 4-Hydroxybenzoate 99%, Aldrich Chemical Company, Inc; (b) Benzyl Benzoate, Aldrich Chemical Company, Inc; (c) Benzyl Alcohol, A.C.S. grade, Aldrich Chemical Company, Inc; and (d) Propyl 4-Hydroxybenzoate 99+%, Aldrich Chemical Company, Inc.

Solvents: Chloroform-d, 99.8 Atom % D, with 0.03% (v/v) Tetramethylsilane (TMS), Aldrich Chemical Company, Inc.

Standard: Toluene, A.C.S. grade, Aldrich Chemical Company, Inc.

Preparation of Standard Solutions

Standard solutions were prepared by adding an appropriate amount of standard reagent to a series of vials containing a known amount of $CDCl_3$. Solid $Cr(AcAc)_3$ was also added to make the solution 0.1 M in Cr^{3+}. Toluene was also added as internal standard. For each sample the moles of toluene was approximately equal to that of the steroid added. Sample vials were stored in a refrigerator. Sample tubes were sealed before obtaining their spectra in order to prevent loss of volatile components. The composition of standard solutions are shown in Table 3.

Table 3

Composition of Standard Solutions

SOLUTION CONSTITUENTS	
Testosterone 17β-cypionate	10.0-151 mg
Toluene	15.4-88.5 mg
$CDCl_3$	1.5-2.2 g
$Cr(acac)_3$	42-50.9 mg
Oxymetholone	ca. 25 mg
$CDCl_3$	1-3.4 g
17α-methyltestosterone	15-130 mg
$Cr(acac)_3$ Toluene	47.1-54.2 mg
$CDCl_3$	9.1-43.2 mg
	2.256-2.291 g
Medroxyprogesterone Acetate	41.7-120.1 mg
$Cr(acac)_3$	52.6-53.2 mg
Toluene	13.4-31.7 mg
$CDCl_3$	1-1.03 g
Nandrolone Decanoate	ca. 23.4 mg
$Cr(acac)_3$	ca. 33.5 mg
$CDCl_3$	0.86-3.2 g

The following procedure [12] was used to obtain the quantitative spectra and integration value for each steroid.

Exponential Multiplication (EM)

Multiplying the FID by an exponentially decaying function weighs the initial portion, which contains the most significant information, more heavily than the tailing portion, which consists mostly of noise. Exponential multiplication enhances the sensitivity (signal-to-noise ratio), but at the expense of resolution since the lines are broadened. EM is not really necessary in most 1H measurements, because the S/N

ratio is good. With ^{13}C, the signal/noise ratio is not as high as in ^{1}H measurements, so EM was used for all spectra.

Fourier Transformation of the Spectrum

The FID is a combination of all of the sine waves whose frequencies and amplitudes correspond to the lines in the NMR spectrum. The time domain FID signal is converted into the frequency domain NMR spectrum by the Fourier transformation (FT).

Phase Correction

The result of the Fourier transform can be recognized as a spectrum, but the phase of lines may not be correct. The spectrum must be phase corrected. Automatic phase correction (PK) command was used for all spectra. Due to, for example, differences between sample tubes, sample compounds, concentration effects, etc., slight variations in the phase correction may occur after PK has been used. In order to optimize the automatic phase correction, an Add Phase subroutine was used in the expansion routine.

Baseline Correction

Baseline correction is critical for quantitative spectra. In order to make all spectra comparable and consistent, fully automatic baseline (FAB) command was used for baseline correction for all spectra. This command picks the peaks in the spectrum, defines the baseline, and fits a third-order polynomial function to it automatically. It then subtracts this function from the spectrum to give a more level baseline.

Integration

Integration was made manually for each peak in spectrum. In the expansion mode, each peak was expanded to fill approximately 2/3 of the screen in width. The integral values were recorded accordingly in integration mode for each peak.

CHAPTER III

RESULTS AND DISCUSSION

Identification of Mixture Components

The separation of components by ^{13}C NMR depends on the equivalent response obtained for all carbon nuclei under quantitative NMR conditions. Under these conditions, peak areas for all resonances within a molecule must equal some integer multiple of the area corresponding to a single nucleus. The minimum number of components in a mixture then can be determined from the quantitative spectrum by counting the number of peak subsets with dissimilar peak intensities [13].

The well-defined relationship between ^{13}C NMR data and chemical structure is ideally suited to the development of computer-aided identification schemes. The Library search algorithms [14-16], pattern recognition techniques [7],[8] and spectral simulation [17-19] have been well developed for interpretating ^{13}C NMR spectra of mixture components. In this project, a library of spectra can be established for all of the steroids of interest. A pattern recognition program may also be developed for qualitative and quantitative analysis purposes. Since qualitative analysis was not a objective of this study, pattern recognition techniques will not be discussed.

A number of preliminary ^{13}C FT NMR experiments were carried out in order to establish the appropriate experimental conditions for this study.

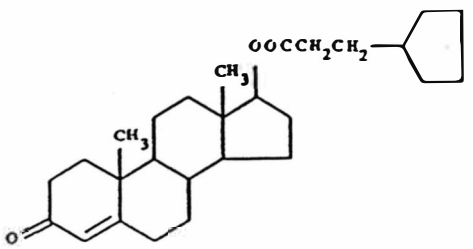
Investigation of Oil and Other Excipients

Steroids injectables are usually dissolved in oils, such as sesame oil, cottonseed

oil, and peanut oil, etc. ^{13}C spectra were obtained for thirteen oils, see appendix B. Comparison of these spectra, reveal their similarities. The peaks are ranged from 13-175 ppm. In this range, a singlet is shown at about 13.5 ppm, while a group of peaks is seen in the range from about 22-34 ppm. Two peaks are seen at 62 and 67 ppm, and another two peaks are shown at 127 and 129, and finally a singlet at 172 ppm. The characteristic peaks for other excipients include C-OH and COOR at about 65-75 ppm and 160-175 ppm respectively.

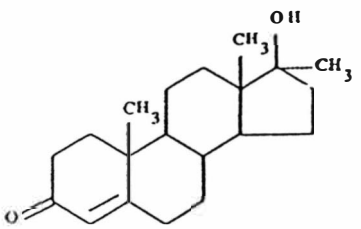
Structure of Steroids and Interpretation of Their Spectra

The structure of steroids studied and their characteristic chemical shifts are listed as follow:



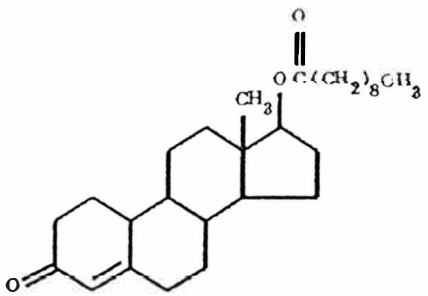
TESTOSTERONE 17-β-CYPIONATE

C-3	198	ppm
C-5	173	ppm
COOR	169.7	ppm
C-4	123	ppm
C-17	81	ppm



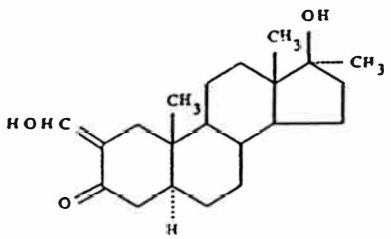
17 α- METHYLTESTOSTERONE

C-3	199	ppm
C-5	171	ppm
C-4	123	ppm
C-17	81	ppm



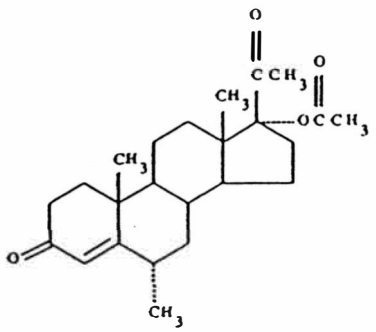
NANDROLONE DECANOATE

C-3	200	ppm
C-5	174	ppm
COOR	167	ppm
C-4	123	ppm
C-17	81	ppm



OXYMETHOLONE

C-3	188	ppm
CHOH	184	ppm
C-2	107	ppm
C-17	81	ppm



MEDROXYPROGESTERONE ACETATE

C-3	202	ppm
C-20	198	ppm
C-5	172	ppm
$COOCH_3$	169	ppm
C-4	119	ppm
C-17	95	ppm

Preliminary Study

^{13}C NMR is not a particularly sensitive method when compared to many other spectroscopic techniques. The time averaging method was used for increasing precision, eg. signal-to-noise ratio (S/N), by taking advantage of a very basic and important difference between the NMR signal and noise [9]. In practice, one scan is taken and stored in a computer of average transients (CAT). The CAT stores the first spectrum; subsequent spectra are added to the sum of all previous ones. When the desired S/N is obtained, scanning is terminated, and the final spectrum is plotted on a X-Y plotter. The relationship of the S/N after n scans and S/N after one scan is described by the following equation:

$$(\frac{S}{N})_n = \sqrt{n}(\frac{S}{N})_1 \qquad \textbf{(2)}$$

where S is signal voltage, N is noise voltage, n is number of scans to be added together.

Based on equation 2, the desired S/N is proportional with square n, in other words, S/N increases as the number of scans increases. Figure 6 shows the effect of the number of scans on signal-to-noise ratio or standard deviation. As the scan numbers increase from 500 to 2500, the standard deviation decreases from ca. 0.18 to 0.06. This indicates the S/N increases. The standard deviation increased when the scan number exceeded 2500. The possible reason that causes the decreased S/N is the evaporation of the solvent. The standard deviations were obtained from 5 trials. 2500 scans were used in all subsequant experiments.

Table 4

Standard Deviations of Integrations as a Function of the Number of Pulses

Number of pulses	Standard Deviation
500	0.185
1000	0.118
2000	0.112
2500	0.065
4000	0.117

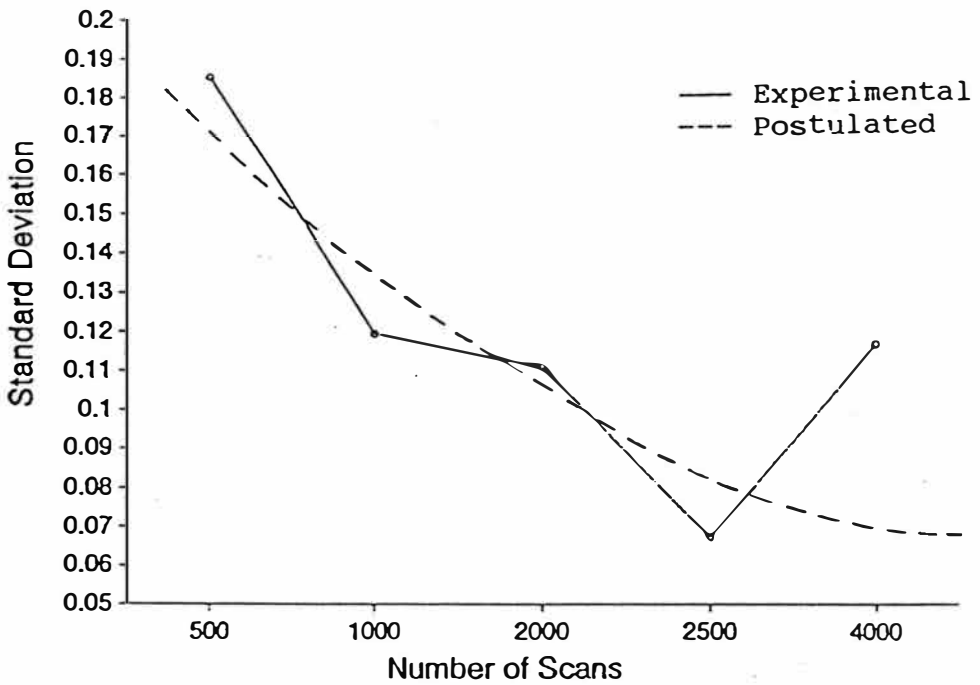


Figure 5. Standard Deviation of Integrals as a Function of the Number of Pulse.

Calibration Plots

Calibration plots were or each peak of all five steroids in order to determine

the linearity of the concentration and the peak area. These plots are shown in appendix C. The correlation coefficients are shown in Table 5. The peak areas were linearly dependent on concentration.

Table 5

Correlation Coefficient

testosterone 17β-cypionate		oxymetholone		medroxyproge-sterone acetate		17α-methyl-testosterone		nandrolone decanoate	
δ (ppm)	C.C.	δ (ppm)	C.C.	δ (ppm)	C.C.	δ (ppm)	C.C.	δ (ppm)	C.C.
198.7	0.9970	188.3	0.9783	202.4	0.9909	198.9	0.9908	200.0	0.9960
173.2	0.9980	183.9	0.9087	198.0	0.9990	170.7	0.9989	173.8	0.9960
170.0	0.9967	107.8	0.7469	172.1	0.9989	123.0	0.9967	166.7	0.9970
123.4	0.9949	81.8	0.1480	169.0	0.9999	80.6	0.9965	124.5	0.9970
81.6	0.9901	53.5	0.9823	119.9	0.9891	53.0	0.9776	82.1	0.9990
52.9	0.9881	50.6	0.9823	95.2	0.9913	49.3	0.9843	49.3	0.9990
49.5	0.9910	45.5	0.9345	51.9	0.9984	44.5	0.9974	42.5	0.9920
41.7	0.9814	40.6	0.9992	49.5	0.9747	37.9	0.9980	40.1	0.9930
38.9	0.9920	39.0	0.9963	45.3	0.9994	35.6	0.9925	36.3	0.9880
37.8	0.9862	37.5	0.9996	39.5	0.9962	34.9	0.9984	35.3	0.9960
35.9	0.9942	36.4	0.9963	37.4	0.9895	33.2	0.9998	34.4	0.9920
35.0	0.9954	35.5	0.9769	34.5	0.9826	32.0	0.9927	31.7	0.9940
34.6	0.9960	31.4	0.9814	34.0	0.9931	30.8	0.9989	30.5	0.9860
33.3	0.9957	28.1	0.9929	32.3	0.9963	25.0	0.9803	29.1	0.9840
31.9	0.9932	25.8	0.9793	29.6	0.9958	22.4	0.9952	27.3	1.0000
31.6	0.9949	23.3	0.9927	28.9	0.9958	19.8	0.9950	26.4	0.9910
30.6	0.9941	20.9	0.9998	24.9	0.9932	16.6	0.9871	25.9	0.9920
26.9	0.9914	13.9	0.9964	22.4	0.9954	13.2	0.9875	24.9	0.9950
24.4	0.9951	11.4	0.9961	19.4	0.9952			23.2	0.9950
22.7	0.9914			16.9	0.9956			22.5	0.9890
19.8	0.9922							13.9	0.9980
16.6	0.9890							11.9	0.9980

C.C.= Correlation Coefficient

Standard Addition Method

The amount of unknown present is calculated by

$$W_{unk} = W_{std} \times \frac{N_{std}}{N_{unk}} \times \frac{M_{unk}}{M_{std}} \times \frac{A_{unk}}{A_{std}} \qquad (3)$$

In this expression, W_{unk} and W_{std} are the weights of the unknown to be calculated and of the standard taken, N_{unk} and N_{std} are the numbers of protons in the groups giving rise to the absorption peaks whose areas are A_{unk} and A_{std}, and M_{unk} and M_{std} are the molecular weights of the two compounds [20].

The analytical results for testosterone 17β-cypionate, medroxyprogesterone acetate and 17α-methyltestosterone are given in Table 6, 7&8. Four runnings were

Table 6

Analytical Results of Testosterone 17β-cypionate by Standard Addition Method

Testosterone 17β-cypionate δ 198.7 ppm Toluene δ 137 ppm					
No. of detns. (n)	added	avg. found (mg)	Std. dev.	C.V.	error (%)
1	150.9	154.7	5.0	3.2	2.5
2	131.2	139.0	7.6	5.5	5.9
3	114.0	120.9	7.1	5.9	6.0
4	100.7	113.0	4.2	3.8	12.2
5	80.0	80.6	8.3	10.3	0.8
6	40.3	46.2	10.6	22.8	14.7
7	20.7	22.8	4.3	19.0	10.3

performed for each trial. The singlet peak due to the C=O at about 200 ppm for each of the steroids was used for calculation. In this experiment, toluene was used as an internal standard. A well-resolved peak due to C-1 of the toluene at about 137 ppm was used for all of the calculations.

Table 7

Analytical Results of Medroxyprogesterone Acetate by Standard Addition Method

Medroxyprogesterone acetate δ 202.4 ppm Toluene δ 137 ppm					
No. of detns. (n)	added found (mg)	avg. Dev. (mg)	Std.	C.V. dev.	error (%)
1	41.7	41.9	2.5	6.0	0.5
2	60.1	58.7	2.3	4.0	-2.2
3	80.6	88.4	2.6	3.0	9.6
4	99.8	100.1	4.8	4.8	0.3

Table 8

Analytical Results of 17α-Methyltestosterone by Standard Addition Method

17α-methyltestosterone δ 202.4 ppm Toluene δ 137 ppm					
No. of detns. (n)	added (mg)	avg. found (mg)	Std. dev.	C.V.	error (%)
1	129.9	131.2	5.3	4.0	1.0
2	110.0	109.8	5.1	4.7	-0.3
3	103.6	104.6	1.6	1.5	1.0
4	94.3	96.4	5.8	6.0	2.2
5	81.0	77.1	3.7	4.8	-4.8
6	50.9	49.0	2.9	6.0	-3.8
7	31.0	31.0	5.0	16.1	0.0

Table 9 shows the results calculated from all of peaks of testosterone 17β-cypionate. In Table 9, the calculations for peaks 198.7, 173.2 and 170 ppm gave errors less than 2.5 %. Results from other peaks gave unsatisfactory high positive bias. The data shows that unprotonated carbons have better results than those of

protonated carbons. In theory, the maximum NOE factor, NOEF=1.99 (this theoretical limit depends on the observed and decoupled nuclear species). In molecules containing more than a few carbons (molecular weights >200) the NOE is usually

Table 9

Analytical Results of All Observed Peaks

Testosterone 17β-cypionate

steroid added 150.9 mg
toluene added 88.5 mg peak used 137.2 ppm

chemical shift (ppm)	avg. found (mg)	stds. dev. (mg)	C.V.(%)	Error (%)
198.7	154.7	5.0	3.2	2.5
173.2	151.5	7.7	5.1	0.4
170	152.2	3.5	2.3	0.9
123.4	206.0	27.7	13.5	36.5
81.6	242.2	1.9	0.8	60.5
52.9	208.5	16.0	7.7	38.1
49.5	210.8	12.8	6.1	39.7
41.7	139.9	6.3	4.5	-7.3
38.9	162.9	6.2	3.8	8.0
37.8	143.3	11.0	7.7	-5.1
35.9	256.9	5.8	2.3	70.2
34.95	266.3	6.6	2.5	76.5
34.6	243.8	19.2	7.9	61.6
33.3	254.3	8.2	1.6	68.2
31.9	279.2	13.1	4.7	5.0
31.6	368.8	25.7	7.0	144.4
30.6	235.0	38.4	8.2	55.7
26.9	262.5	8.3	3.1	74.0
24.4	321.1	23.9	7.4	112.8
22.7	239.1	21.8	9.1	58.4
19.8	249.6	28.6	11.5	65.4
16.6	173.4	15.8	9.1	14.9
11.1	148.0	15.8	10.7	-1.9

(but not always) complete for all protonated carbons. Nonprotonated carbons achieve NOEF values of 0.8 to ~2.0. [21] In steroids, the NOEF for unprotonated carbons is about 1.3 to 1.6 which is smaller than that of protonated carbons. The NOE is probably quenched for the nonprotonated carbons. However NOE is probably not fully quenched for the protonated carbons. This may explain the high positive error for the protonated carbons.

The reproducibility of the peak integrals in a single sample has shown that the precision of the integral measurement appears to be the major limiting factor in the overall precision of the assay.

CHAPTER IV

CONCLUSION AND SUGGESTIONS FOR FURTHER WORK

The results of the studies presented here indicate that accurate qualitative and quantitative analyses for steroids by ^{13}C NMR are possible. The NOE effect for unprotonated carbons can be quenched by using shiftless shift reagent $Cr(AcAc)_3$ along with the inverse gated decoupling technique. A 3-s time delay was employed between the end of each data acquisition and the beginning of the next pulse with the decoupler being turned on only during the acquisition. This condition is not satisfactory when protonated carbons are used for quantitative analysis. It should be possible to further eliminate the NOE effect of the protonated carbons with longer relaxation time. The analysis time is estimated at about 2 hours with very little sample preparation time.

An identification method is also needed to establish. A pattern recognition system of steroids of interests will be developed. Simulated libraries may also be constructed from structural data of steroids. A search program can be developed for identification.

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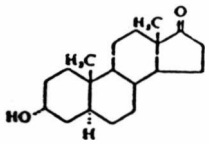
Appendix A

List of Steroid Hormones and Other Steroidal Synthetics

Names & synonyms: ANDROSTERONE;
cis-androsterone;
3α-hydroxy-17-androstanone;
androstane-3α-ol-17-one.

Formulae:

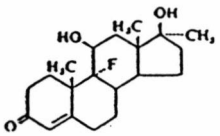


$C_{19}H_{30}O_2$

Molecular weight: 290.4
Melting point(oC): 185-185.5
Specific rotation: (α)15/D+85- +90 (150 mg. in 10 ml. dioxane)
Absorption max:

Names & synonyms: FLUOXYMESTERONE;
(α)-fluoro- 11 ß-hydroxy-17α-
methyltestosterone;
9α-fluoro-11ß,17ß-dihydroxy-17α-methyl-
4-androsten-3-one.

Formulae:

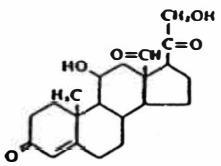


$C_{20}H_{29}FO_3$

Molecular weight: 336.4
Melting point(oC): 270
Specific rotation:(α)25/D+107- +109(alcohol)
Absorption max:240mμ(ε=16,700) alcohol

Names & synonyms: ALDOSTERONE;electrocortin;
18-oxocorticosterone;
18-formyl-11ß,21-dihydroxy-4-pregnene-3,20-dione.

Formulae:

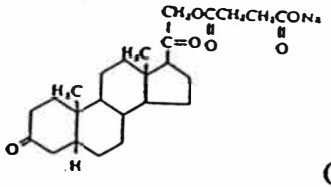


$C_{21}H_{28}O_5$

Molecular weight: 360.4
Melting point(oC): 108-112(hydra); 164(anhydrous)
Specific rotation: (α)25/d+161 (10 mg. in 10 ml. chloroform)
Absorption max: 240 mμ (log ϵ=4.20 monohydr.; ϵ mol. 15,000 anhydr.)

Names & synonyms: HYDROXYDIONE SODIUM;
21-hydroxypregane-3,20-dione-21-sodium hemisuccinate.

Formulae:

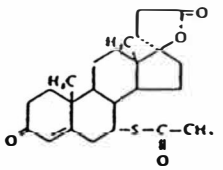


$C_{25}H_{35}O_6Na$

Molecular weight: 454.5
Melting point(oC): 193-203
Specific rotation: (α)25/D +95 (chloroform) for free acid.
Absorption max: 280 mμ (ϵ=93.2)

Names & synonyms: SPIRONOLACTONE;
3-(3-ono-7α-acetylthio-17ß-hydroxy-4-androsten-17α-yl)-propionic acid γlactone.

Formulae:

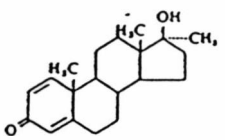


$C_{24}H_{32}O_4S$

Molecular weight: 416.5
Melting point(oC): 135(preliminary)-202
Specific rotation: (α)25/D-34 (chloroform)
Absorption max: ϵ^{238}=20,200

Names & synonyms: METHANDROSTENOLONE;
17α-methyl-17ß-hydroxy-1,4-androstadien-3-one.

Formulae:

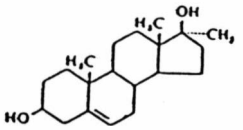


$C_{20}H_{28}O_2$

Molecular weight: 300.4
Melting point(°C): 166-167
Specific rotation:(α)20/D +9- +17 (100 mg. in 10 ml. alcohol)
Absorption max:

Names & synonyms: METHYLANDROSTENEDIOL;
MAD;
methandriol;
17α-methyl-5-androsten-3ß, 17ß-diol.

Formulae:

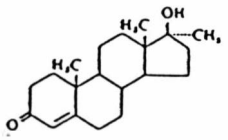


$C_{20}H_{32}O_2$

Molecular weight: 304.4
Melting point(oC): 205-207
Specific rotation: (α)20/D-73(100 mg. in 10. alcohol)
Absorption max:

Names & synonyms: METHYL TESTOSTERONE;
17-methyl testosterone;
17α-methyl-Δ4-androsten-17-ß-ol-3-one;
17(ß)-hydroxy-17(α)-methyl-4-androsten-3-one.

Formulae:

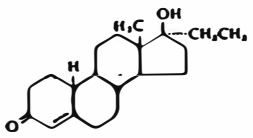


$C_{20}H_{30}O_2$

Molecular weight: 302.4
Melting point(oC): 161-166
Specific rotation:(α)25/D+69- +75 (100mg. in 10 ml. dioxane)
Absorption max:

Names & synonyms: NORETHANDROLONE;
17α-ethyl-19-nortestosterone;
17α-ethyl-17-hydroxy-4-norandrosten-3-one;
17α-ethyl-17-hydroxy-19-norandrost-4-en-3-one.

Formulae:

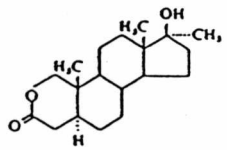


$C_{20}H_{30}O_2$

Molecular weight: 302.4
Melting point(oC): 130-136
Specific rotation: (α) 25/D +21 (dioxane)
Absorption max: 240 mμ (ε=16,500)

Names & synonyms: OXANDROLONE;
17ß-hydroxy-17α-methyl-2-oxa-5α-androstane-3-one.

Formulae:

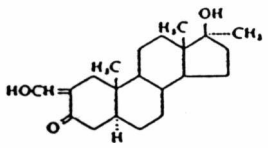


$C_{19}H_{30}O_3$

Molecular weight: 306.4
Melting point(°C): 230-233
Specific rotation: (α) 25/D -21 (1%) in chloroform)
Absorption max: none

Names & synonyms: OXYMETHOLONE;
17-ß-hydroxy-2-hydroxymethylene-17α-methyl-3-androstanone;
2-hydroxymethylene-17-α-methyl-dihydrotestosterone.

Formulae:



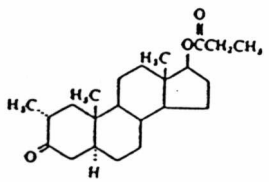
$C_{21}H_{32}O_3$

Molecular weight: 332.4
Melting point(°C): 182
Specific rotation: (α)25/d= +36 (200 mg. in 10 ml. dioxane)

Absorption max: E_1^1 =547 at 315 mμ (in alkaline methanol made 0.01 N with NaOH)

Names & synonyms: PROMETHOLONE;
2α-methyl-dihydro-testosterone propionate;
2α-methyl-5α-androstane-17ß-ol-3-one-propionate;

Formulae:

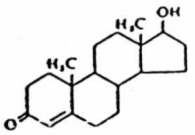


$C_{23}H_{36}O_3$

Molecular weight: 360.5
Melting point(°C): 124-130
Specific rotation: (α) 25/D +22- +29 (200 mg. in 10 ml. chloroform)
Absorption max: without significant absorption from 220-300 mμ (methanol)

Names & synonyms: TESTOSTERONE;
trans-testosterone;
Δ^4-androsten-17-ß-ol-3-one;
17ß-hydroxy-4-androsten-3-one.

Formulae:

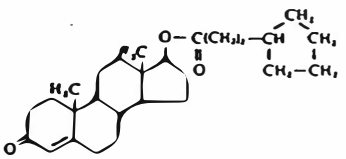


$C_{19}H_{28}O_2$

Molecular weight: 288.4
Melting point(°C): 151-156
Specific rotation: (α) 24/D+109 (400 mg. in 10 ml. alcohol)
Absorption max: 238 mu

Names & synonyms: TESTOSTERONE CYPIONATE;
testosterone cyclopentylpropionate;
17ß-hydroxy-4-androsten-3-one, cyclopentanepropionate.

Formulae:

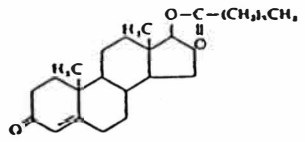


$C_{27}H_{40}O_3$

Molecular weight: 412.6
Melting point(°C): 100-102
Specific rotation: $(\alpha)_D+88.5\pm3.5$ ($CHCl_3$)
Absorption max: λ_{max}241 mμ (ε 16,125)

Names & synonyms: TESTOSTERONE ENANTHATE;
testerone heptanoate;
17ß-hydroxyandrost-4-en-3-one-17-enanthate.

Formulae:

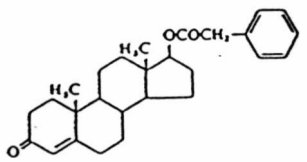


$C_{26}H_{40}O_3$

Molecular weight: 400.6
Melting point(°C): 34-39
Specific rotation: (α)25/D +77- +82 (2% in dioxane)
Absorption max: 241 mμ (in ethanol)

Names & synonyms: TESTOSTERONE PHENYLACETATE;
17ß-hydroxy-4-androsten-3-one phenyl acetate;
testosterone α-toluate.

Formulae:

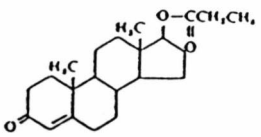


$C_{27}H_{34}O_3$

Molecular weight: 406.5
Melting point(°C):129-131
Specific rotation:(α)25/D +101 ±3 (1% in chloroform)
Absorption max: 241 mμ (in ethanol)

Names & synonyms: TESTOSTERONE PROPIONATE;
Δ^4-androstene-17-ß-propionate-3-one.

Formulae:



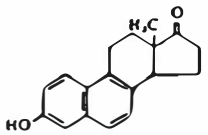
$C_{22}H_{32}O_3$

Molecular weight: 344.4
Melting point(°C): 118-122
Specific rotation: (α)25/D+83- +90 (100 mg. in 10 ml. dioxane)
Absorption max:

ESTROGENS

Names & synonyms: EQUILENIN;
3-hydroxy-17-keto-$\Delta^{1,3,5\text{-}10,6,8}$ estrapentaene;
1,3,5-10,6,8-estrapentaen-3-ol-17-one.

Formulae:

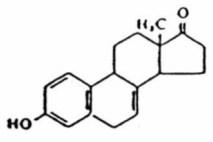


$C_{18}H_{18}O_2$

Molecular weight: 266.3
Melting point(°C): 258-259
Specific rotation: (α)25/D +89 (dioxane)
Absorption max: 231, 270, 282, 292, 325, 340 mμ

Names & synonyms: EQUILIN;
3-hydroxy-17-keto-$\Delta^{1,3,5-10,7}$ estratetraene;
1,3,5,7-estratetraen-3-ol-17-one.

Formulae:

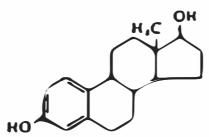


$C_{18}H_{20}O_2$

Molecular weight: 268.3
Melting point(°C): 236-240
Specific rotation: (α)25/D +308 (200 mg. in 10 ml. dioxane);
+325 (200 mg. in 10 ml. alcohol).
Absorption max: 283-285 mμ

Names & synonyms: ESTRADIOL (formerly called α-estradiol);
zz-estradiol;
dihydrofolliculin;
dihydroxyestrin;
1,3,5-estratriene-3,17ß-diol;
3,17-epidigydroxyestratriene.

Formulae:

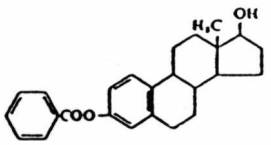


$C_{18}H_{24}O_2$

Molecular weight: 272.3
Melting point(°C): 173-179
Specific rotation: (α)25/D +76- +83 (100 mg. in 10 ml. dioxane)
Absorption max: 225,280 mμ

Names & synonyms: ESTRADIOL BENZOATE;
ß-estradiol-3-benzoate;
estradil monobenzoate.

Formulae:

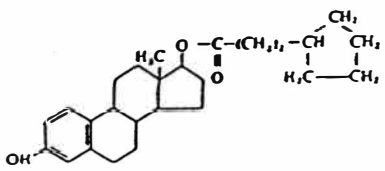


$C_{25}H_{28}O_3$

Molecular weight: 376.4
Melting point(°C): 191-196
Specific rotation: (α)25/D +58- +63 (200 mg. in 10 ml. dioxane)
Absorption max:

Names & synonyms: ESTRADIOL CYPIONATE;
estradiol cyclopentylpropionate;
ß-estradiol 17-cyclopentanepropionate;
1,3,5(10)-estratriene-3,17ß-diol, 17-cyclopentanepropionate.

Formulae:

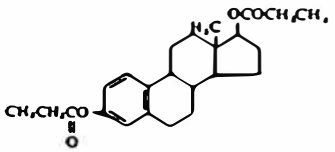


$C_{26}H_{36}O_3$

Molecular weight: 396.6
Melting point(°C): 151-154
Specific rotation: $(\alpha)_D$ +41.5±3.5 (dioxane)
Absorption max: 223mμ

Names & synonyms: ESTRADIOL DIPROPIONATE;
α-estradiol dipropionate;
17ß-estradiol dipropionate.

Formulae:

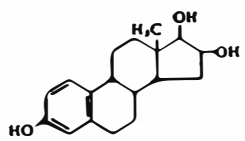


$C_{24}H_{32}O_4$

Molecular weight: 384.5
Melting point(°C): 104-109
Specific rotation: (α)25/D +39±2 (1%) in dioxane)
Absorption max: 268 mμ

Names & synonyms: ESTRIOL;
trihydroxyestrin;
$\Delta^{1,3,5-10}$-estratriene-3-16-cis-17-tran-diol;
1,3,5-estratriene-3,16α, 17ß-triol.

Formulae:

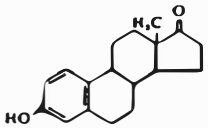


$C_{18}H_{24}O_3$

Molecular weight: 288.3
Melting point(°C): 282
Specific rotation: (α)25/D +53- +63 (40 mg. in 1 ml. dioxane)
Absorption max: 280 mμ

Names & synonyms: ESTRONE;
folliculin;
ketohydroxyestrin;
1,3,5-estratrien-3-ol-17-one.

Formulae:

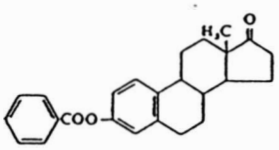


$C_{18}H_{22}O_2$

Molecular weight: 270.3
Melting point(°C): 258-262
Specific rotation: (α)25/D+158-+168 (100 mg. in 10 ml. dioxane)
Absorption max: 283-285 mμ

Names & synonyms: ESTRONE BENZOATE

Formulae:

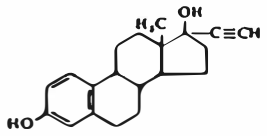


$C_{25}H_{26}O_3$

Molecular weight: 374.4
Melting point(°C): 220
Specific rotation: (α)25/D+120 (dioxane)
Absorption max:

Names & synonyms: ETHYNYL ESTRADIOL;
17-ethinyl estradiol;
17α-ethynyl-1,3,5-estratriene-3,17ß-diol.

Formulae:

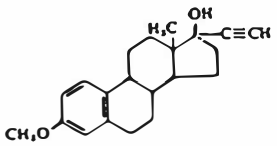


$C_{20}H_{24}O_2$

Molecular weight: 296.4
Melting point(°C): 141-146
Specific rotation: (α)25/D+1- +10 (100 mg. in 10 ml. dioxane)
Absorption max: 248 mμ

Names & synonyms: MESTRANOL;
ethynylestradiol 3-methyl ether;
3-methoxy-17α-ethynyl-1,3,5(10)-estratriene-17ß-ol;
3-methoxy-19-nor-17α-pregna-1,3,5,trien-20-yn-17-ol.

Formulae:



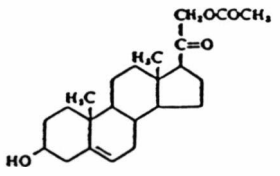
$C_{21}H_{26}O_2$

Molecular weight: 310.4
Melting point(°C): 148-154
Specific rotation: (α)25/D +2 to +8 (200 mg. in 10 ml. dioxane)
Absorption max: 278 to 287 mμ (methanol)

PROGESTOGENS AND PROGESTINS (INCLUDING 19-NORSTEROID COMPOUNDS)

Names & synonyms: ACETOXYPREGNENOLONE;
21-acetoxypregnenolone;
prebediolone acetate;
Δ3-pregene-3ß, 21-diol-20-one-21-monoacetate;
21-acetoxy-5-pregnene-3-ol-20-one;
3-hydroxy-21-acetoxy-5-pregnen-20-one.

Formulae:

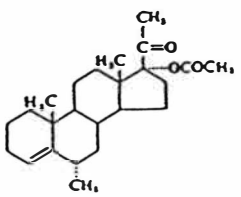


$C_{23}H_{34}O_4$

Molecular weight: 374.5
Melting point(°C): 184-185
Specific rotation: (α)20/D+37-+43 (dioxane)
Absorption max:

Names & synonyms: ANAGESTONE ACETATE;
6α-methyl-4-pregnen-17α-ol-20-one acetate;
17α-acetoxy-6α-methylpregn-4-en-20-one;
17α-acetoxy-6α-methyl-4-pregnen-20-one.

Formulae:

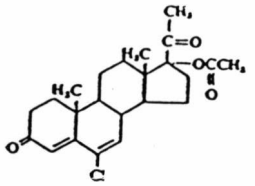


$C_{24}H_{36}O_3$

Molecular weight: 372.6
Melting point(°C): 172-178
Specific rotation: (α)25/D+40 to +45 (10 mg. in 10 ml. chloroform)
Absorption max:

Names & synonyms: CHLORMADINONE ACETATE;
6-chloro-Δ^6-dehydro-17α-acetoxyprogesterone);
6-chloro-$\Delta^{4,6}$-pregnadiene-17α-ol-3,20-dioneacetate.

Formulae:

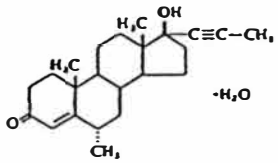


$C_{23}H_{29}ClO_4$

Molecular weight: 404.9
Melting point(°C): 204-212
Specific rotation: (α)25/D 0 to -6 (200 mg. in 10 ml. chloroform)
Absorption max: 284 mμ(methanol)
Log ϵ=4.34±0.02

Names & synonyms: DIMETHISTERONE;
6α,21-dimethylethisterone;
6α,21-dimethyl-17ß-hydroxy-17α-pregn-4-en-20-yn-3-one;
17ß-hydroxy-6α-methyl-17α-(prop-1-ynyl)-androst-4-ene-3-one.

Formulae:

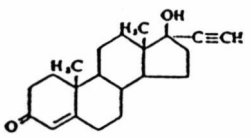


$C_{23}H_{32}O_2H_2O$

Molecular weight: 358.5
Melting point(°C): App. 100 (dec.)
Specific rotation: (α)20/D+16.5 to +18.5 (2% solution in chloroform) (calculated to the anhydrous basis)
Absorption max: App. 240 mμ (anhydrous ethanol)
$E^{1\%}_1$ cm=443

Names & synonyms: ETHISTERONE;
anhydrohydroxyprogesterone;
ethinyl testosterone;
pregneninolone;
17α ethynyl testosterone;
17α-ethynyl-17ß-hydroxy-4-androsten-3-one.

Formulae:

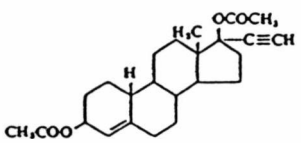


$C_{21}H_{28}O_2$

Molecular weight: 312.4
Melting point(°C): 266-273
Specific rotation: (α)25/D-32° (100 mg. in 10 ml. pyridine)
Absorption max: 241 mμ (methanol)

Names & synonyms: ETHYNODIOL DIACETATE;
17α-ethynyl-4-estrene-3ß, 17ß-diol-17-diacetate;
19-nor-17α-pregn-4-en-20-yne-3ß,17-diol diacetate.

Formulae:

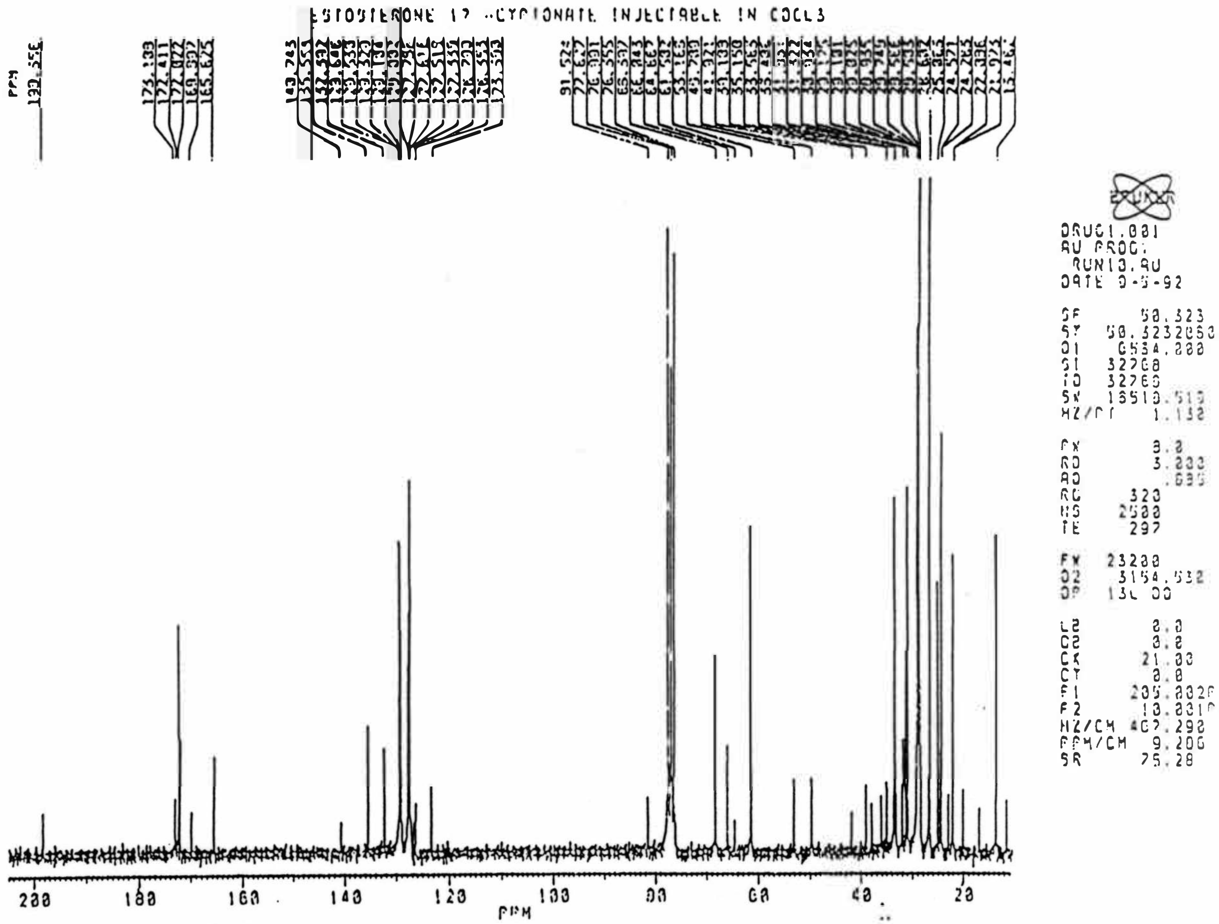


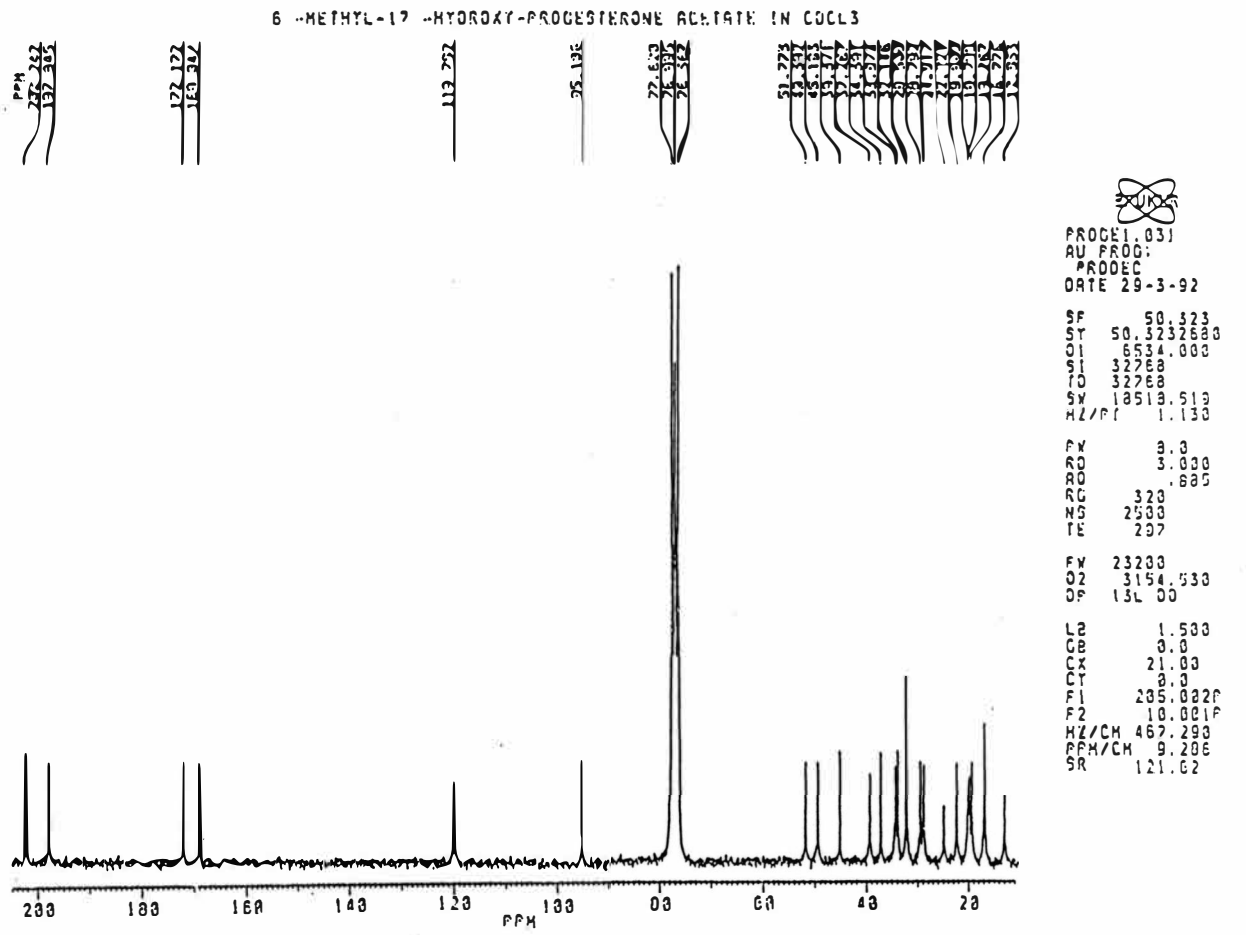
$C_{24}H_{32}O_4$

Molecular weight: 384.5
Melting point(°C): 126-132
Specific rotation: (α)25/D-74 (1% in chloroform)
Absorption max: None

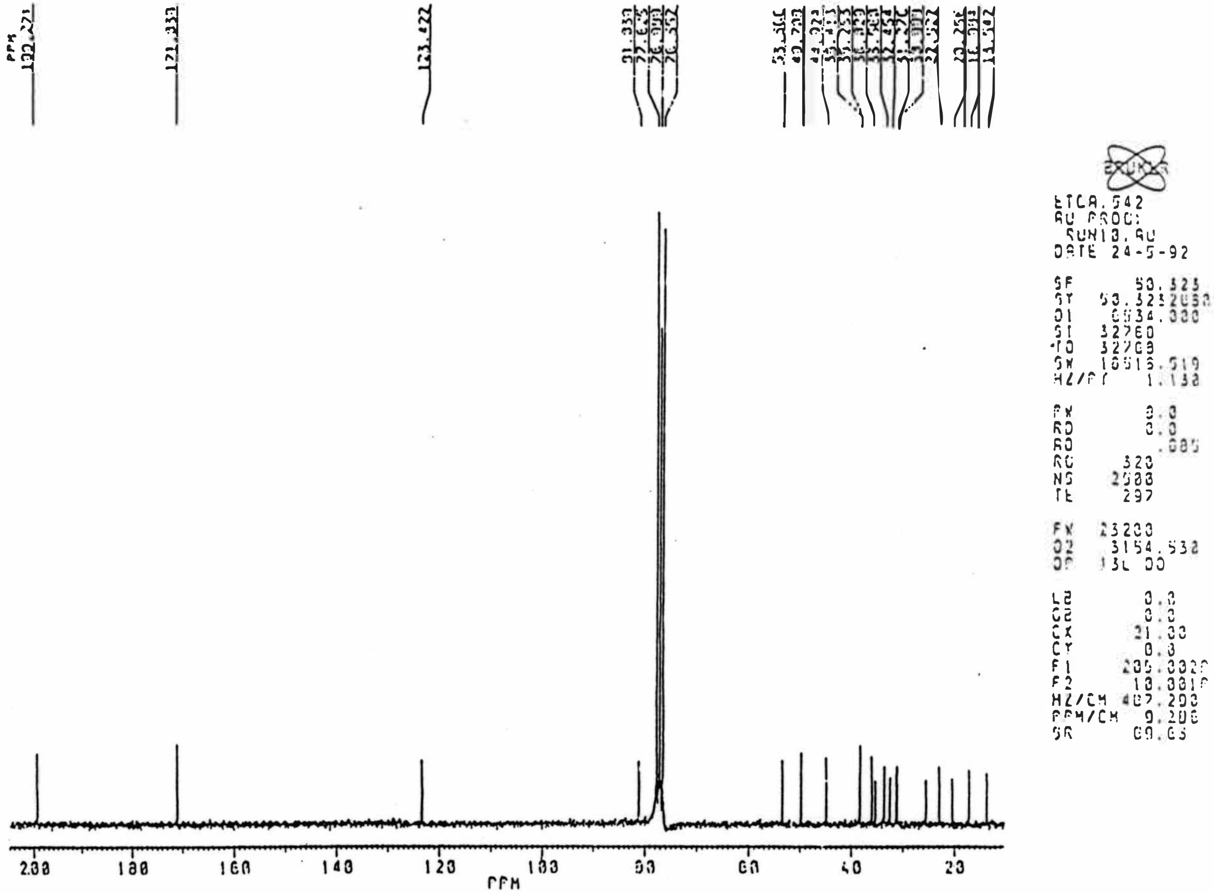
Appendix B

^{13}C Spectra

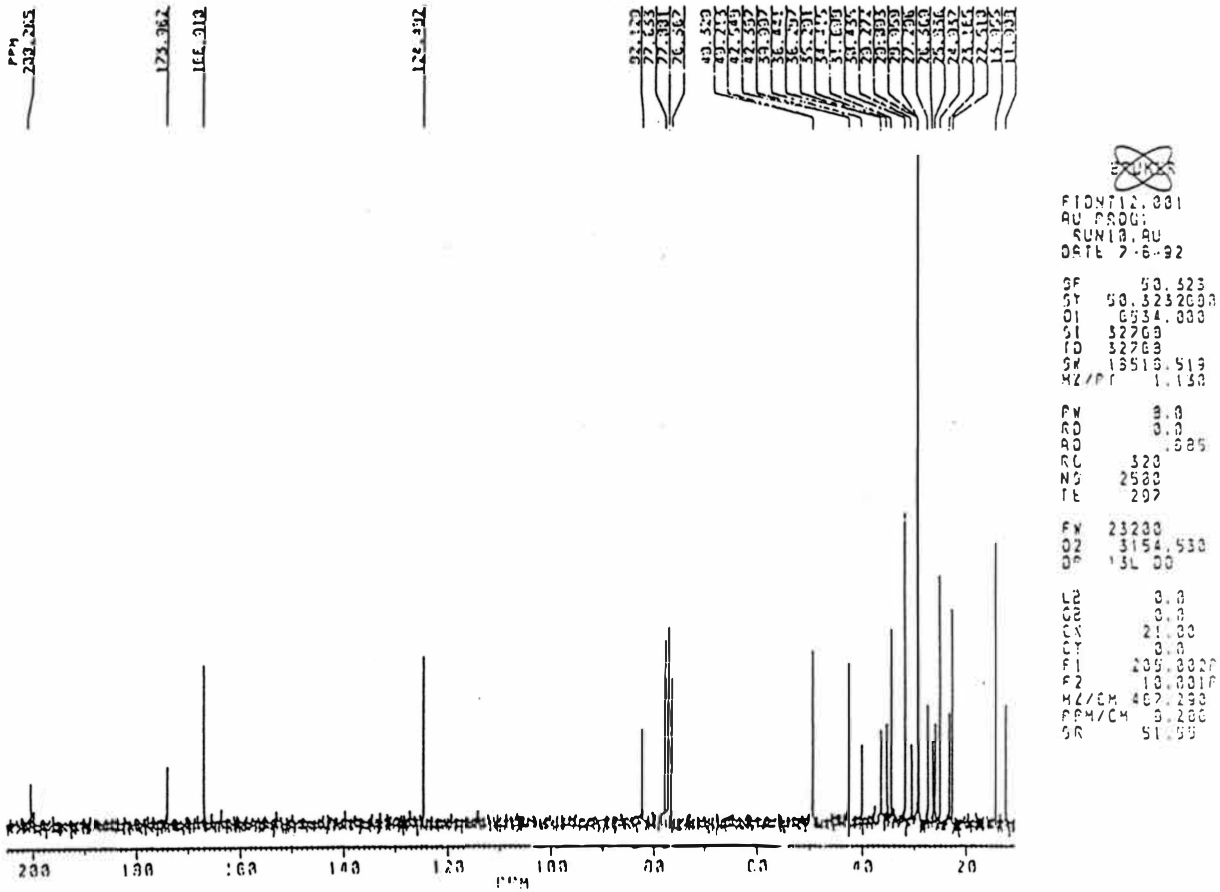

ESTOSTERONE 17-CYPIONATE INJECTABLE IN CDCL3
DRUG1.001
AU PROG:
RUN13.AU
DATE 9-5-92
SF 50.323
200 180 160 140 120 100 80 60 40 20
PPM

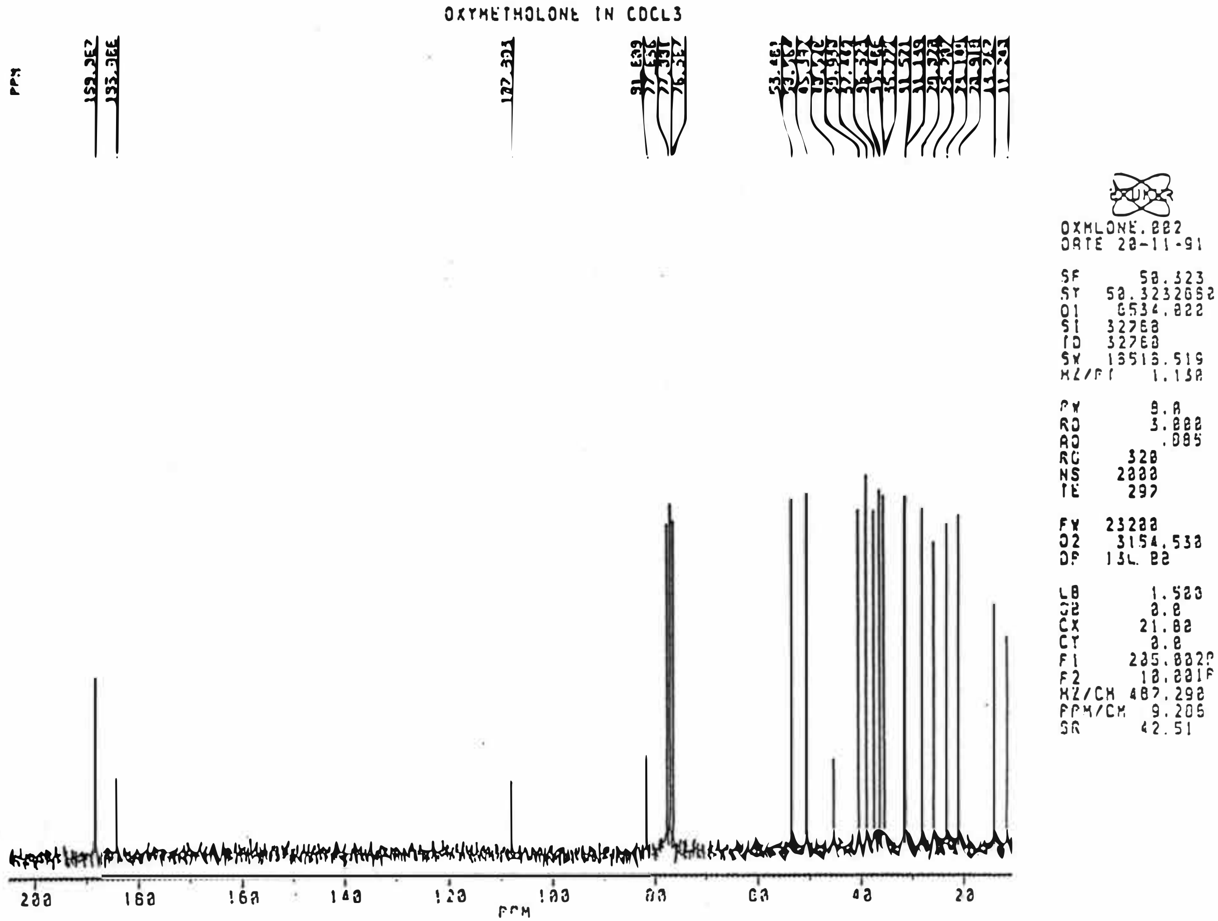

6 -METHYL-17 -HYDROXY-PROGESTERONE ACETATE IN CDCL3
PPM
122.122
113.252
95.136
51.223
PROGE1.031
AU PROG:
PRODEC
DATE 29-3-92
SF 50.323
ST 50.3232680
O1 6534.000
SI 32768
TD 32768
SW 10518.518
HZ/PT 1.130
PW 8.0
RD 3.000
AQ .680
RG 320
NS 2500
TE 297
FW 23200
O2 3154.530
DF 13L.00
L2 1.500
GB 0.0
GX 21.00
GT 0.0
F1 235.0020
F2 10.0010
HZ/CM 467.293
PPM/CM 9.286
SR 121.62
200
180
160
140
120
100
80
60
40
20
PPM

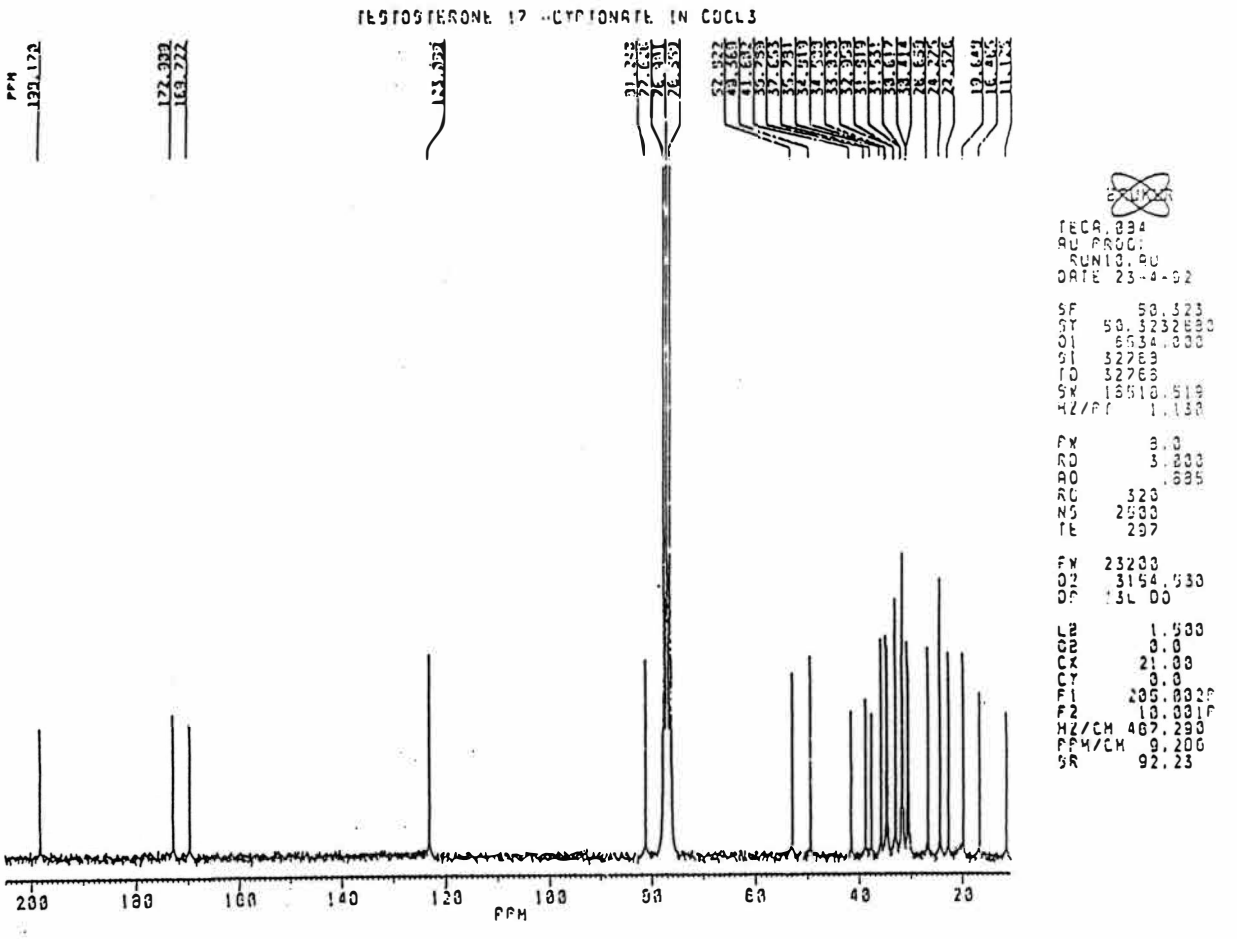
17α-METHYLTESTOSTERONE IN CDCl3

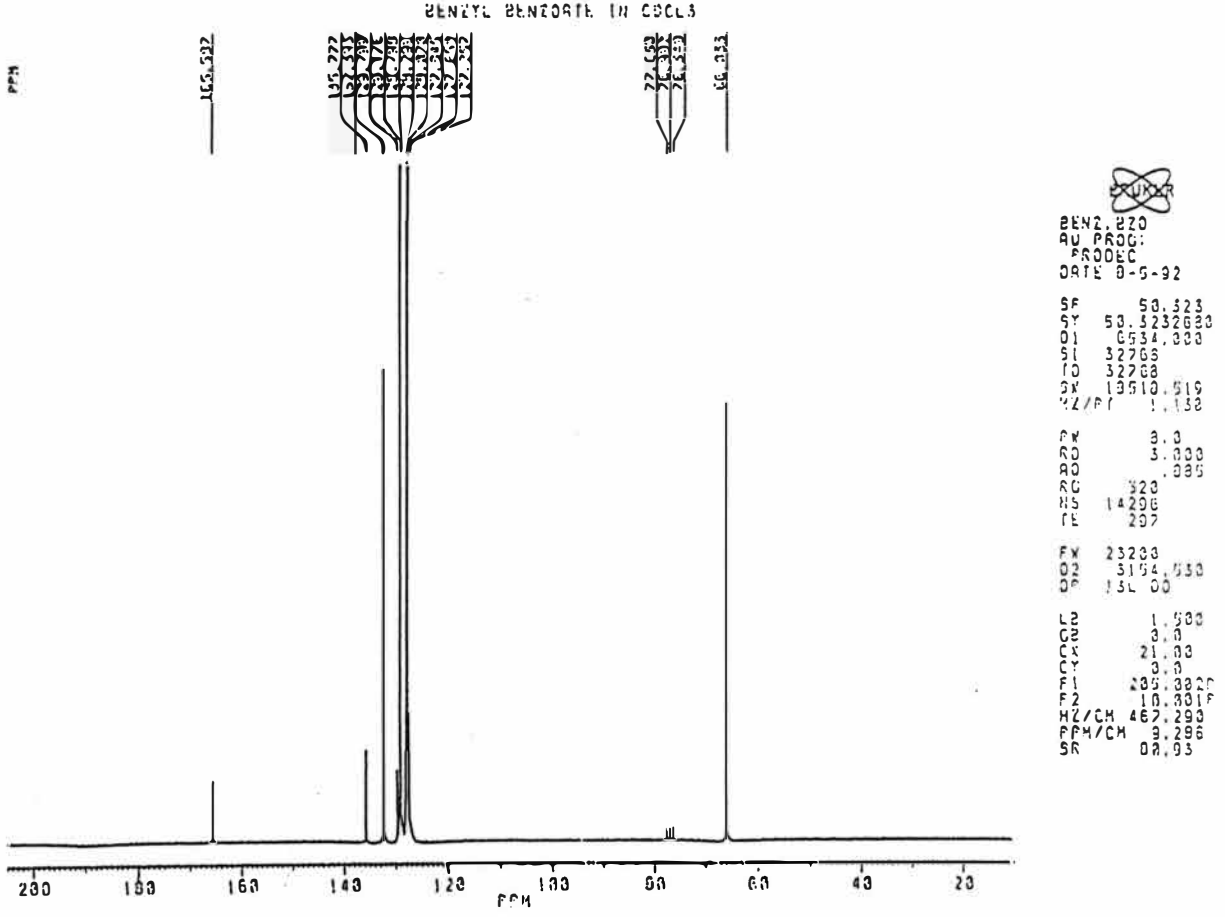


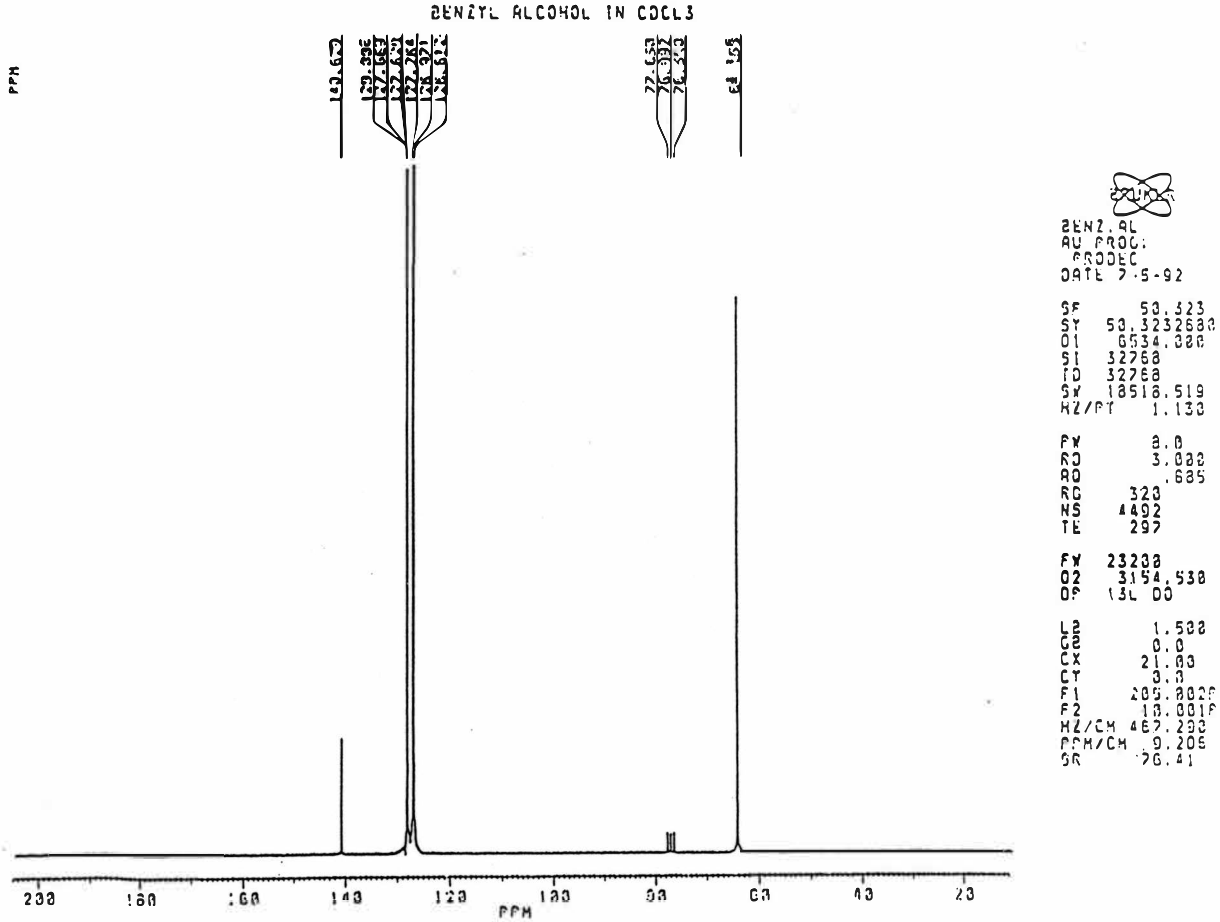
19-NORTESTOSTERONE 17-DECANOATE IN CDCL3

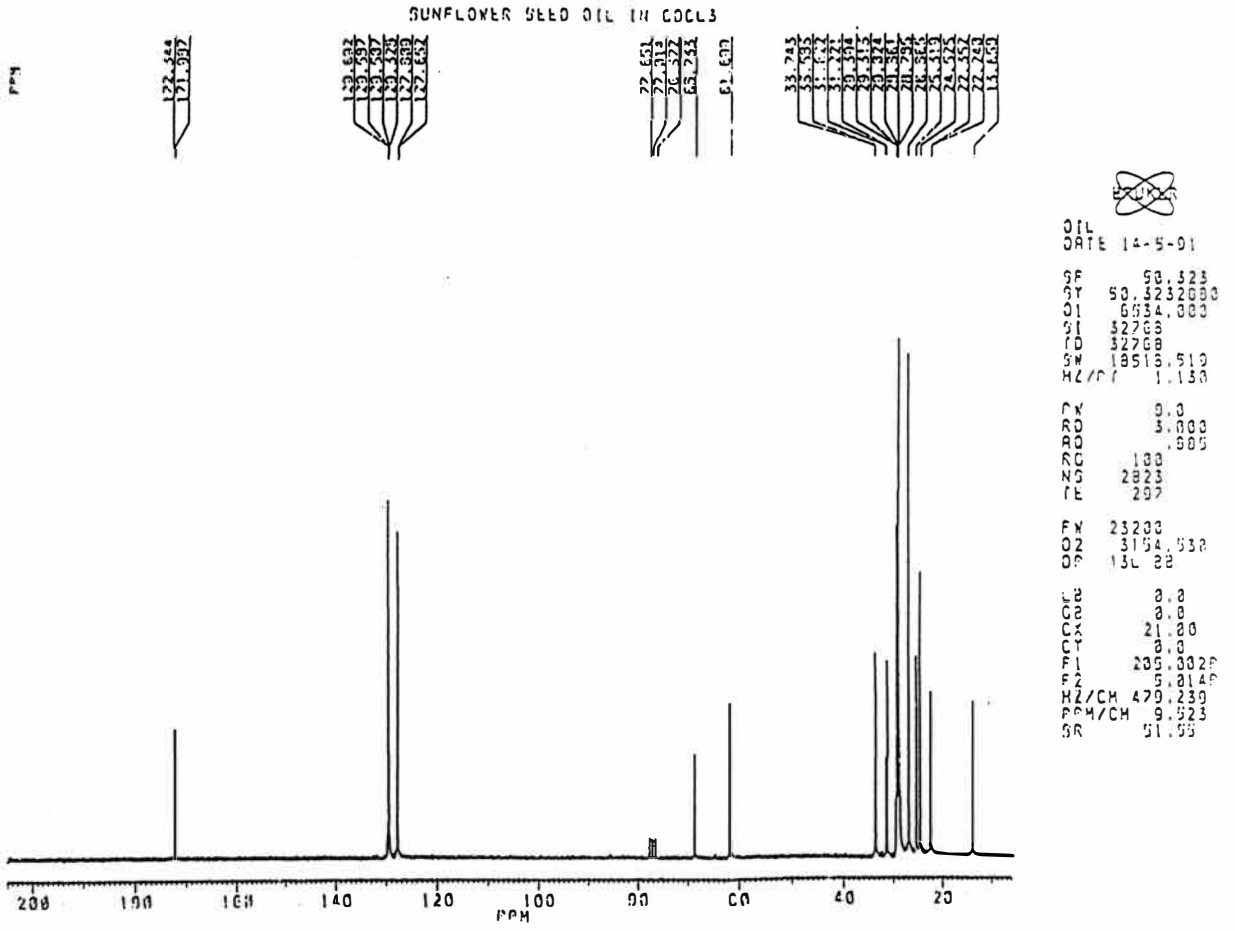


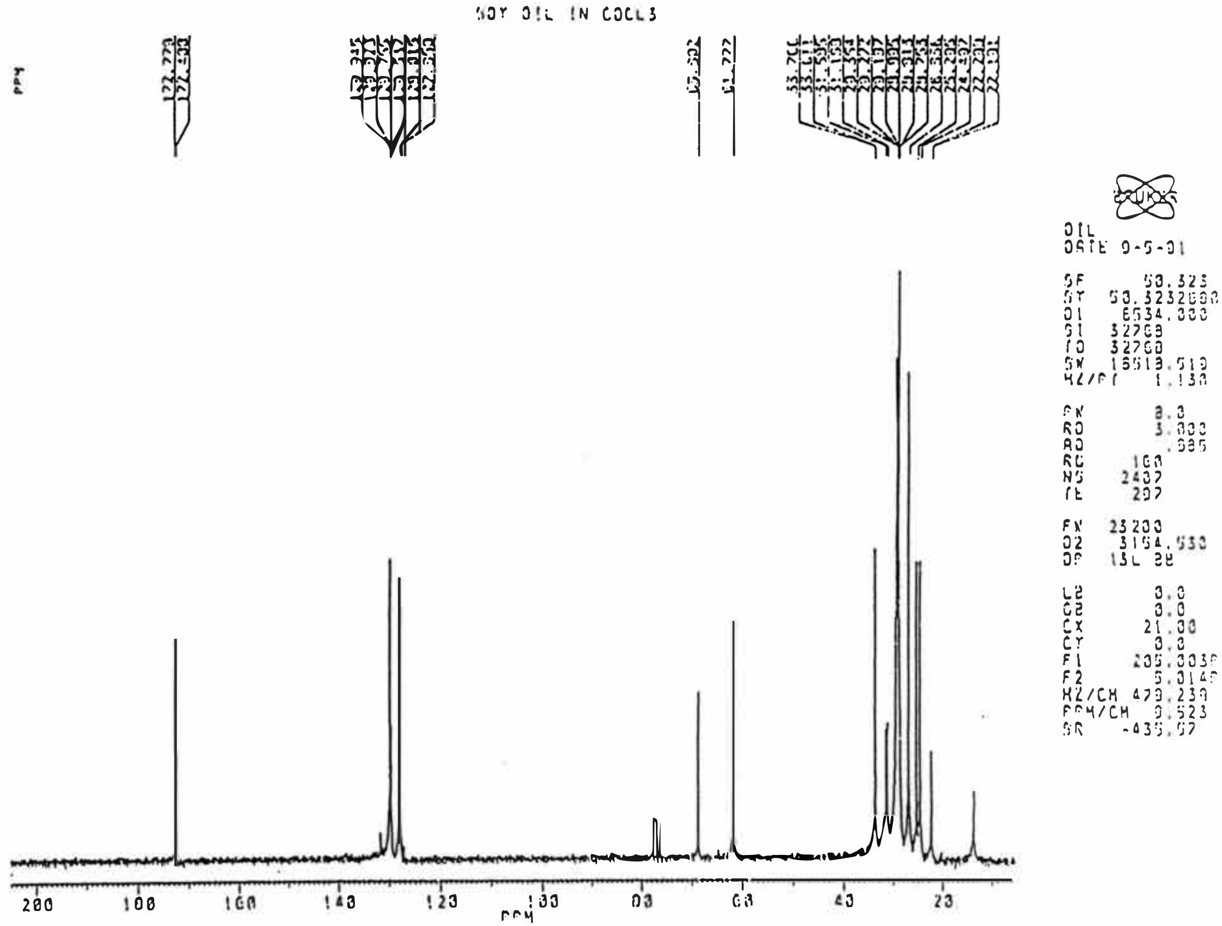

OXYMETHOLONE IN CDCL3
PPM
OXMLONE.002
DATE 20-11-91
SF 50.323
SY 50.3232062
O1 6534.022
SI 32768
TD 32768
SW 16515.519
HZ/PT 1.130
PW 9.0
RD 3.000
AQ .085
RG 320
NS 2000
TE 297
FW 23200
O2 3154.530
DP 13L 02
LB 1.500
GB 0.0
CX 21.00
CY 0.0
F1 205.002P
F2 10.001P
HZ/CM 487.290
PPM/CM 9.205
SR 42.51
200
180
160
140
120
100
80
60
40
20
PPM


TESTOSTERONE 17 -CYPIONATE IN CDCL3
PPM
170.172
122.333
169.722
123.553
31.222
27.626
26.901
26.352
52.822
49.365
41.687
39.758
37.655
35.781
34.519
34.253
33.333
32.969
31.515
31.531
30.617
18.814
28.654
24.324
22.526
12.647
16.462
11.124
TECA.834
RU PROCI
RUN10.RU
DATE 23-4-92
SF 50.323
OT 50.3232880
OI 6034.000
SI 32768
TD 32768
SW 19510.519
HZ/PT 1.190
PW 8.0
RD 3.000
AQ .685
RG 320
NS 2500
TE 297
FW 23200
O2 3154.530
DE 136.00
GB 1.500
GB 0.0
GX 21.00
GY 0.0
F1 205.8020
F2 10.0310
HZ/CM 467.290
PPM/CM 9.200
SR 92.23
200
180
160
140
120
100
80
60
40
20
PPM

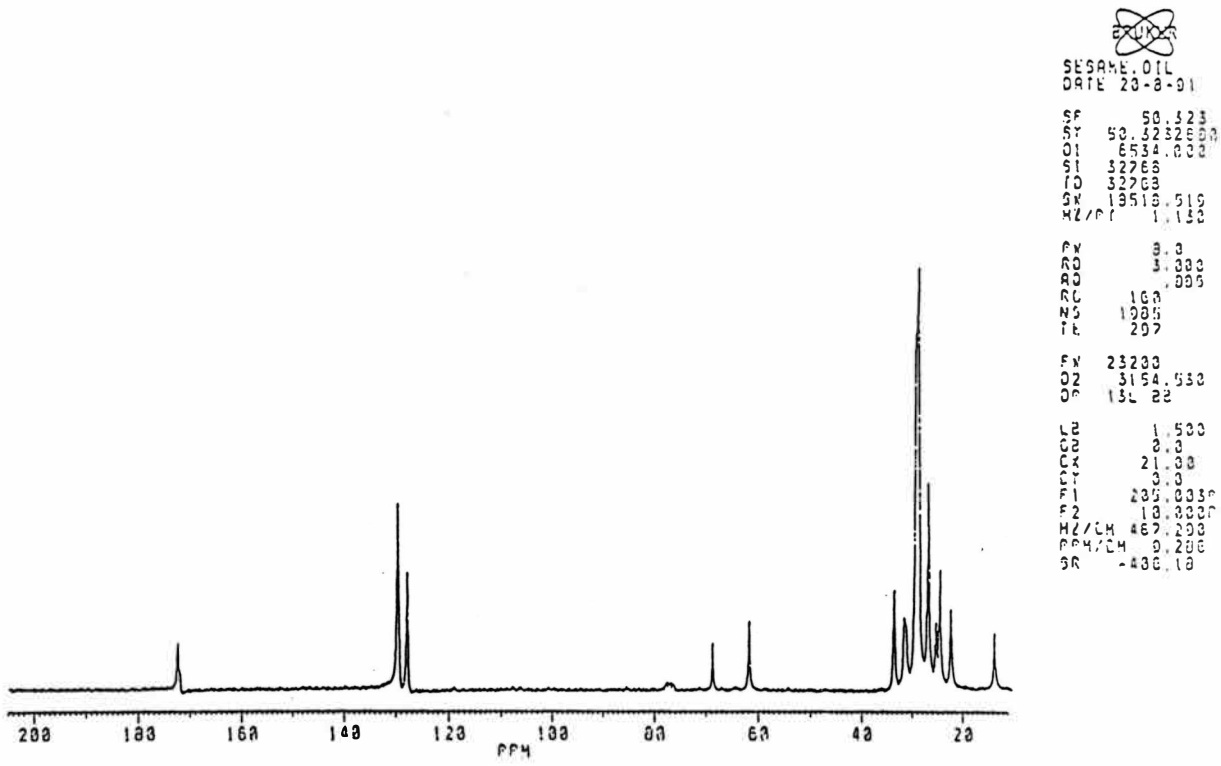

BENZYL BENZOATE IN CDCL3
PPM
165.322
66.353
230
180
160
140
120
100
80
60
40
20
PPM
BRUKER
BENZ.220
AU PROG:
PRODEC
DATE 8-5-92
SF 50.323
SY 50.3232030
DI 6554.000
SI 32768
TD 32768
SW 13510.919
HZ/PT 1.158
PW 8.0
RD 3.000
AQ .986
RG 320
NS 14208
DE 297
FW 23200
O2 3154.050
DP USER
LB 1.500
GB 3.0
GX 21.00
GY 3.0
FI 285.3822
F2 10.3018
HZ/CM 462.290
PPM/CM 9.296
SR 00.03

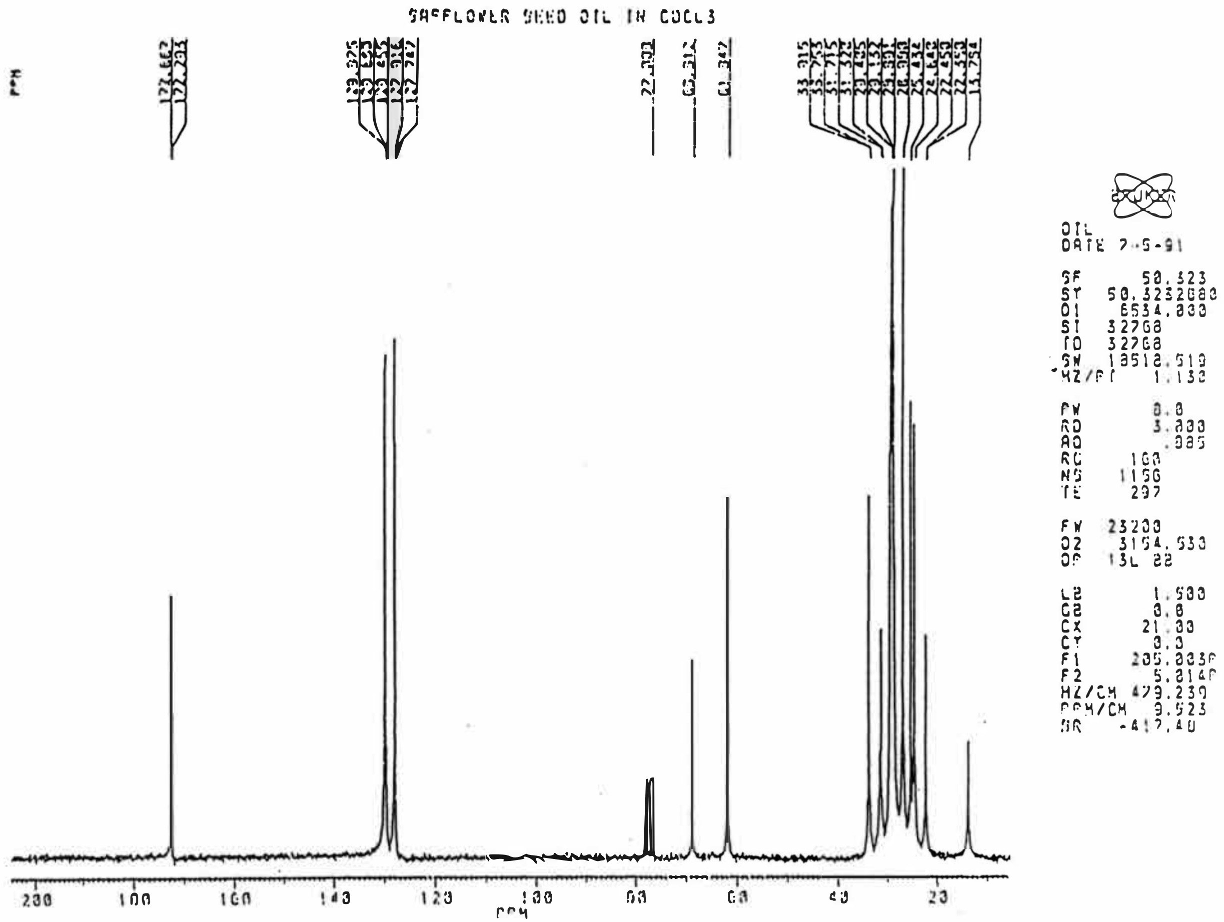

BENZYL ALCOHOL IN CDCL3
PPM
140.673
128.336
127.667
77.653
76.382
76.553
64.155
BENZ.AL
AU PROG:
PRODEC
DATE 7-5-92
SF 50.323
SY 50.3232680
O1 6534.000
SI 32768
TD 32768
SW 18518.519
HZ/PT 1.130
PW 8.0
RD 3.000
AQ .885
RG 320
NS 4492
TE 297
FW 23200
O2 3154.530
DP 13L DO
LB 1.500
GB 0.0
CX 21.00
CY 3.0
F1 205.002P
F2 10.001P
HZ/CM 467.290
PPM/CM 9.205
SR 76.41
200
180
160
140
120
100
80
60
40
20
PPM

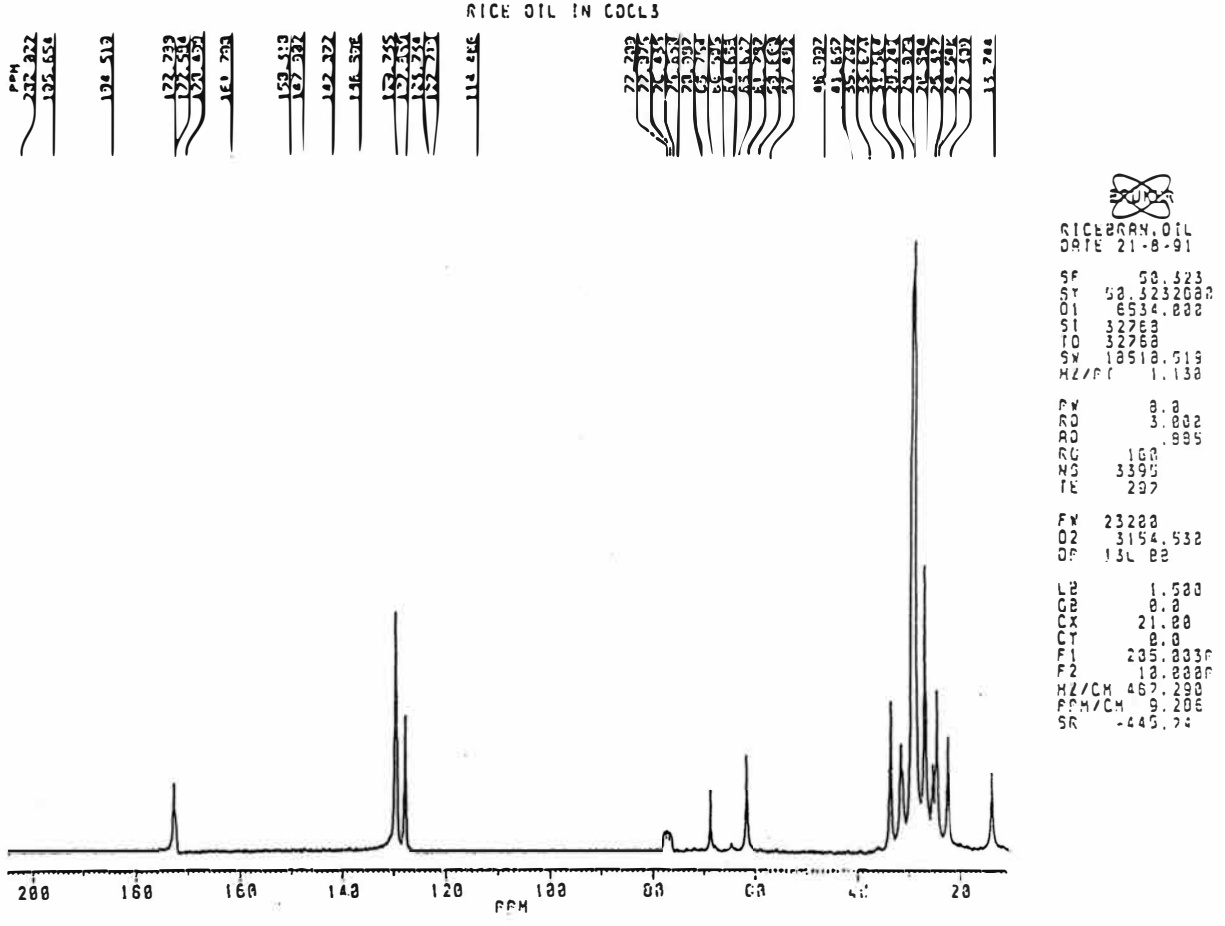

SUNFLOWER SEED OIL IN CDCL3
PPM
122.344
171.397
133.632
133.597
133.537
133.478
132.830
132.652
72.651
72.312
26.322
63.251
61.603
33.745
33.695
31.642
31.477
29.354
29.312
29.274
21.361
20.794
26.564
25.113
24.525
22.452
22.249
13.659
OIL
DATE 14-5-01
SF 50.323
SF 50.3232880
O1 6034.300
SI 32768
TD 32768
SW 18518.519
HZ/PT 1.130
PW 9.0
RD 3.000
RG .985
NS 2823
TE 297
FW 23200
O2 3154.538
CDCL3 21.80
PPM/CM 9.523
SR 51.55
200
180
160
140
120
100
80
60
40
20
PPM

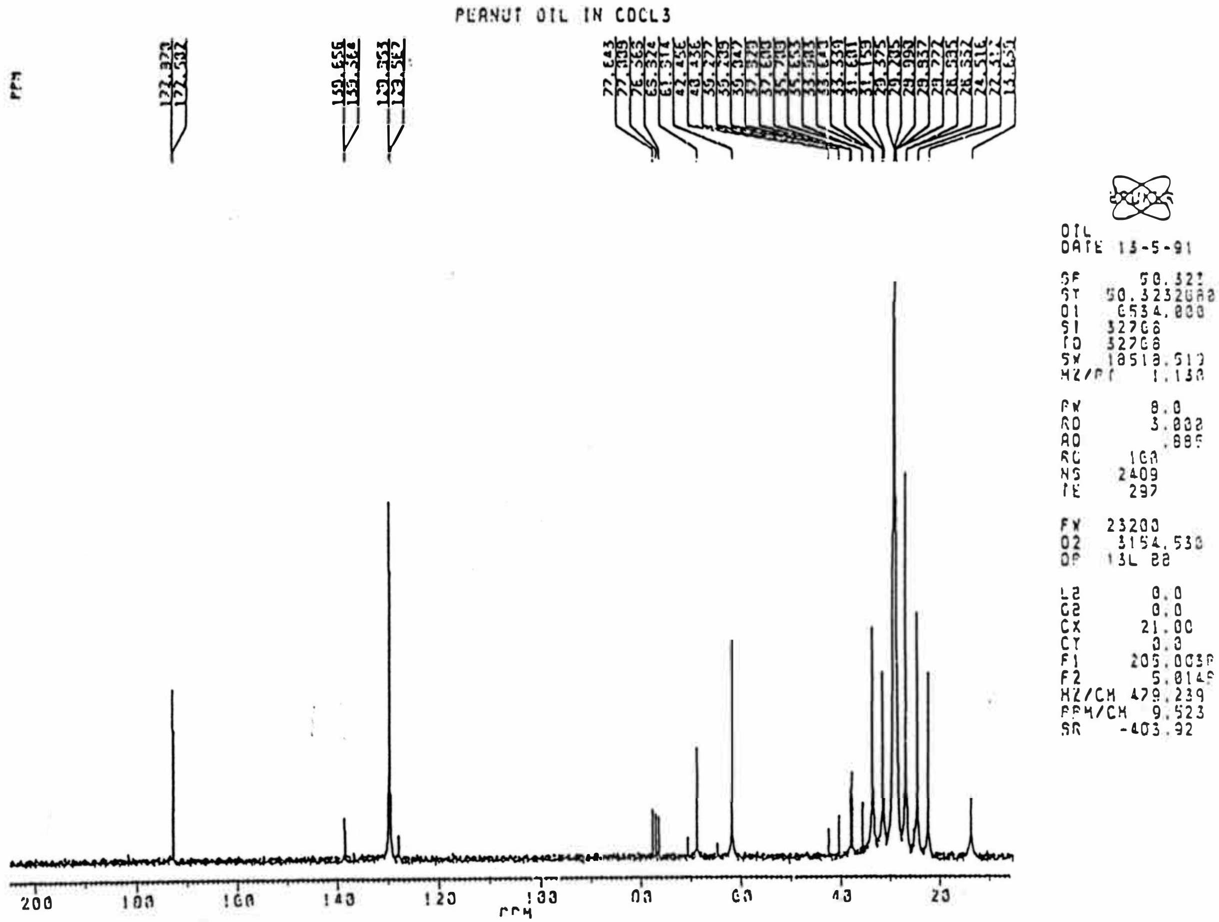

SOY OIL IN CDCL3
PPM
OIL
DATE 9-5-91
PPM

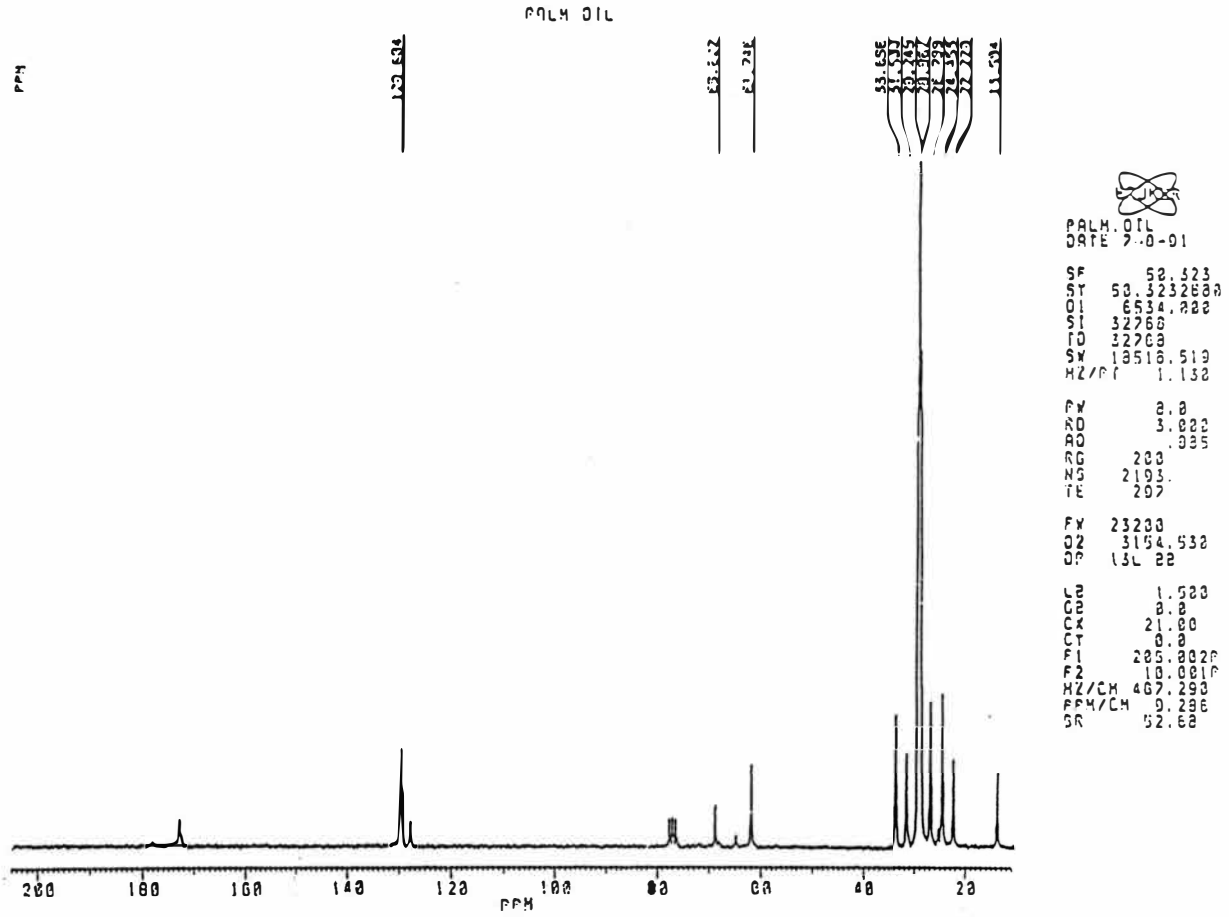
SESAME OIL IN CDCL3

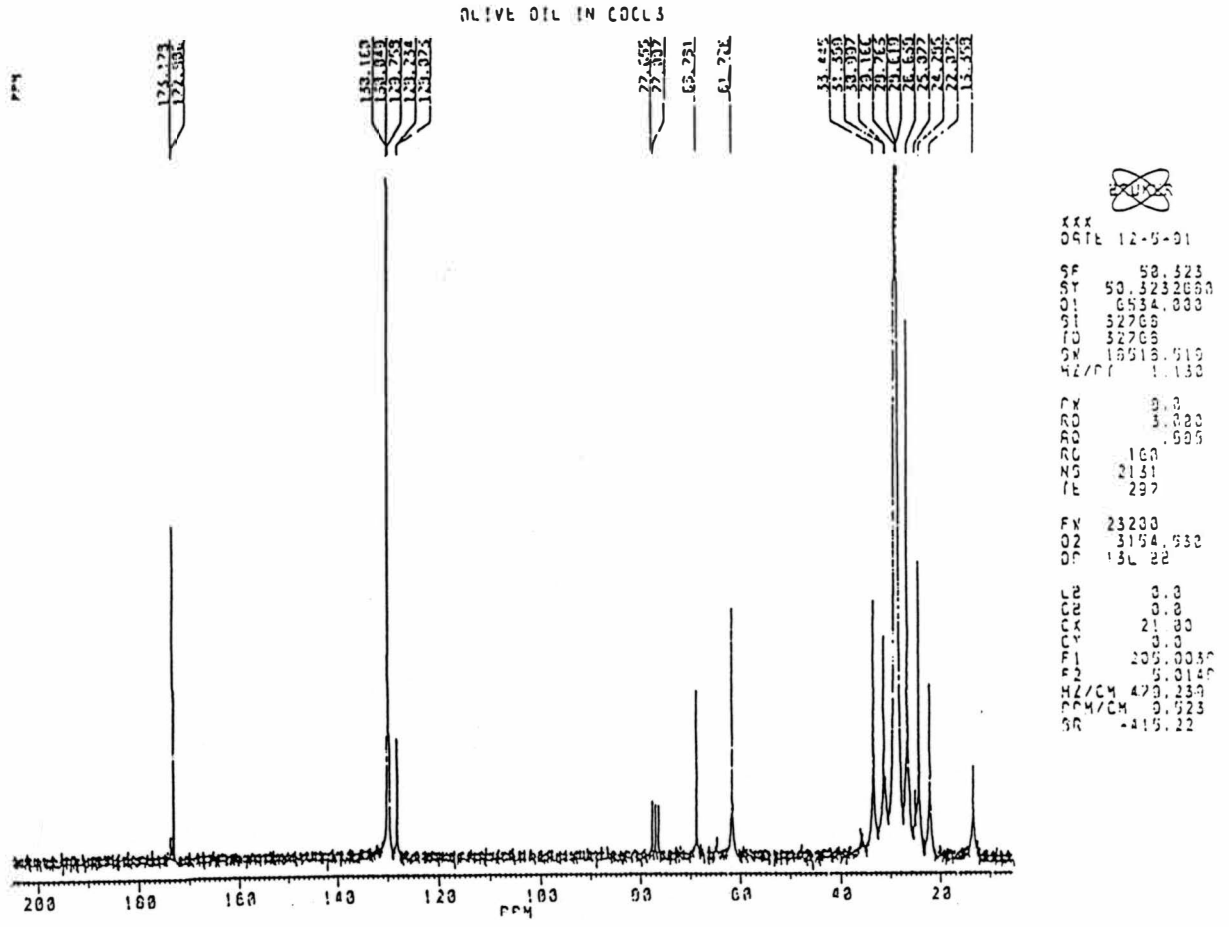


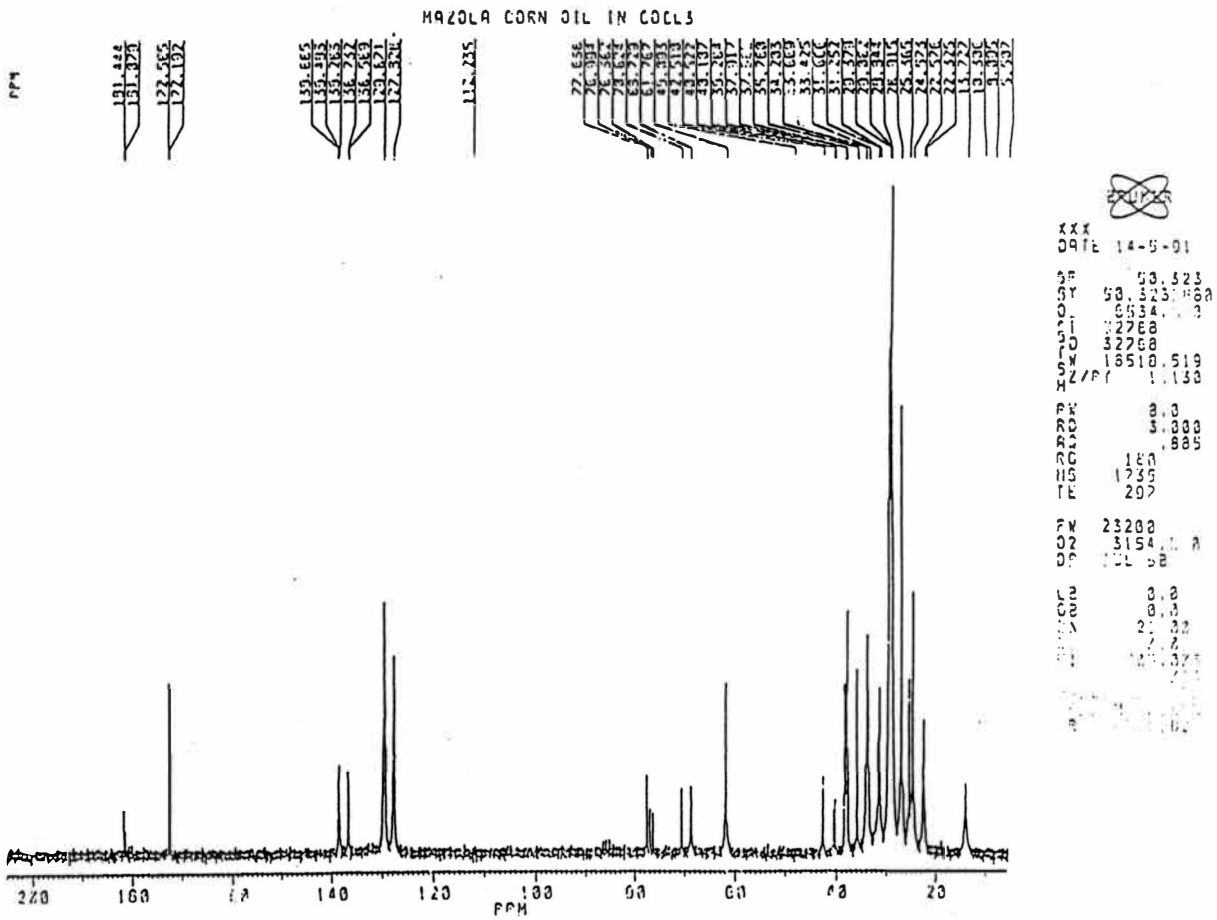

SAFFLOWER SEED OIL IN CDCL3
PPM
OIL
DATE 7-5-91
SF 50.323
SY 50.3232080
O1 6534.000
SI 32768
TD 32768
SW 13512.519
HZ/PT 1.132
PW 0.0
RD 3.000
AQ .005
RG 100
NS 1156
TE 297
FW 23200
O2 3154.530
DP 13L 22
LB 1.500
GB 0.0
CX 21.00
CY 0.0
F1 205.003P
F2 5.014P
HZ/CM 479.239
PPM/CM 9.523
SR -417.40
200
180
160
140
120
100
80
60
40
20
PPM

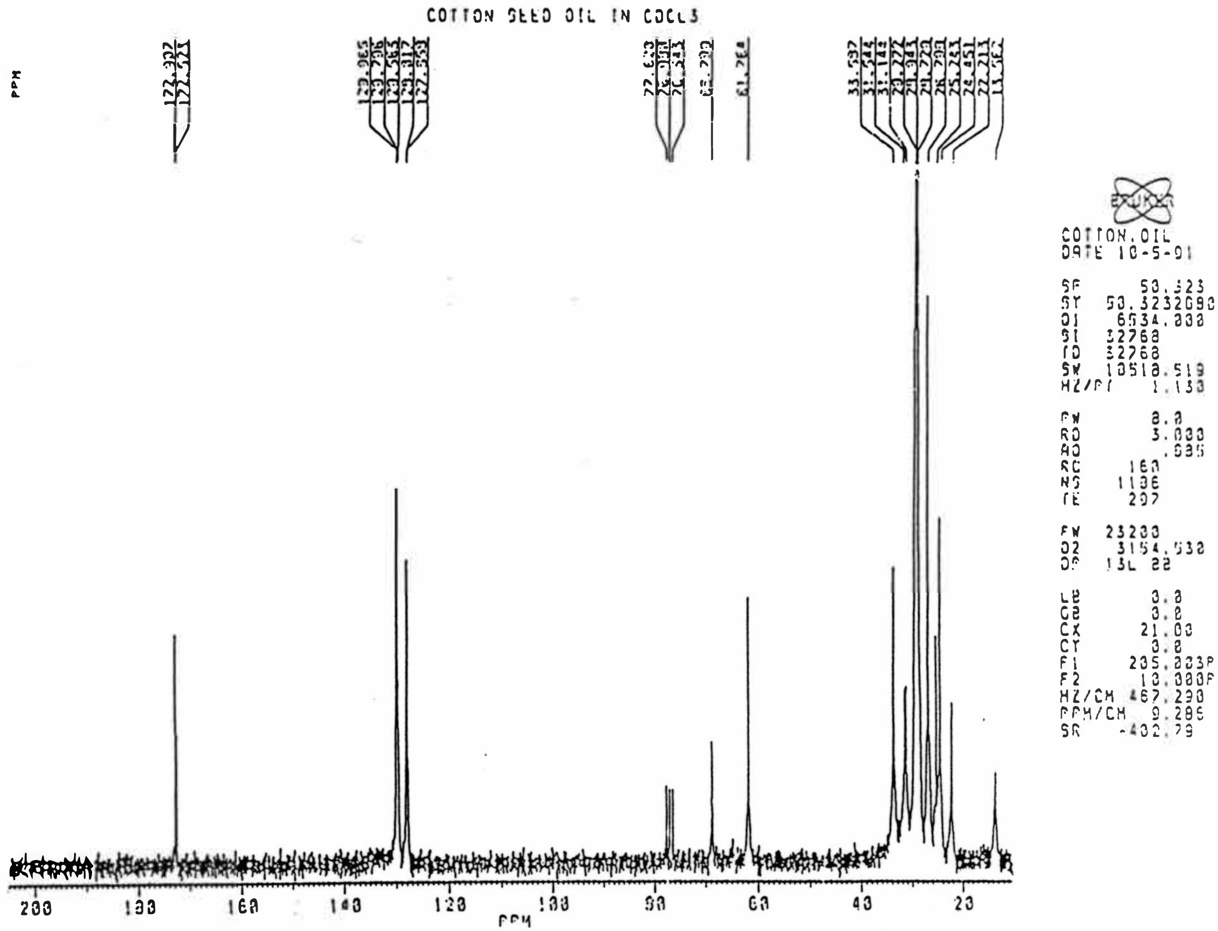

RICE OIL IN CDCL3
PPM
RICEBRAN.OIL
DATE 21-8-91
200
160
140
120
100
80
60
40
20
PPM

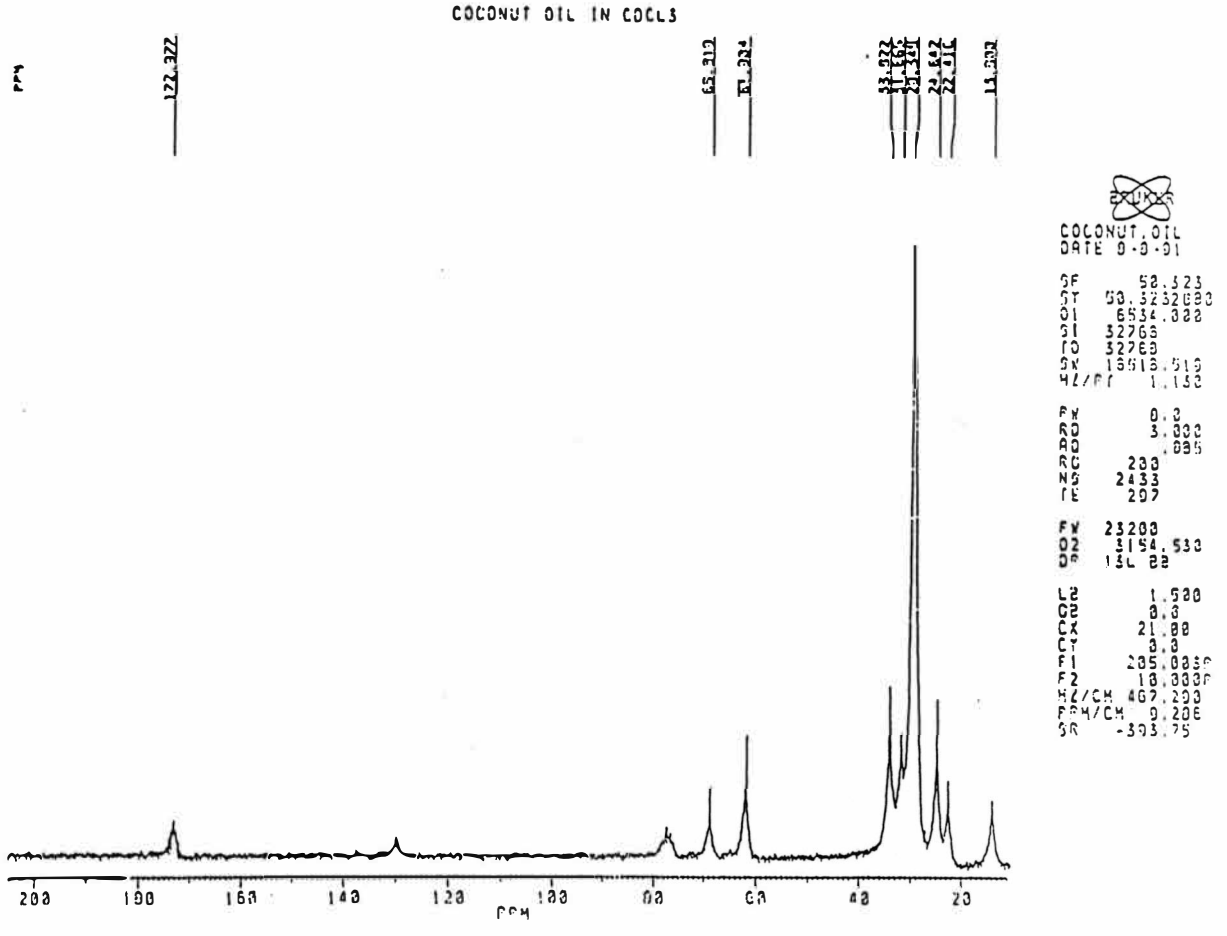

PEANUT OIL IN CDCL3
OIL
DATE 13-5-91
SF 50.32
SY 50.3232
O1 6534.000
SI 32768
TD 32768
SW 10518.519
HZ/PT 1.130
PW 8.0
RD 3.002
AQ .885
RG 160
NS 2409
TE 297
FW 23200
O2 3154.530
DP 13L 22
LB 0.0
GB 0.0
CX 21.00
CY 0.0
F1 205.003P
F2 5.014P
HZ/CM 479.239
PPM/CM 9.523
SR -403.92
200
180
160
140
120
100
80
60
40
20
PPM

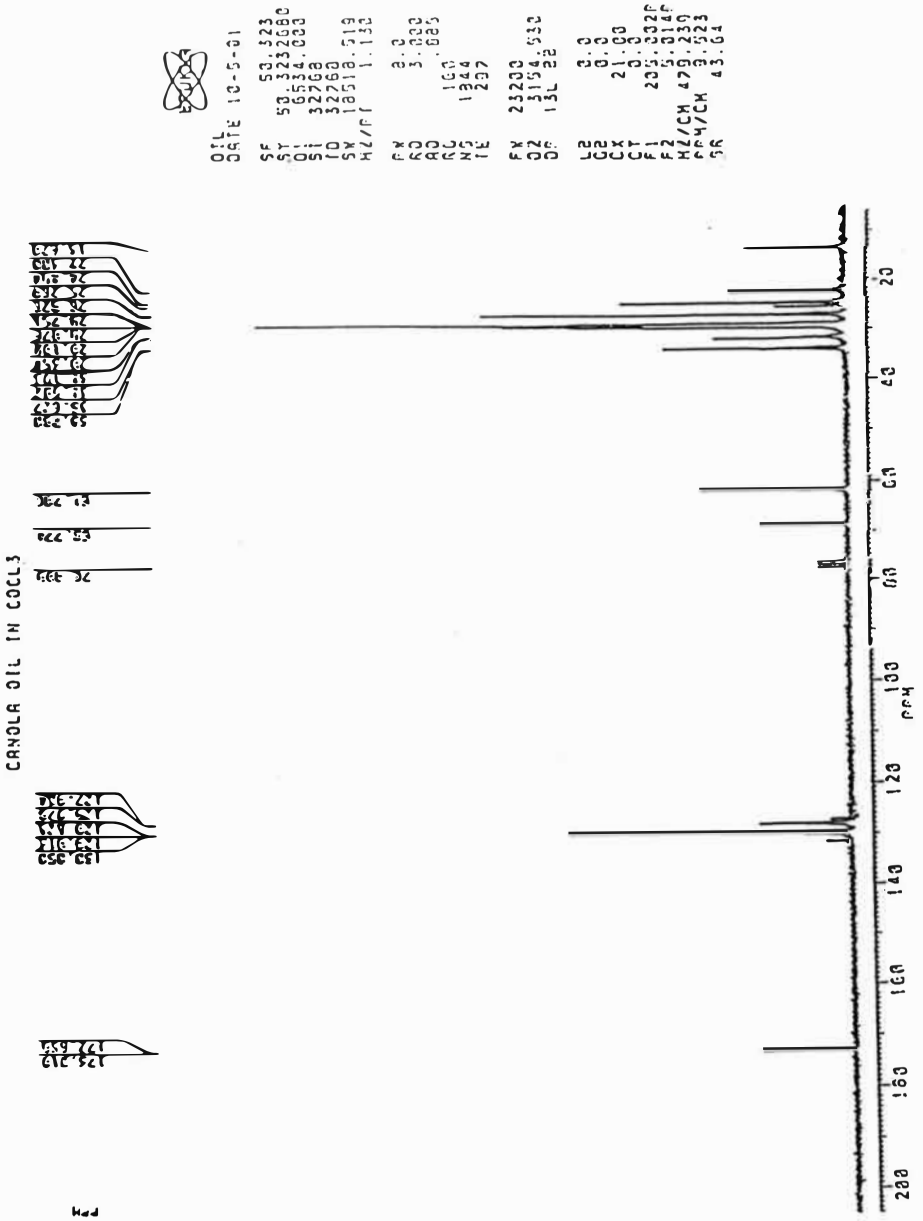

PALM OIL
PPM
129.934
63.642
61.284
33.658
31.933
29.765
29.387
29.239
24.853
22.723
14.094
PALM.OIL
DATE 7.0-01
SF 52.523
SI 32768
SW 13516.519
HZ/PT 1.152
PW 8.0
RD 3.022
AQ .985
RG 203
NS 2193.
TE 297
FW 23200
O2 3154.532
DP 13L 22
CX 21.00
F1 205.8028
F2 10.0018
HZ/CM 467.293
PPM/CM 8.896
SR 52.68
200
180
160
140
120
100
80
60
40
20
PPM

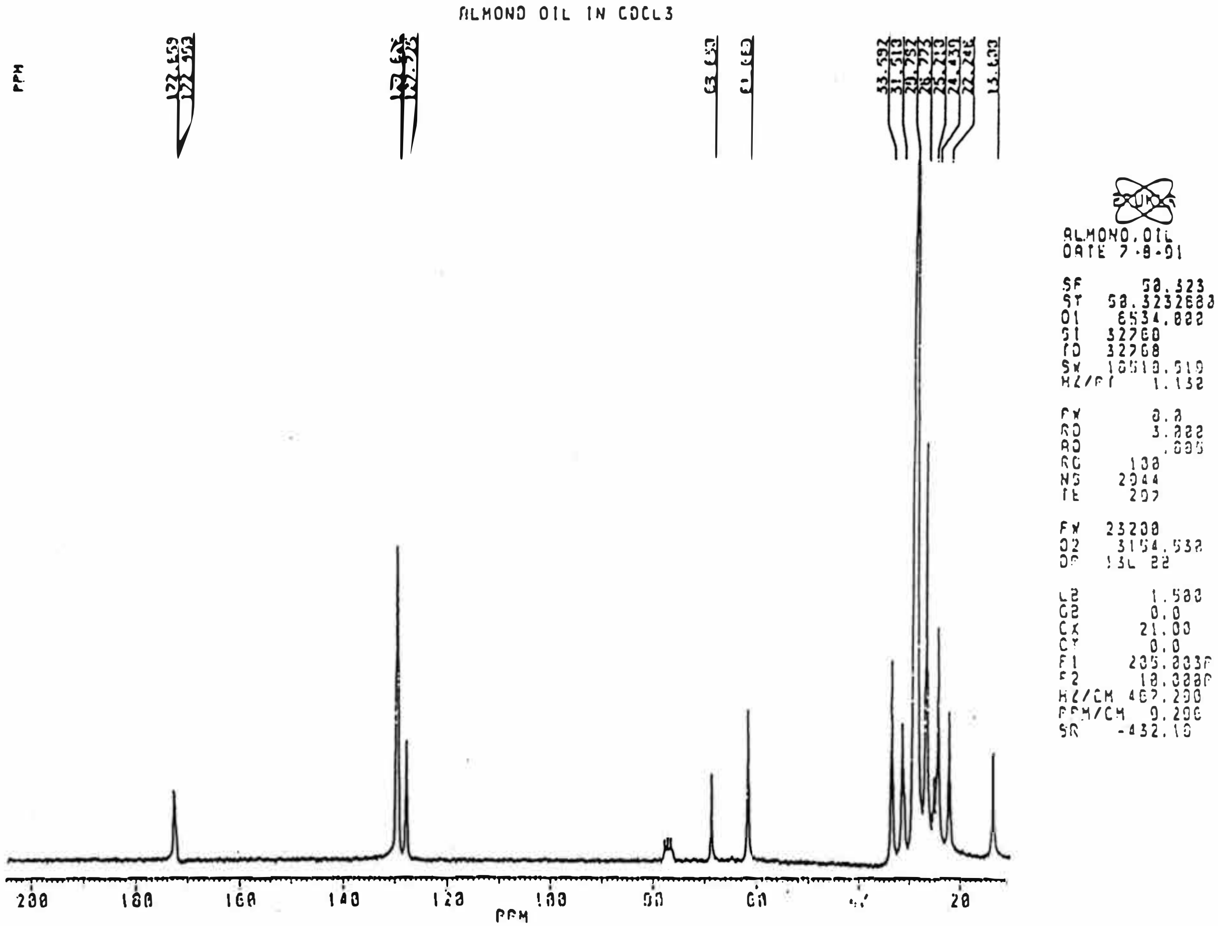

OLIVE OIL IN CDCL3
PPM
200
180
160
140
120
100
80
60
40
20
PPM


MAZOLA CORN OIL IN CDCL3
PPM
220
160
140
120
100
80
60
40
20
DATE 14-5-01


COTTON SEED OIL IN CDCL3
PPM
COTTON.OIL
DATE 10-5-91
SF 50.323
SY 50.3232090
O1 6534.000
SI 32768
TD 32768
SW 10518.519
HZ/PT 1.130
PW 8.0
RD 3.000
AQ .005
RG 160
NS 1106
TE 297
FW 23200
O2 3154.530
DP 13L 22
LB 0.0
GB 0.0
CX 21.00
CY 0.0
F1 205.003P
F2 10.000P
HZ/CM 467.290
PPM/CM 9.286
SR -402.79
200
180
160
140
120
100
80
60
40
20
PPM

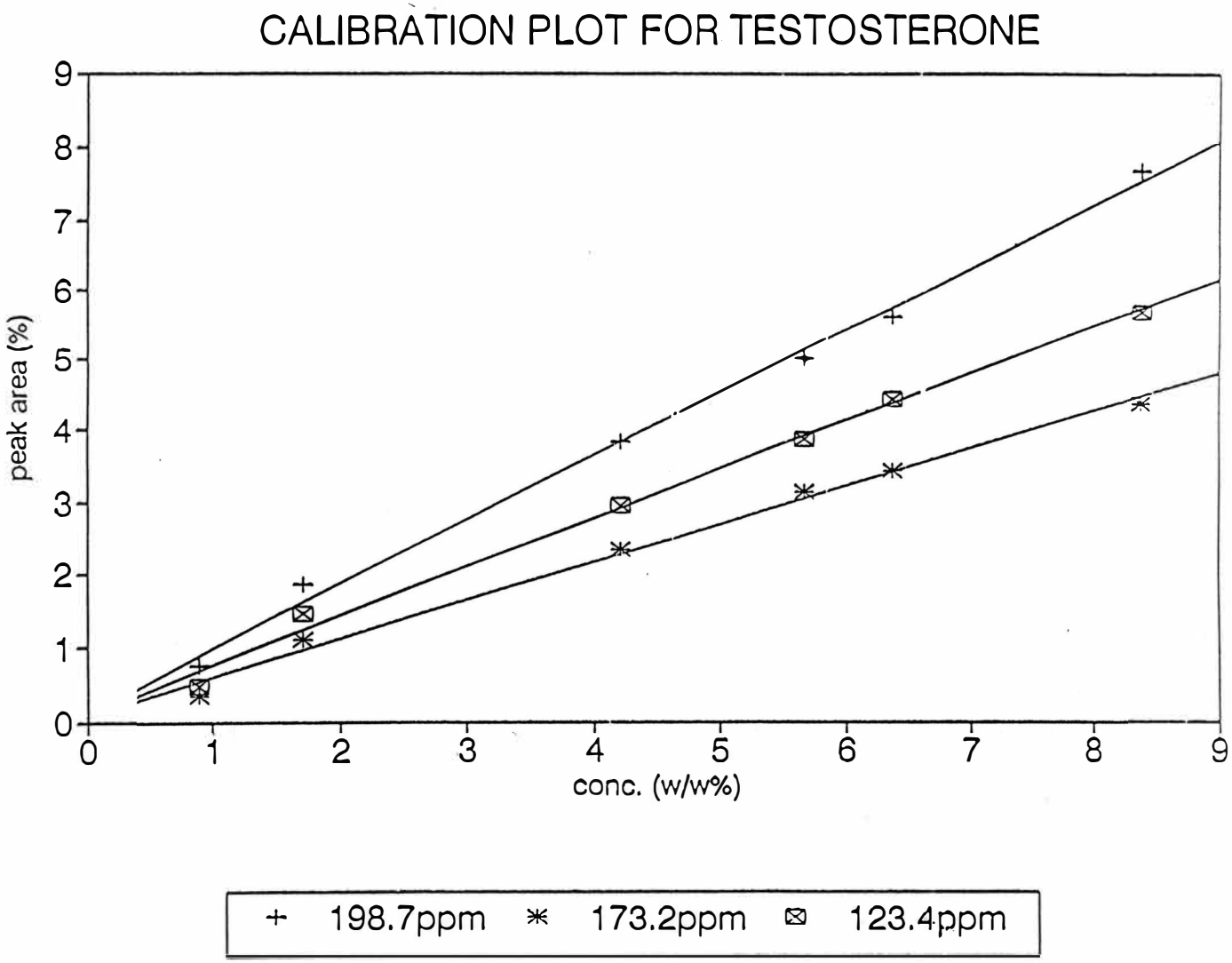

COCONUT OIL IN CDCL3
122.322
65.312
31.884
33.922
31.663
29.349
24.842
22.612
14.092
COCONUT.OIL
DATE 8-8-91
SF 50.323
ST 50.3232080
SI 65536.000
SI 32768
TD 32768
SW 16518.519
HZ/PT 1.132
PW 8.3
RD 3.000
AQ .885
RG 230
NS 2433
TE 297
FW 23200
O2 3154.530
DE 13L 88
L2 1.500
GO2 8.3
CX 21.80
CY 8.3
F1 205.0050
F2 10.0000
HZ/CM 467.200
PPM/CM 9.280
SR -393.75
200
180
160
140
120
100
80
60
40
20
PPM

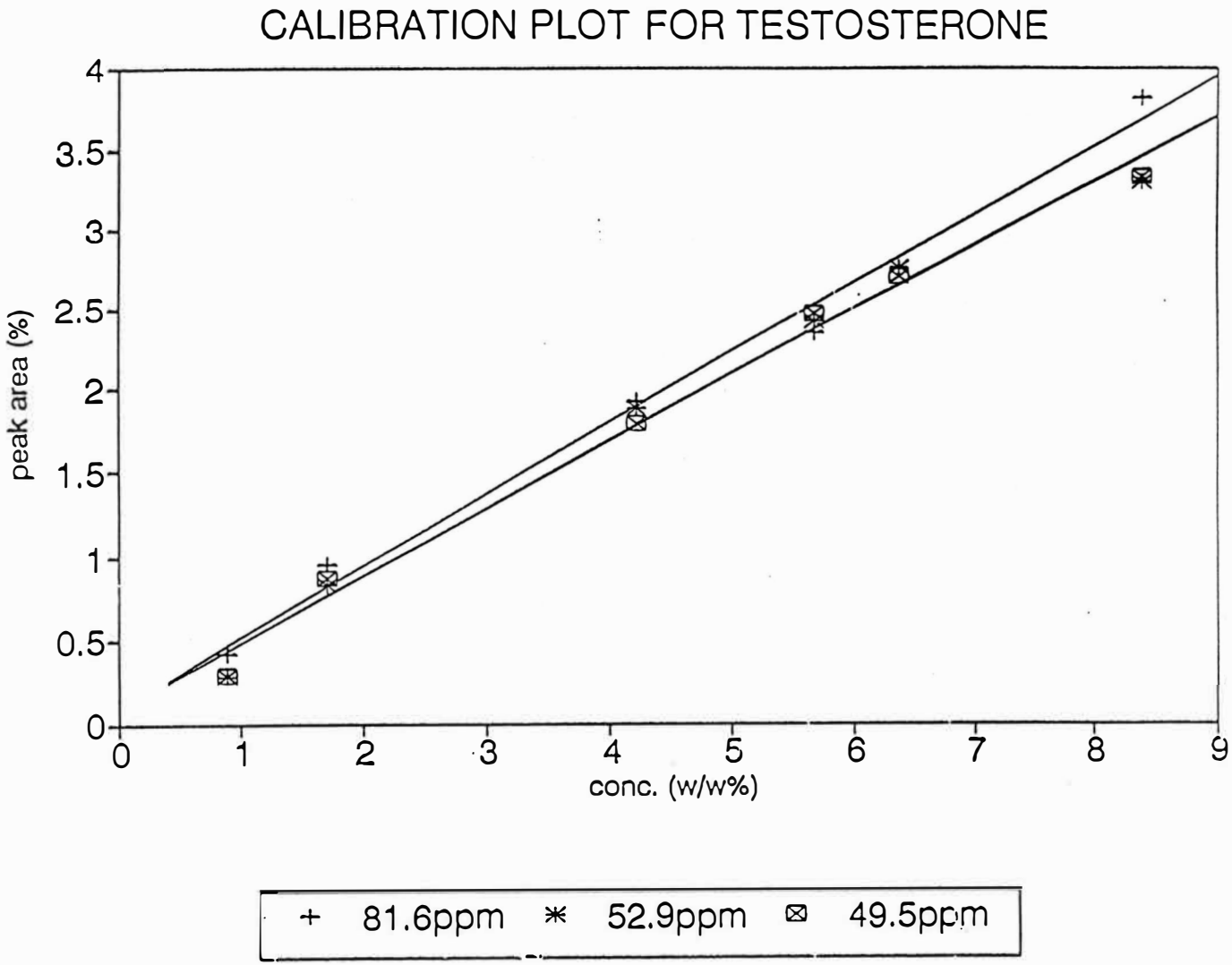

CANOLA OIL IN CDCL3
PPM

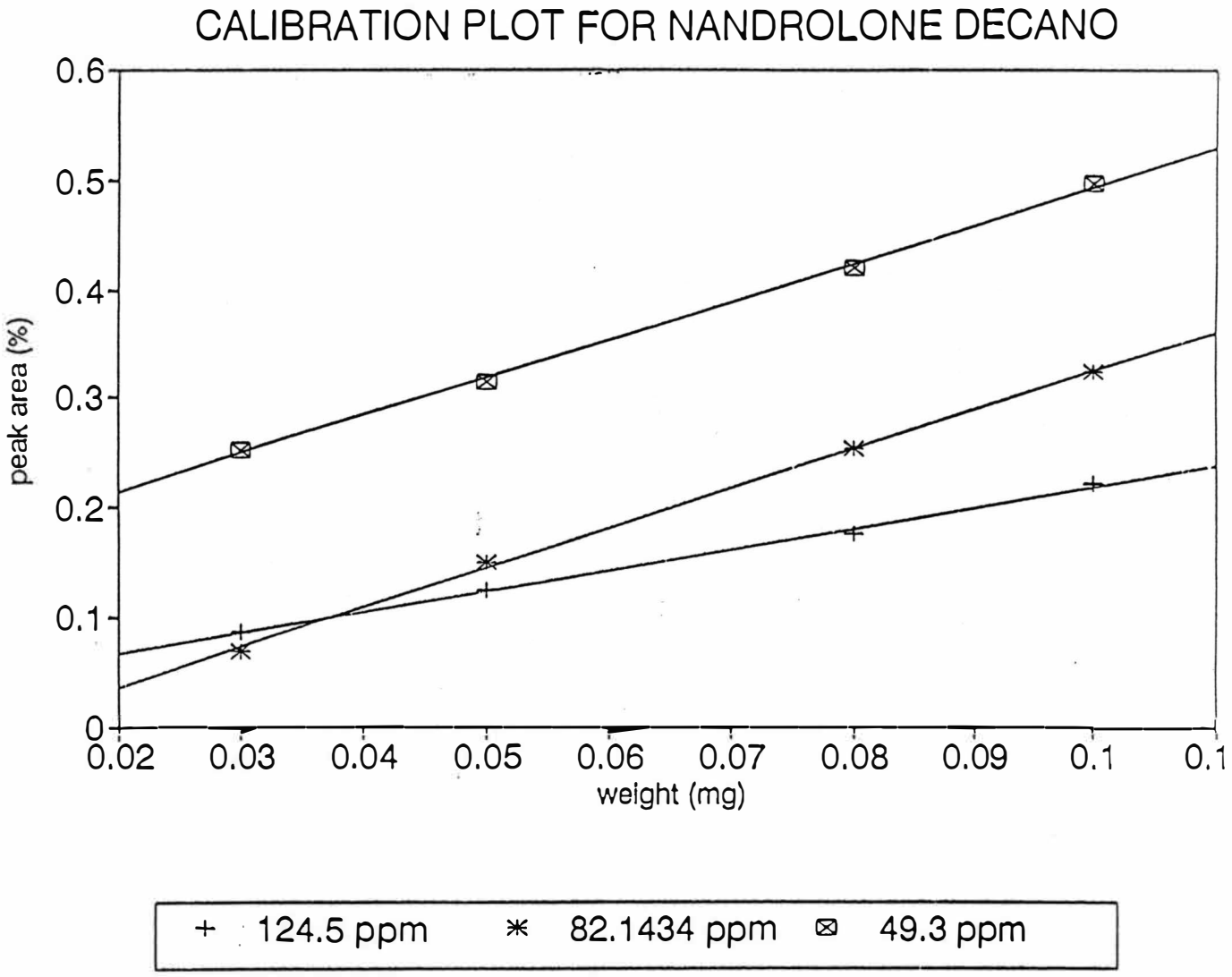

ALMOND OIL IN CDCL3
PPM
33.592
31.513
22.24
ALMOND.OIL
DATE 7-8-91
SF 50.323
ST 50.3232680
O1 6534.082
SI 32768
TD 32768
HZ/PT 1.132
RD 3.822
RG 100
NS 2944
FW 23200
O2 3154.538
L2 1.500
G2 0.0
CX 21.00
CY 0.0
F1 205.003P
F2 10.000P
HZ/CM 467.200
PPM/CM 9.200
SR -432.10
200
180
160
140
120
100
80
60
20
PPM

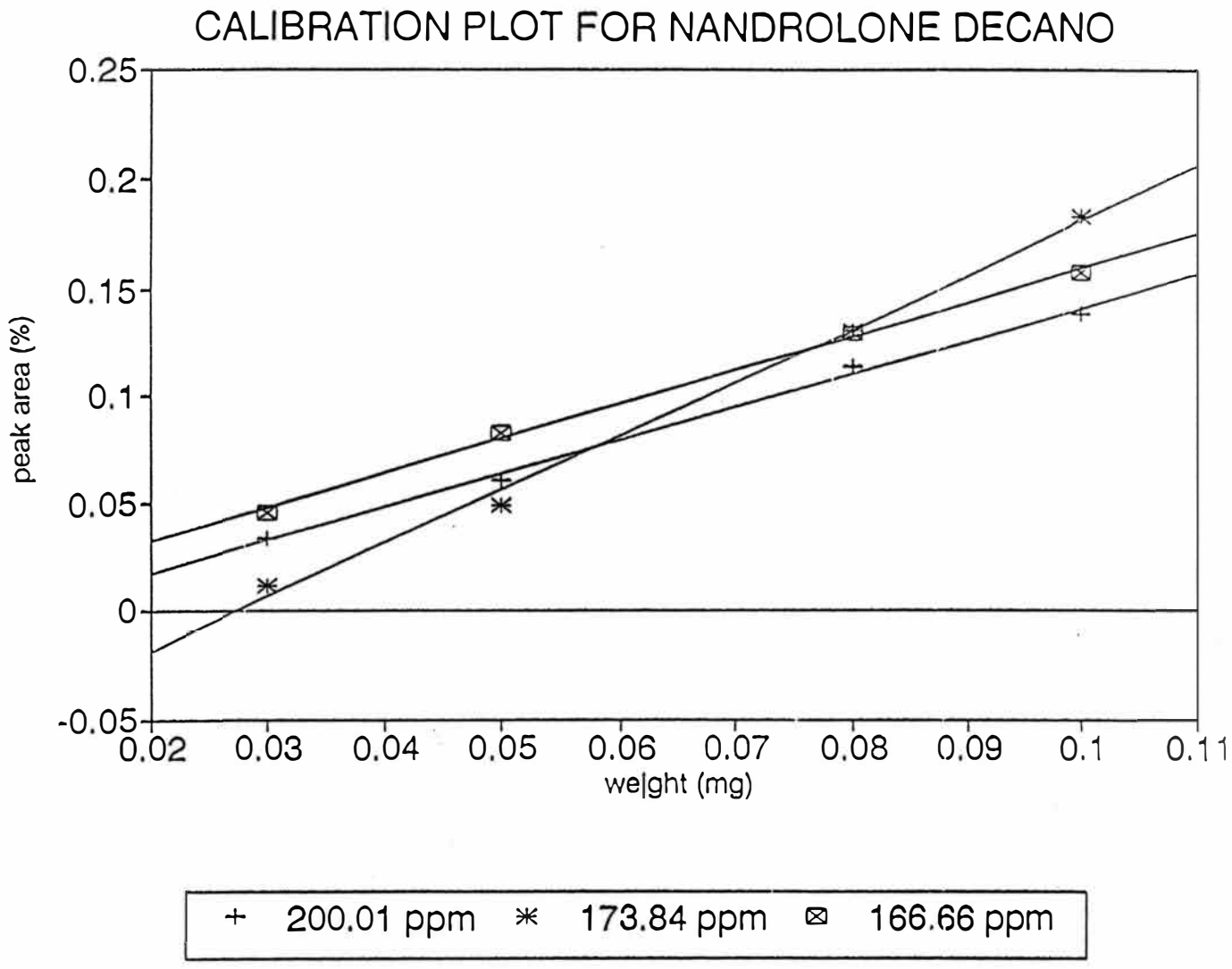
Appendix C

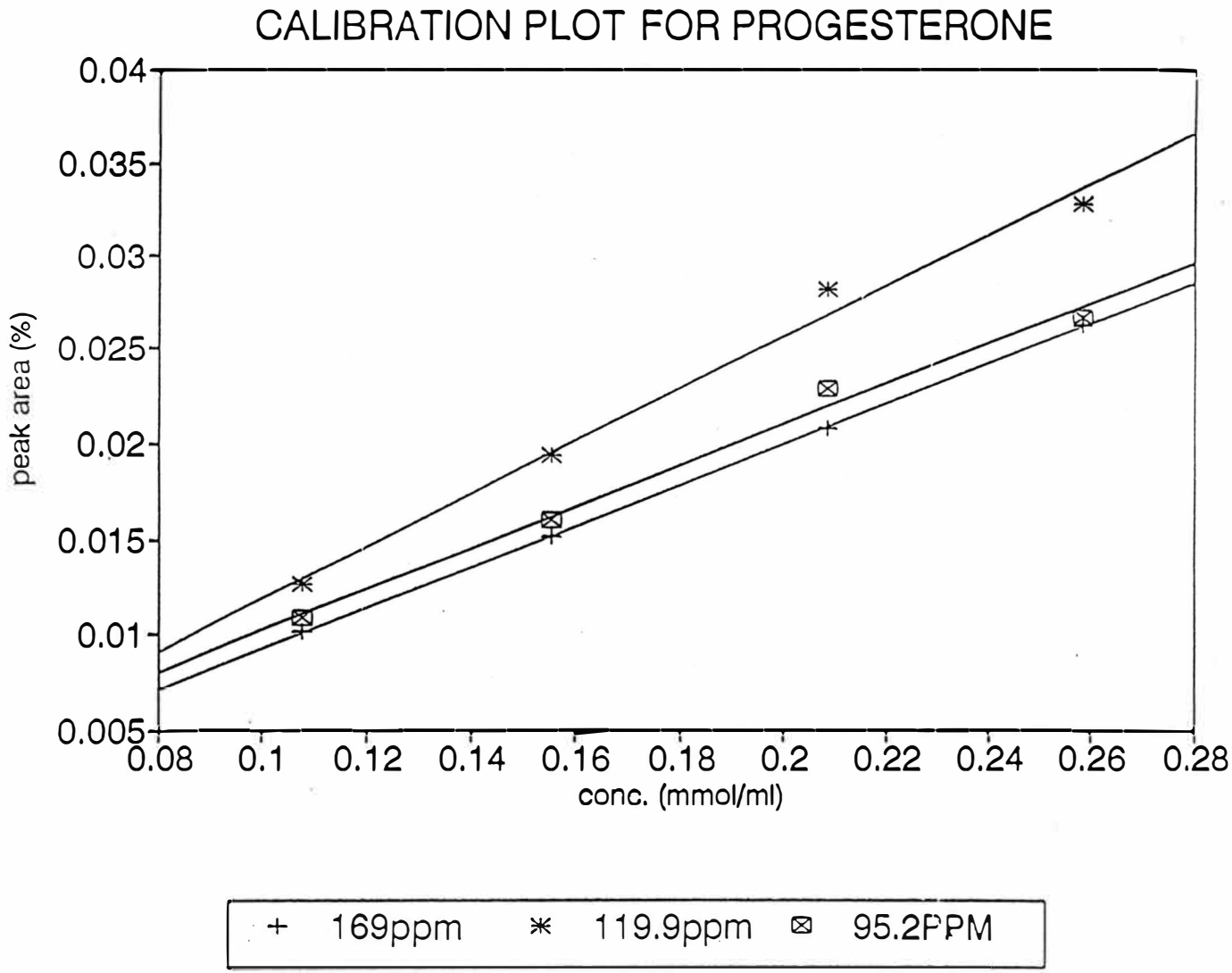
Calibration Plots

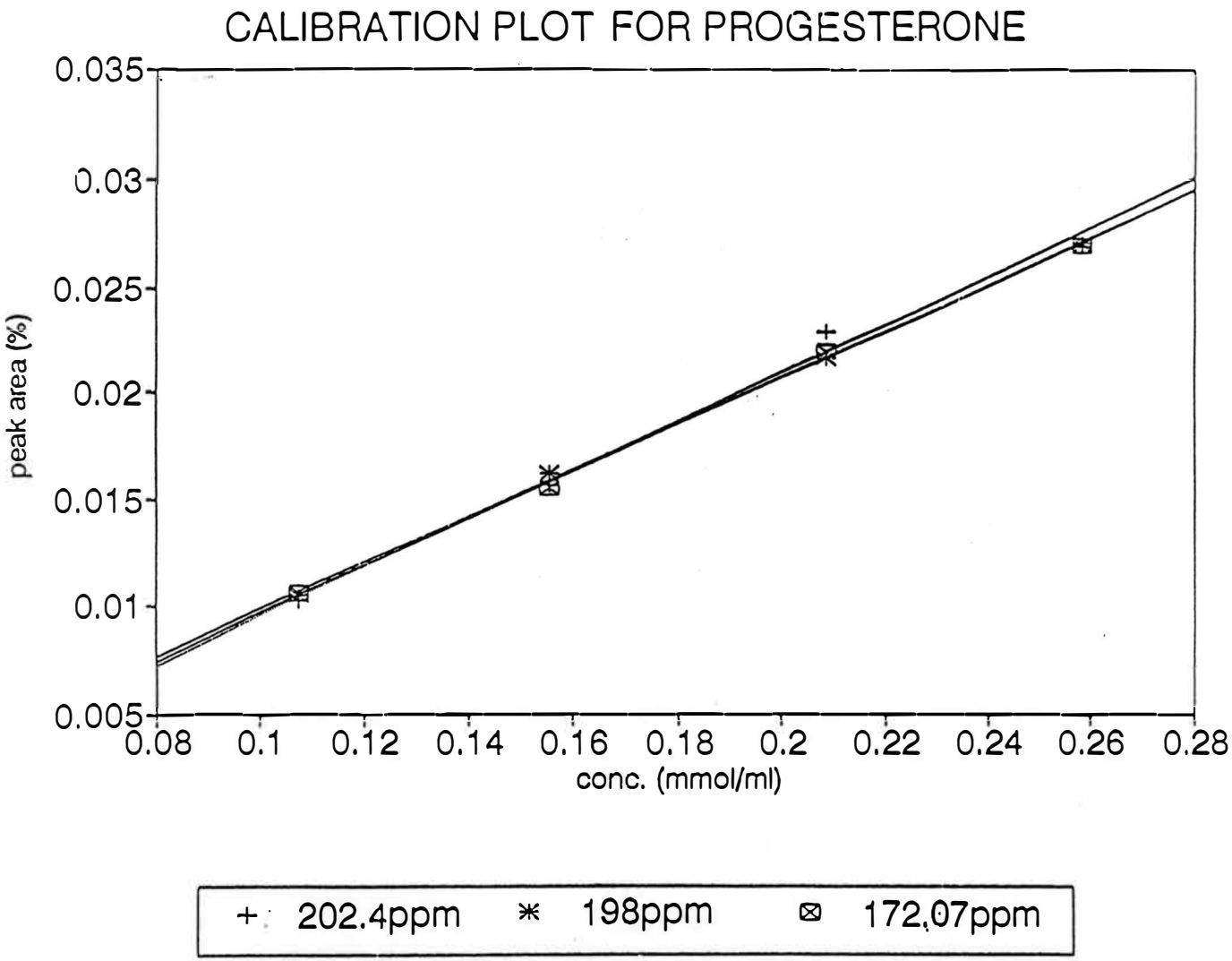

CALIBRATION PLOT FOR TESTOSTERONE
peak area (%)
conc. (w/w%)
0
1
2
3
4
5
6
7
8
9
+ 198.7ppm
* 173.2ppm
⊠ 123.4ppm

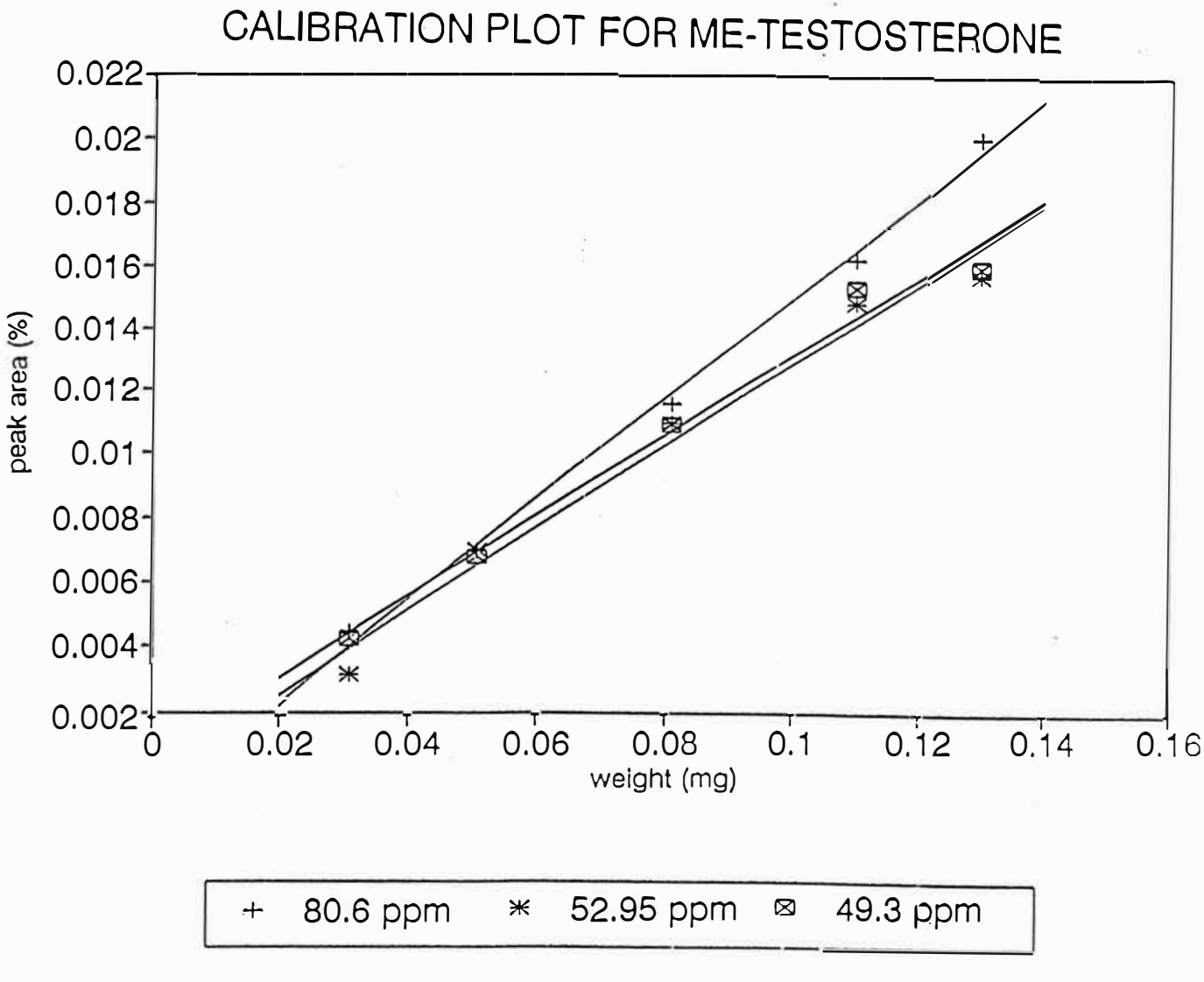

CALIBRATION PLOT FOR TESTOSTERONE
peak area (%)
conc. (w/w%)
0
0.5
1
1.5
2
2.5
3
3.5
4
0
1
2
3
4
5
6
7
8
9
+ 81.6ppm
* 52.9ppm
⊠ 49.5ppm

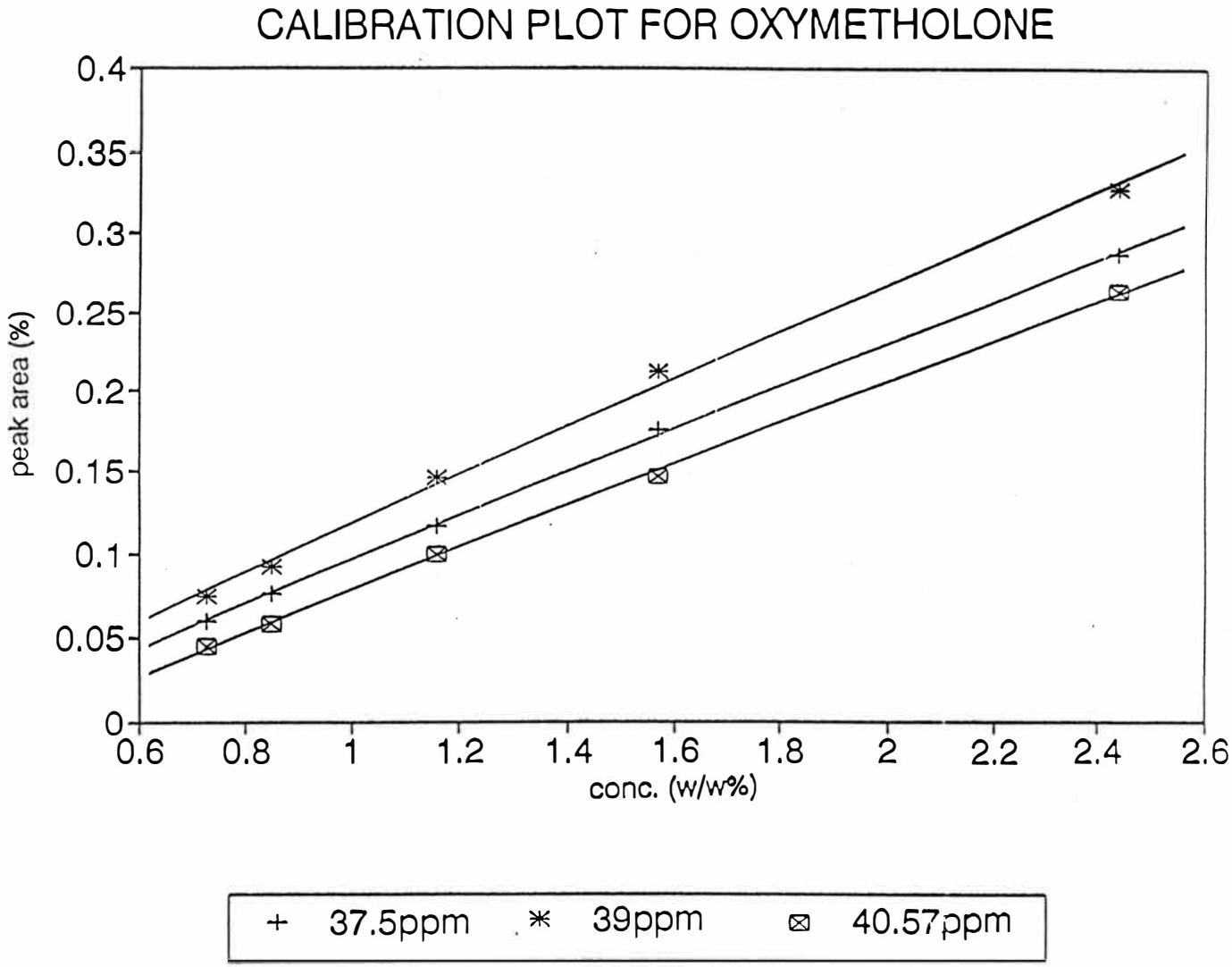

CALIBRATION PLOT FOR NANDROLONE DECANO
peak area (%)
0.6
0.5
0.4
0.3
0.2
0.1
0
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.1
0.1
weight (mg)
+ 124.5 ppm
* 82.1434 ppm
⊠ 49.3 ppm

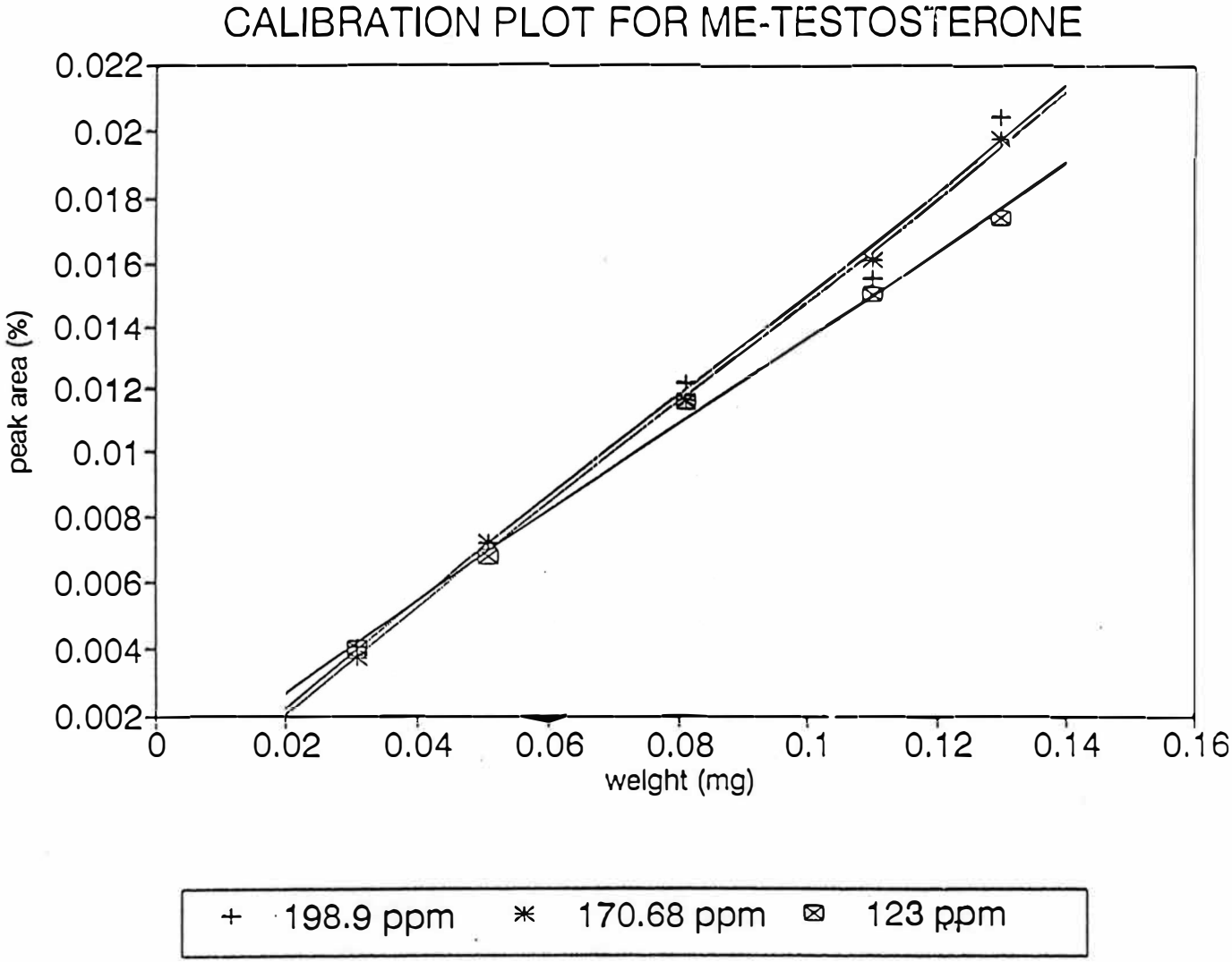

CALIBRATION PLOT FOR NANDROLONE DECANO
0.25
0.2
0.15
0.1
0.05
0
-0.05
peak area (%)
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0.1
0.11
weight (mg)
+ 200.01 ppm
* 173.84 ppm
⊠ 166.66 ppm

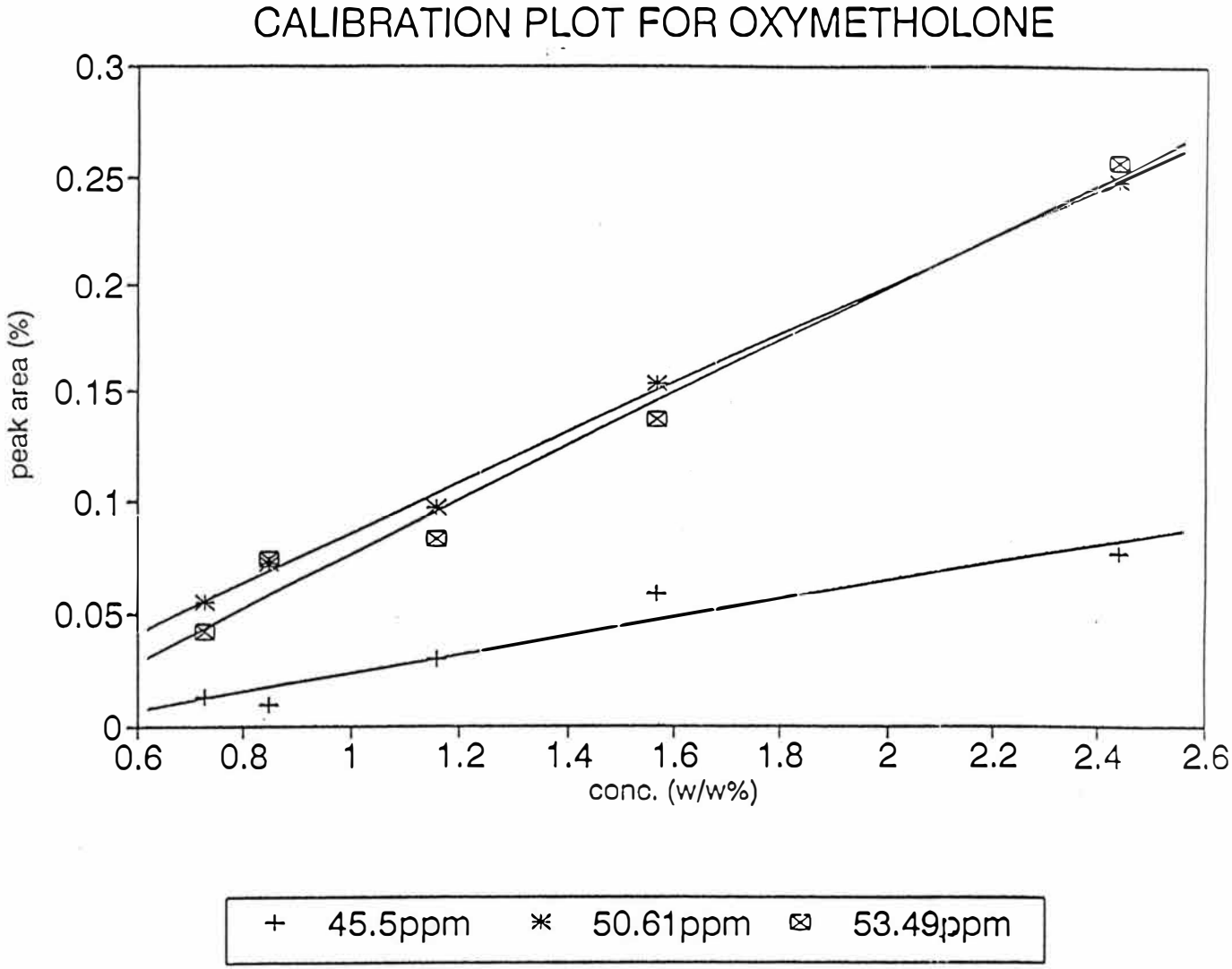

CALIBRATION PLOT FOR PROGESTERONE
peak area (%)
0.04
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0.08
0.1
0.12
0.14
0.16
0.18
0.2
0.22
0.24
0.26
0.28
conc. (mmol/ml)
+ 169ppm
* 119.9ppm
⊠ 95.2PPM


CALIBRATION PLOT FOR PROGESTERONE
peak area (%)
0.035
0.03
0.025
0.02
0.015
0.01
0.005
0.08
0.1
0.12
0.14
0.16
0.18
0.2
0.22
0.24
0.26
0.28
conc. (mmol/ml)
+ 202.4ppm
* 198ppm
⊠ 172.07ppm


CALIBRATION PLOT FOR ME-TESTOSTERONE
peak area (%)
0.022
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
weight (mg)
80.6 ppm
52.95 ppm
49.3 ppm


CALIBRATION PLOT FOR OXYMETHOLONE
peak area (%)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
2.4
2.6
conc. (w/w%)
+ 37.5ppm
* 39ppm
⊠ 40.57ppm


CALIBRATION PLOT FOR ME-TESTOSTERONE
peak area (%)
0.022
0.02
0.018
0.016
0.014
0.012
0.01
0.008
0.006
0.004
0.002
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
welght (mg)
+ 198.9 ppm
* 170.68 ppm
⊠ 123 ppm


CALIBRATION PLOT FOR OXYMETHOLONE
peak area (%)
0.3
0.25
0.2
0.15
0.1
0.05
0
0.6
0.8
1
1.2
1.4
1.6
1.8
2
2.2
2.4
2.6
conc. (w/w%)
+ 45.5ppm
* 50.61ppm
⊠ 53.49ppm

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