

Carbon isotopic (^{13}C and ^{14}C) composition of synthetic estrogens and progestogens

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ABSTRACT

RATIONALE:

Steroids are potent hormones that are found in many environments. Yet, contributions from synthetic and endogenous sources are largely uncharacterized. The goal of this study was to evaluate whether carbon isotopes could be used to distinguish between synthetic and endogenous steroids in wastewater and other environmental matrices.

METHODS:

Estrogens and progestogens were isolated from oral contraceptive pills using semi-preparative liquid chromatography/diode array detection (LC/DAD). Compound purity was confirmed by gas chromatography-flame ionization detection (GC-FID), gas chromatography/time-of-flight mass spectrometry (GC/TOF-MS) and liquid chromatography/mass spectrometry using negative electrospray ionization (LC/ESI-MS). ^{13}C content was determined by gas chromatography/isotope ratio mass spectrometry (GC/IRMS) and ^{14}C was measured by accelerator mass spectrometry (AMS).

RESULTS:

Synthetic estrogens and progestogens are ^{13}C depleted ($\delta^{13}\text{C}_{\text{estrogen}} = -30.0 \pm 0.9 \text{ ‰}$; $\delta^{13}\text{C}_{\text{progestogen}} = -30.3 \pm 2.6 \text{ ‰}$) compared to endogenous hormones ($\delta^{13}\text{C} \sim -16 \text{ ‰}$ to -26 ‰). The ^{14}C content of the majority of synthetic hormones is consistent with synthesis from C_3 plant-based precursors, amended with “fossil” carbon in the case of EE_2 and norethindrone acetate. Exceptions are progestogens that contain an ethyl group at carbon position 13 and have entirely “fossil” ^{14}C signatures.

CONCLUSIONS:

Carbon isotope measurements have the potential to distinguish between synthetic and endogenous hormones in the environment. Our results suggest that ^{13}C could be used to discriminate endogenous from synthetic estrogens in animal waste, wastewater effluent, and natural waters. In contrast, ^{13}C and ^{14}C together may prove useful for tracking synthetic progestogens.

INTRODUCTION

In the last two decades, thousands of studies have attempted to characterize the concentration and toxicity of endocrine disrupting chemicals (EDCs) in aquatic environments. Much of this research has focused on steroidal hormones such as estrogens, which are particularly potent EDCs, capable of negatively impacting the normal functioning of aquatic organisms and human populations at extremely low (sub ng L⁻¹) concentrations^[1, 2].

Steroidal hormones include a variety of familiar compounds (e.g., testosterone, progesterone, and estrogen) that are naturally produced by all vertebrates to support growth and development. Many of these so-called endogenous hormones are also synthesized for use in contraceptive, veterinary, scientific, and medical applications^[3].

Both endogenous and synthetic estrogens can enter surface waters by a variety of pathways. Major sources include wastewater treatment plant effluent, septic systems, and livestock operations. Biodegradation is responsible for significant estrogen reductions in treatment plants, septic systems, and natural waters. Yet ~15 % of the estrogen flux typically escapes treatment and is discharged directly to receiving waters^[4]. The specific organisms and mechanisms that support hormone degradation are largely unknown, but they are clearly important for managing the risks associated with EDC pollution in natural waters.

Although many studies have characterized estrogen concentrations in receiving waters, few have specifically characterized the proportions derived from synthetic versus endogenous sources. This information is valuable for evaluating and apportioning “problem” sources, designing effective treatment schemes, and better understanding the environmental fate of synthetic and endogenous estrogens in terms of the mechanisms and byproducts of biodegradation.

Synthetic pharmaceutical hormones often have unique chemical structures that improve their pharmacokinetic profiles. For example, the active estrogen in most oral contraceptive pills, 17 α -ethynylestradiol (EE₂), contains a characteristic ethynyl group, which sets it apart from endogenous estrogens, extends its half-life in the body, and facilitates its detection in the environment by chemical means (e.g., GC/MS or LC/MS). In other cases, synthetic hormones have identical chemical structures as their endogenous counterparts, making chemical discrimination difficult. For example, some synthetic estrogens administered to cattle or used in human hormone replacement therapy are chemically identical to endogenous estrogens. Fortunately, natural abundance isotope measurements can help distinguish between the two. In fact, stable carbon isotopes (¹²C and ¹³C) have already been used to characterize the provenance of certain chemicals for a variety of purposes, such as verifying product labels, protecting against pharmaceutical fraud, and detecting performance enhancing substance abuse^[5-7].

This last application takes advantage of the fact that endogenous steroids typically contain significantly more ¹³C than synthetic steroids^[8-11]. The present study was designed to test whether synthetic estrogens and progestogens, such as those found in oral contraceptive pills and commercial preparations, are similarly depleted in ¹³C.

Additionally, we hypothesized that coupled radiocarbon (¹⁴C) measurements could improve our ability to characterize the source signatures of synthetic hormones. Radiocarbon (5730 year half-life) is a powerful tracer of “fossil” carbon since petroleum and natural gas no longer contain ¹⁴C while recently fixed CO₂ contains much higher levels of ¹⁴C. This distinction has been useful in a variety of applications, including characterizing the fate of fossil fuel CO₂ and discriminating between natural and synthetic chemicals in the environment.

Here we present a method for isolating the steroidal hormones from oral contraceptive pills and report results for compound-specific ^{13}C and ^{14}C measurements. The goal of this study was to characterize the ^{13}C and ^{14}C signature of numerous synthetic estrogens and progestogens (Figure 1) in order to evaluate whether carbon isotopes could be used to help elucidate the sources of these hormones in complex environmental systems.

EXPERIMENTAL

A semi-preparative liquid chromatographic method was developed to isolate pure EE_2 and progestogens from contraceptive pills for carbon isotope (^{13}C and ^{14}C) analysis (Figure 2). Nine types of oral contraceptive pills and seven commercially available authentic steroid hormone standards were investigated. For the purposes of this study, we assume that these standards are representative of mass-produced synthetic estrogens and progestogens used in medical and veterinary applications. Standards were purchased from Sigma-Aldrich (St. Louis, MO, USA) (estrone (E_1), $\geq 99\%$; 17β -estradiol (E_2), $\geq 98\%$; estriol (E_3), 98% ; 17α -ethynylestradiol (EE_2), $\geq 98\%$; progesterone, $\geq 99\%$; desogestrel, 99.7% ; levonorgestrel, $\geq 99\%$). All solvents were Chromasolv grade from Sigma-Aldrich, and all glassware and filters were baked at $450\text{ }^\circ\text{C}$ for 5 h prior to use.

Pill preparation

Oral contraceptive pills were crushed with an agate mortar and pestle, extracted by sonication in 15 mL methanol for 25 min, and filtered through a GF/F filter (Whatman, Maidstone, Kent, UK). The filtrate was reduced to dryness under vacuum (300 mbar; $60\text{ }^\circ\text{C}$) then dissolved in dichloromethane (10 mL), washed with MilliQ water (Millipore, Billerica, MA, USA; $3 \times 5\text{ mL}$; pH 5), and dried over baked anhydrous Na_2SO_4 ($450\text{ }^\circ\text{C}$; 5 h). This extract was then reduced to dryness under N_2 ($40\text{ }^\circ\text{C}$) and finally reconstituted in 500 μL 70:30 methanol/MilliQ water.

Liquid chromatography, coupled to mass spectrometry (LC/MS) and UV-visible diode array detection (LC/DAD), was used to confirm the identities of steroidal hormones in each extract. The LC/MS instrument (Agilent 6130 single quadrupole mass spectrometer; Santa Clara, CA, USA) was operated in negative electrospray ionization (ESI), full scan (m/z 120 – 400) mode. The LC/DAD instrument (Agilent 1260) monitored three wavelengths (210, 254, and 280 nm) and collected full UV-visible (210 – 400 nm) spectra at the base and apex of each chromatographic peak.

Fraction collection

For compound isolation, the LC/DAD system was configured to collect fractions corresponding to the individual EE_2 and progestogen peaks. Separation was achieved on a Hypersil GOLD C18 aQ column (Thermo Fisher Scientific, Waltham, MA, USA; $5\text{ }\mu\text{m}$, $250 \times 4.6\text{ mm}$) using gradient elution (70 – 100 % methanol; $2\text{ }\% \text{ min}^{-1}$) at a flow rate of 1.5 mL min^{-1} and a column temperature of $25\text{ }^\circ\text{C}$. In all cases EE_2 eluted first (4.55 – 5.10 min), followed by the progestogens: levonorgestrel (5.40 – 5.90 min), norethindrone acetate (6.35 – 6.95 min), norgestimate “a” (8.35 – 8.90 min), norgestimate “b” (8.90 – 9.45 min), and desogestrel (14.00 – 14.50 min). Fractions were collected from two to four individual 100 μL injections, then they were combined and stored at $-20\text{ }^\circ\text{C}$. Clean solvent (70:30 methanol/MilliQ water) was also injected ($11 \times 100\text{ }\mu\text{L}$) so that corresponding fractions could be used to correct for organic

interferences (“column bleed”) present in the mobile phase and released from the column during each time interval.

Fraction clean-up

All sample and column bleed fractions were reduced to dryness under N₂ (40 °C) and transferred to a ¹⁴C-clean laminar flow hood for clean up on 250 mg of fully activated (450 °C; 8 h) silica gel (100 – 200 mesh). After sample loading, hexane (2 mL), ethyl acetate (3 mL), and methanol (2 mL) were successively passed through the silica gel column. All compounds of interest eluted in the ethyl acetate fraction, from which a small aliquot (17 µL or ~ 0.5 %) was removed to confirm fraction purity by GC with flame ionization detection (FID) and GC coupled with time-of-flight mass spectrometry (TOF-MS). Methanol fractions were also analyzed to confirm that target compounds eluted completely in the ethyl acetate fraction.

Fraction purity and blank assessment

In addition to the LC/DAD and LC/MS analyses mentioned above, GC-FID and GC/TOF-MS analyses confirmed the identity and purity of each ethyl acetate fraction. Moreover, a blank ethyl acetate fraction collected from the silica gel clean-up step and all of the column bleed fractions were indistinguishable from a GC blank injection. Non-active sugar pills included in oral contraceptive pill packaging were processed alongside sample pills and confirmed that cross-contamination was not a problem during pill preparation. The six column bleed fractions were subsequently quantified and analyzed for ¹⁴C.

Quantification and combustion

The remainders of each ethyl acetate fraction were transferred to a pre-baked (850 °C; 5 h) quartz tube and blown dry under N₂ (40 °C). Pre-baked CuO (850 °C; 5 h) was added and each tube was evacuated and sealed on a vacuum line, then combusted at 850 °C for 5 h. The resulting CO₂ was quantified manometrically and split into three aliquots. One aliquot was measured for stable carbon isotopic composition (δ¹³C value) on a VG PRISM series II mass spectrometer (VG Isotech, defunct) at the National Ocean Sciences Accelerator Mass Spectrometry (NOSAMS) facility at Woods Hole Oceanographic Institution (Woods Hole, MA, USA). The second aliquot was used for ¹⁴C analysis on the compact accelerator mass spectrometer MICADAS equipped with a gas ion source for small samples [12-15] at the Laboratory for Ion Beam Physics at ETH (Zürich, Switzerland). The third aliquot was archived.

Small amounts (~ 1 mg) of authentic estrogen and progestogen standards were also submitted to NOSAMS for ¹³C and ¹⁴C analysis without preprocessing. All radiocarbon data are reported according to accepted conventions [16, 17].

Column bleed corrections

By the end of sample processing, the column bleed fraction corresponding to the EE₂ time window contained 0.9 µg carbon per LC run, or 3.7 % of a typical EE₂ sample. The ¹⁴C content of this fraction (Δ¹⁴C = -947 ± 8 ‰) was used to correct sample EE₂ Δ¹⁴C values for contributions of carbon carried by the LC mobile phase. Progestogen samples were corrected similarly using the appropriate column bleed fractions, which contained ~ 0.5 µg carbon (Δ¹⁴C = -825 ± 27 ‰) per LC run, or no more than 1.5 % of the smallest progestogen sample.

The reported Δ¹⁴C values are also corrected for instrumental blanks and normalized using the oxalic acid standard OX-II; reported errors represent propagated errors from all corrections.

Due to insufficient carbon in column bleed fractions, $\delta^{13}\text{C}$ values (all referenced to VPDB) are only corrected for instrumental blanks.

RESULTS AND DISCUSSION

Estrogens

The ^{14}C content of EE_2 isolated from oral contraceptive pills ($\Delta^{14}\text{C} = -189 \pm 18 \text{‰}$; Table 1, Figure 3) suggests that EE_2 is synthesized from primarily plant-based steroidal starting materials amended with small amounts of fossil (natural gas or petrochemical) carbon. The mean ^{13}C content of EE_2 ($\delta^{13}\text{C} = -29.4 \pm 0.3 \text{‰}$; Table 1, Figure 3) is consistent with known steroidal precursors (β -sitosterol, stigmasterol, diosgenin; Figure 1) found in C_3 plants such as soybean (*Glycine max*) and wild yam (*Discorea* spp.)^[18-21].

It is possible to quantify the fraction of EE_2 carbon derived from fossil (f_{fossil}) and modern (f_{modern}) sources using the following equations,

$$f_{\text{fossil}} + f_{\text{modern}} = 1 \quad (1)$$

$$\Delta^{14}\text{C}_{\text{measured}} = f_{\text{fossil}}(\Delta^{14}\text{C}_{\text{fossil}}) + f_{\text{modern}}(\Delta^{14}\text{C}_{\text{modern}}) \quad (2)$$

where we assume that $\Delta^{14}\text{C}_{\text{fossil}} = -1000 \text{‰}$ and $\Delta^{14}\text{C}_{\text{modern}} = 50 \text{‰}$. Measured $\Delta^{14}\text{C}$ values indicate that, on average, 23 % (or 4.5 out of 20) of the carbon atoms in EE_2 are derived from fossil sources. If we assume that both ethynyl carbon atoms in EE_2 are petrochemical, then the plant-based precursor compounds must also contain some fossil carbon atoms. These fossil carbons are likely derived from CO_2 amendments to commercial greenhouses from natural gas heating exhaust^[22, 23] and/or from plants grown in areas heavily impacted by fossil fuel emissions^[24]. It is interesting to note that Eglinton et al.^[25] found similar ^{14}C content ($\Delta^{14}\text{C} = -113 \text{‰}$) in a sample of *Crassula argentea* grown in a greenhouse heated by natural gas.

In contrast, synthetic estrogens that lack an ethynyl group (such as E_1 , E_2 , and E_3) exhibit an entirely modern ^{14}C signature (Figure 3), implying that fossil CO_2 amendment is not universal in steroid precursor plant cultivation and that, by itself, ^{14}C cannot distinguish between synthetic estrogens (made for pharmaceutical, scientific, and veterinary applications) and their endogenous counterparts derived from dietary (and primarily modern) carbon.

In this case, ^{13}C seems to hold greater promise for discriminating between synthetic and natural sources since endogenous steroids contain significantly more ^{13}C than the synthetic estrogens ($\delta^{13}\text{C} = -30.0 \pm 0.9 \text{‰}$; Table 1, Figure 3) measured in this study. In general, the $\delta^{13}\text{C}$ values of synthetic steroids ($\delta^{13}\text{C} \sim -27 \text{‰}$ to -34‰) are more ^{13}C depleted than their endogenous counterparts ($\delta^{13}\text{C} \sim -16 \text{‰}$ to -26‰) because synthetic steroid precursors are typically derived from C_3 plants whereas endogenous steroids reflect dietary mixtures of C_3 (e.g., wheat, soybean, fruit, vegetables) and C_4 plants (e.g., corn, sugarcane)^[8-11, 26, 27].

In fact, the unique ^{13}C signature of synthetic steroids is currently used to test athletes for doping with synthetic androgens^[28] and to detect the treatment of cattle with synthetic estrogens^[29]. Our results suggest that ^{13}C could also be used to discriminate endogenous from synthetic estrogens in animal waste, wastewater effluent, and natural waters.

The fact that synthetic estrogens (including E_1 , E_2 , E_3 , and EE_2) are made from mostly plant-derived precursors also highlights an important distinction between “synthetic” and “anthropogenic” chemicals^[30]; that is, if synthetic chemicals with natural counterparts (such as steroids) are made from plants, then the typical isotope approaches that rely on ^{14}C may fail to

detect some synthetic chemicals. This calls into question approaches that use fossil carbon content (^{14}C) alone as a proxy for anthropogenic inputs to the environment. Therefore, ^{14}C data should be regarded as providing a minimum estimate of contributions from synthetic sources.

Progestogens

Like the estrogens, synthetic progestogens contain significantly less ^{13}C ($\delta^{13}\text{C} = -30.3 \pm 2.6 \text{ ‰}$; Table 2, Figure 3) than endogenous steroids. In contrast, however, most progestogens are composed entirely of fossil carbon ($\Delta^{14}\text{C} = -994 \pm 11 \text{ ‰}$; Table 2, Figure 3). The two exceptions are norethindrone acetate ($\Delta^{14}\text{C} = -136 \text{ ‰}$) and progesterone ($\Delta^{14}\text{C} = 54 \text{ ‰}$). Like the estrogens, these two progestogens have ^{14}C contents that suggest they were synthesized from plant-derived steroidal precursors. Notably, norethindrone acetate and progesterone also share a common structural feature – a methyl group at the chiral C-13 position – with both estrogens and steroidal precursor compounds (see Figure 1).

The measured $\Delta^{14}\text{C}$ of norethindrone acetate indicates that 4 out of its 22 carbon atoms are fossil-derived (as per Equation 1 and 2). It is therefore likely that the ethynyl and acetyl groups are composed of fossil carbon while the steroid backbone derives from modern C_3 plant precursors. Yet the measured $\delta^{13}\text{C}$ value of norethindrone acetate (-36.4 ‰) would suggest that these fossil carbon atoms derive from sources with anomalously low ^{13}C content. Since ^{14}C constrains the fraction of carbon from each source ($f_{\text{fossil}} = 0.18$; $f_{\text{steroid backbone}} = 0.82$), we can use the ^{13}C analogue of Equation 2 to determine a range of $\delta^{13}\text{C}$ values for the fossil carbon component of norethindrone acetate:

$$\delta^{13}\text{C}_{\text{measured}} = f_{\text{fossil}} (\delta^{13}\text{C}_{\text{fossil}}) + f_{\text{steroid backbone}} (\delta^{13}\text{C}_{\text{steroid backbone}}) \quad (3)$$

If we assume that the steroid backbone contains carbon with typical synthetic steroid $\delta^{13}\text{C}$ values (-27 ‰ to -34 ‰), then the calculated range of $\delta^{13}\text{C}_{\text{fossil}}$ (-79 ‰ to -47 ‰) suggests that the fossil (i.e., ethynyl and acetyl) carbon in norethindrone acetate is most likely derived from either biogenic methane, which typically has very low $\delta^{13}\text{C}$ values (-110 ‰ to -50 ‰)^[31], or as the result of a strongly fractionating synthetic reaction.

The progestogens that have entirely fossil ^{14}C signatures all share an ethyl group at C-13. Indeed, total synthesis from petrochemical precursors appears to be the preferred synthetic pathway for progestogens with this structural similarity^[19, 20]. These results are also consistent with reports that many 19-norsteroids (such as the progestogens found in oral contraceptive pills) are currently synthesized from non-steroidal petrochemical precursors^[32]. Therefore, together, ^{13}C and ^{14}C may prove useful for tracking this group of synthetic progestogens in human and animal urine, wastewaters, and a range of aquatic systems.

Environmental Forensics

In complex environments (e.g., soils and natural waters), compound-specific isotope measurements have the potential to provide information about the sources and environmental transformations of contaminants, provided that source signatures are sufficiently unique and fractionation factors are known^[33, 34]. However, it would be difficult to investigate steroidal hormone transformation mechanisms using ^{13}C because the fractionation of isotopes at reactive bonds would be “diluted” by the non-reactive carbon atoms in these large molecules^[33]. Still, in cases where the isotope signatures of hormone sources are sufficiently distinct, it should be possible to apportion source inputs using compound-specific isotope measurements.

Moreover, dual (or multiple) isotope analyses can provide additional constraints on source signatures. When combined with ^{13}C measurements, compound-specific ^{14}C data typically provide an added level of specificity^[35] and are a particularly powerful tracer of “fossil” carbon^[36]. Yet in the case of plant-based synthetic chemicals such as estrogens and some progestogens, where ^{13}C content is the strongest indicator of synthetic origin, the considerable expense and effort involved in ^{14}C measurements would not be justified. This is fortuitous because ^{13}C analyses require ~ 100-fold less material, which makes steroid-specific isotope measurements feasible even in complex environmental matrices.

Given typical environmental estrogen concentrations^[37-44], we estimate that compound-specific ^{13}C analysis of individual estrogens would require extraction of ~ 1 – 5 L of wastewater effluent, ~ 100 – 200 g of sewage-impacted coastal sediments, and ~ 200 – 300 L of sewage-impacted coastal waters. By pooling the free, conjugated, and halogenated forms of estrogens, it should be possible to further reduce sample sizes by a factor of two to five^[45, 46].

Taken together, carbon isotopes have proven to be a valuable tool for distinguishing between natural and synthetic chlorinated organic compounds in the ocean^[30, 47], apportioning sources of combustion-derived polycyclic aromatic hydrocarbons^[48, 49] and detecting hormone abuse in human athletes^[7] and cattle operations^[29]. The different $\delta^{13}\text{C}$ signatures of synthetic and endogenous steroidal hormones open up the possibility for characterizing steroid sources and fate in wastewater treatment plants, rivers, lakes, and the coastal ocean.

CONCLUSIONS

We found that synthetic estrogens and progestogens in oral contraceptive pills and commercially synthesized standards contain significantly less ^{13}C than their endogenous counterparts. The majority of synthetic hormones appear to be made from C_3 plant-based precursors, amended with ~20 % fossil carbon in the case of EE_2 and norethindrone acetate. Exceptions are progestogens that contain an ethyl group at carbon position 13 and are entirely synthesized from fossil precursors. Thus, there is potential to use carbon isotopes to quantify inputs of synthetic hormones to the environment, which would improve our understanding of hormone sources and fates and inform the design of effective mitigation solutions.

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Table 1. Estrogen-specific carbon isotope values for oral contraceptive pills and authentic standards

Sample	Compound	Ethynyl group at C-17 position [‡]	$\delta^{13}\text{C}$ (‰) ^a	$\Delta^{14}\text{C}$ (‰) [°]	Lab No.
1 _{EE2}	EE ₂	Y	-29.7	-199 ± 12	ETH-43740
2 _{EE2}	EE ₂	Y	-29.2	-173 ± 11	ETH-43741
3 _{EE2}	EE ₂	Y	-29.6	-205 ± 14	ETH-43742
4 _{EE2}	EE ₂	Y	-28.7	-209 ± 23	ETH-43743
5 _{EE2}	EE ₂	Y	-29.4	-159 ± 13	ETH-43756
6 _{EE2}	EE ₂	Y	-29.5	-186 ± 14	ETH-43758
7 _{EE2}	EE ₂	Y	-29.5	-193 ± 13	ETH-43759
std _{EE2p} [†]	EE ₂	Y	-30.7	-170 ± 13	ETH-43754
std _{EE2} [§]	EE ₂	Y	-31.7	-47 ± 3	OS-92452
std _{E1} [§]	E ₁	N	-30.9	48 ± 4	OS-92449
std _{E2} [§]	E ₂	N	-30.8	47 ± 5	OS-92450
std _{E3} [§]	E ₃	N	-30.5	50 ± 3	OS-92451
Average			-30.0 ± 0.9	-116 ± 108	

[‡]see Figure 1 for steroid carbon position numbering

^ainstrumental precision for $\delta^{13}\text{C}$ measurements is ± 0.1 ‰

[°] $\Delta^{14}\text{C}$ errors (± 1 SD) reflect propagated instrumental errors and column bleed corrections

[†]authentic standard processed alongside oral contraceptive pill samples

[§]authentic standards analyzed at NOSAMS without pre-processing; $\Delta^{14}\text{C}$ errors reflect only instrumental errors; $\delta^{13}\text{C}$ values were measured on a VG Optima SIRMS using a dual inlet source configuration

Table 2. Progestogen-specific carbon isotope values for oral contraceptive pills and authentic standards

Sample [*]	Compound	Substituent at C-13 position [‡]	$\delta^{13}\text{C}$ (‰) ^a	$\Delta^{14}\text{C}$ (‰) [°]	Lab No.
1 _{NL}	levonorgesetrel	ethyl	-27.7	-998 ± 4	ETH-43755
3 _{NA}	norethindrone acetate	methyl	-36.4	-136 ± 12	ETH-43750
4 _{DS}	desogestrel	ethyl	-31.3	-969 ± 7	ETH-43757
6 _{NL}	levonorgestrel	ethyl	-27.4	-1001 ± 2 [†]	ETH-43751
7 _{NTa}	norgestimate “a”	ethyl	-29.9	-994 ± 5	ETH-43752
7 _{NTb}	norgestimate “b”	ethyl	-29.8	-999 ± 2	ETH-43753
std _{NL} [§]	levonorgestrel	ethyl	-29.9	-996 ± 1	OS-92455
std _{PR} [§]	progesterone	methyl	-31.2	54 ± 4	OS-92453
std _{DS} [§]	desogestrel	ethyl	-29.5	-999 ± 1	OS-92454
Average			-30.3 ± 2.6	-782 ± 423	

^{*} samples 2 and 5 not measured

[‡] see Figure 1 for steroid carbon position numbering

^a instrumental precision for $\delta^{13}\text{C}$ measurements is ± 0.1 ‰

[°] $\Delta^{14}\text{C}$ errors (± 1 SD) reflect propagated instrumental errors and column bleed corrections

[†] calculated assuming age of 50,000 y BP

[§] authentic standards analyzed at NOSAMS without pre-processing; $\Delta^{14}\text{C}$ errors reflect only instrumental errors; $\delta^{13}\text{C}$ values were measured on a VG Optima SIRMS using a dual inlet source configuration

FIGURE CAPTIONS

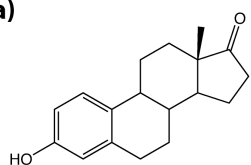
Figure 1. Chemical structures of (a) estrogens, (b) progestogens, and (c) steroidal precursors. Generic steroid carbon position numbers are shown on the structure of β -sitosterol.

Figure 2. Flow chart for isolating, confirming purity, and analyzing the carbon isotopic composition of estrogens and progestogens in oral contraceptive pills.

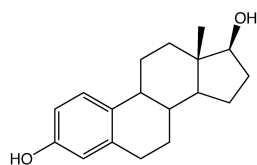
Figure 3. Characteristic carbon isotope values for a variety of estrogens (orange and brown symbols) and progestogens (blue and purple symbols) isolated from oral contraceptive pills (circles) or purchased as authentic standards (diamonds). Average end-member isotope values are shown in grey. These include bulk C_3 and C_4 plant tissue^[50,51], soybean fatty acids^[52], inferred soybean β -sitosterol^[52, 53], bulk yam tissue^[21], endogenous steroids^[10], atmospheric CO_2 ^[54], greenhouse-grown *C. argentea*^[25], petroleum^[55], and biogenic methane^[31]. Data points are labeled with sample numbers, and subscripts indicate the specific estrogen or progestogen according to Tables 1 and 2. Note that C_3 and C_4 plants, petroleum, and methane have larger natural $\delta^{13}C$ ranges than shown^[56].

Figure 1.

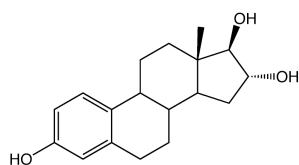
(a)



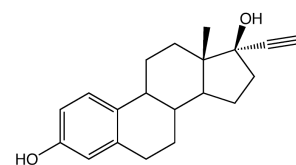
Estrone (E₁), MW 270.4



17β-Estradiol (E₂), MW 272.4

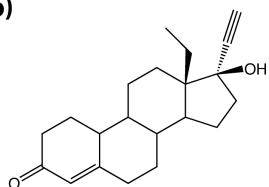


Estriol (E₃), MW 288.4

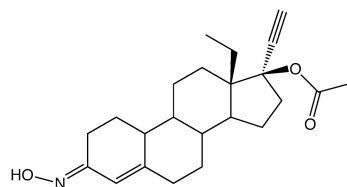


17α-Ethynylestradiol (EE₂), MW 296.4

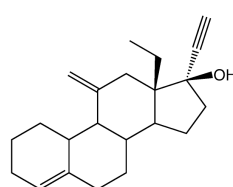
(b)



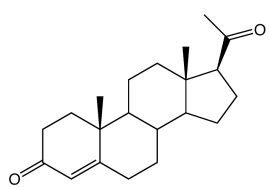
Levonorgestrel, MW 312.4



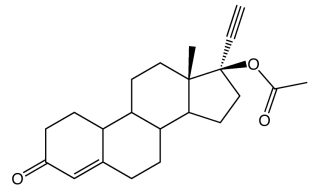
Norgestimate, MW 369.5



Desogestrel, MW 310.5

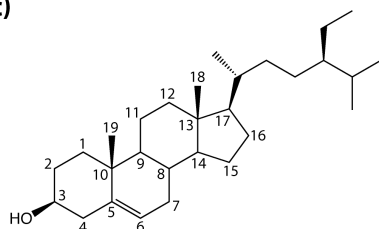


Progesterone, MW 314.5

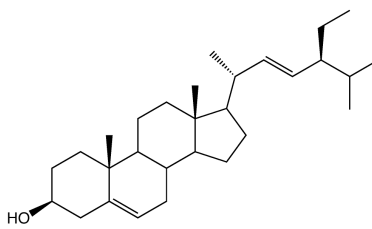


Norethindrone acetate, MW 340.5

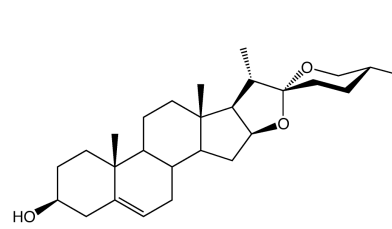
(c)



β-Sitosterol, MW 414.7



Stigmasterol, MW 412.7



Diosgenin, MW 414.6

Figure 2.

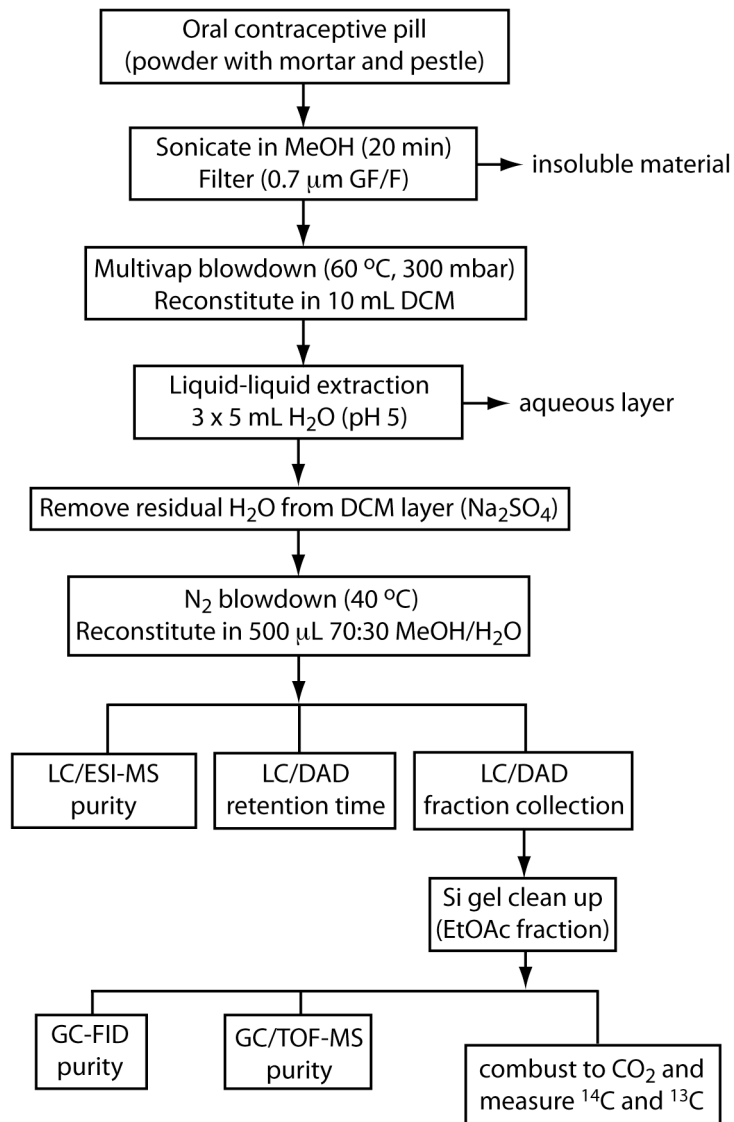


Figure 3.

