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Compound specific detection of endogenous steroid abuse in athletes

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The problem of doping represents a public health and equity issue. The detection of doping practices, however, is itself an issue that requires the development of reliable, non-invasive and cost-effective analytical methods. Endogenous steroid abuse presents a particular problem for doping control laboratories to determine the origin of steroids as being from the *body* or the *bottle*. In an effort to increase circulating levels of biologically active steroids, the abuse of prohormones – steroids capable of being metabolised to testosterone (T) – has provided a significant challenge to identify what specific steroid has been administered. Metabolic schemes provided in the literature propose dehydroepiandrosterone (DHEA; androst-5-ene-3β,17β-diol), androstenedione (ADIONE; androst-4-ene-3,17-diol), 4-androstenediol (4-ADIOL; androst-4-ene-3β,17β-diol) and 5-androstenediol (5-ADIOL; androst-5-ene-3β,17β-diol) to each be excreted as androsterone (A; 5α-androstane-3α-ol-17-one) and etiocholanolone (Et; 5β-androstane-3α-ol-17-one) in urine [1], hence requiring discrete markers of their administration for more effective doping control.

Experimental

DHEA (KAIZEN Inc., Los Angeles, CA, USA [Lot #37033]), ADIONE (ONE-LIFE, Santa Monica, CA, USA [Lot #569]) and 19nor-ADIONE (KAIZEN Inc., Los Angeles, CA, USA [Lot #208006]) were obtained in lots of 60 or 100 capsules, from which 10 were randomly selected for identification, purity and $\delta^{13}C$ analysis using NMR, GC-MS and GC-C-IRMS respectively. 4- and 5-ADIOL (2 g each from Steraloids Inc., Newport, RI, USA) were

obtained as reference materials. Testosterone enanthate (Schering, Germany) was obtained as an injectable preparation (250 mg in non-allergenic oil). Single and multiple administrations of DHEA (ECN-98-42), ADIONE, 4-ADIOL, 5-ADIOL (ECN-05-99) and testosterone enanthate (ECN-04-99) to two healthy males (21 and 30 years old) were approved by the Human Ethics Committee of Southern Cross University, Lismore NSW, Australia. During each of the administration studies, a managed diet was implemented to minimise variations in urinary steroid ^{13}C content.

Urinary steroids originating from the free and glucuronide forms were analysed by GC-MS and GC-C-IRMS according to previously reported procedures [2]. Testosterone was selectively purified for $\delta^{13}C$ analysis using HPLC [3]. Urinary steroids originating from sulfoconjugates were selectively isolated using ion-paired extraction and hydrolysed to their free form using a peer-reviewed method [4]. GC-C-IRMS co-elution of A, DHEA and epiA necessitated the use of HPLC purification, using conditions provided previously [3], to separate and collect individual fractions (Figure 1) containing DHEA (F1 = 10:30 to 11:12), epiA (F2 = 11:12 to 11:42), Et and A (F3 = 11:42 to 12:45).

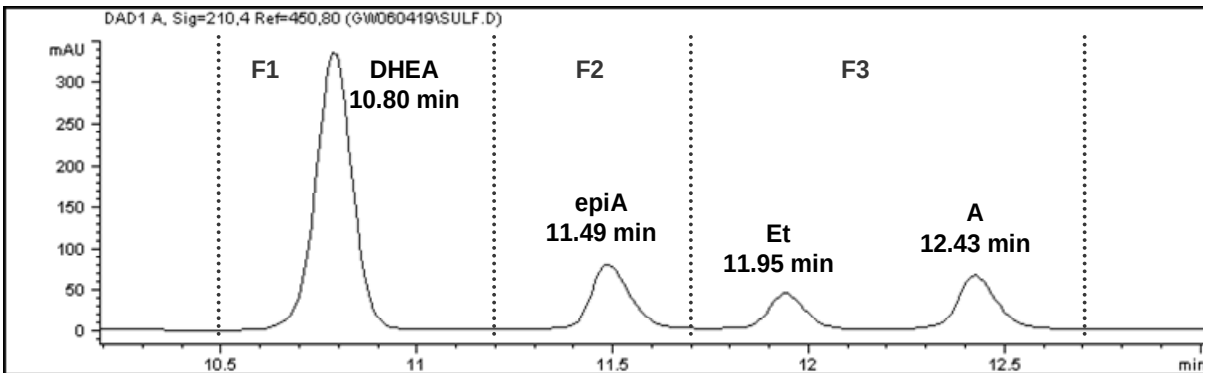


Figure 1: HPLC profile (λ=210 nm) of urinary steroids obtained from the sulfoconjugate fraction (approximately 2 μg) selectively purified for GC-C-IRMS analysis.

Results and discussion

This research has shown that overloaded profiles of A and Et represent the primary indicator of prohormone administration. The new knowledge contributed to endogenous steroid analysis concerning compound specific detection of abuse is summarised by:

Dehydroepiandrosterone (DHEA)

The first application of "looking outside the metabolic box" was described previously [2] to investigate the excretion of 3α,5-cyclo-5α-androstane-6β-ol-17-one (3α,5-cyclo) following DHEA administration. This discovery has not merely provided an answer to the question of DHEA abuse, but more importantly, exposed the limitations that research confined to the analysis of known metabolites places on doping control. Furthermore, the measurement of a urinary by-product originating from ingested synthetic material expands the scope of steroid analysis from a "metabolite-only" domain to a true urinalysis procedure. GC-MS screening concentrations for 3α,5-cyclo of greater than 100 ng/mL will identify samples that require GC-C-IRMS confirmation based on the ^{13}C depleted content of the 3α,5-cyclo marker.

Androstenedione (ADIONE)

Initiating marker discovery represents the most significant challenge to achieving results. Predictive MS approaches can provide practical solutions for anti-doping scientists to investigate new steroids. This "desktop" activity assisted the characterisation of the *tris*-TMS derivative of 4-hydroxyandrostenedione (4OH-ADIONE; androst-4-ene-4-ol-3,17-dione; Figure 2), a metabolite induced by ADIONE administration. A 17-fold increase in the response factor of 4OH-ADIONE relative to 17α-MeT (internal standard) was observed for subject 1 at 18 hours post-administration (Figure 3). At the same time subject 2 displayed a far greater abundance of this peak with an increase of 33-fold (Figure 3). Indeed, the magnitude of the *m/z* 518 ion at 18 hours demonstrated chromatographic overload that resulted in a distorted peak shape and later retention time (15.5 min). The increases in relative response of this compound with ADIONE administration were greater than those observed for the C_6-hydroxylated metabolites reported previously [5-6].

GC-MS screening concentrations for 4OH-ADIONE of greater than 40 ng/mL will identify samples that require GC-C-IRMS confirmation based on the ^{13}C depleted content of the 4OH-ADIONE metabolite [7]. The endogenous nature of 4OH-ADIONE and 3α,5-cyclo was demonstrated, yet their presence was neglected by previous detection strategies that focussed only on known steroids.

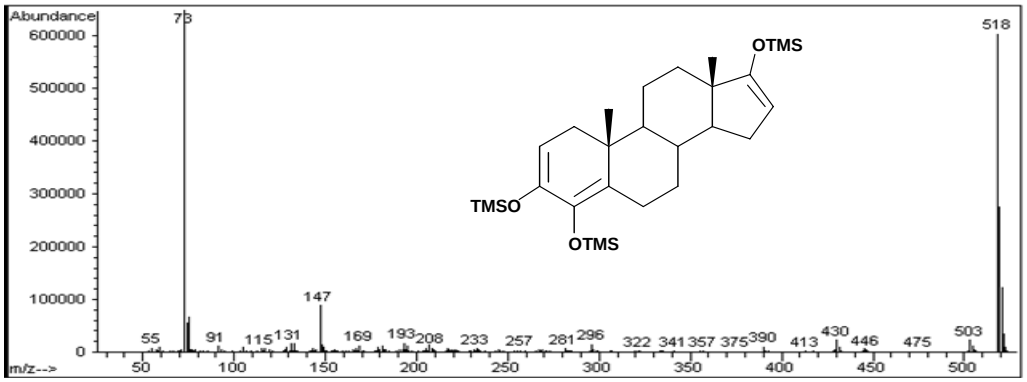
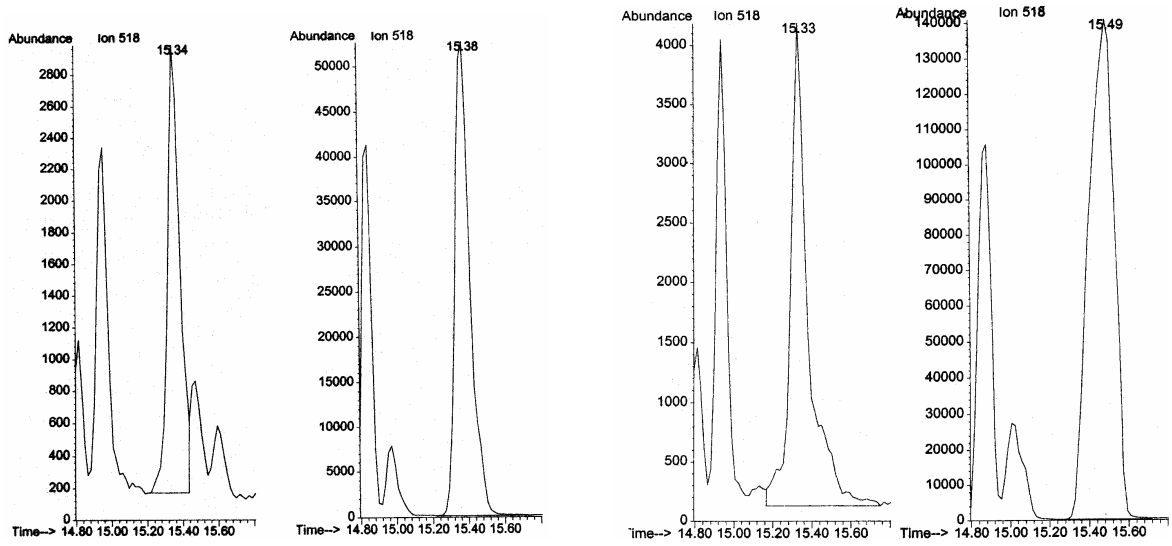


Figure 2: EI full scan mass spectrum of response at 15.4 min (RRt = 1.14 to 17α-MeT) identified to be *tris*-TMS 4OH-ADIONE obtained from subject 1.



t = 0 hours t = 18 hours t = 0 hours t = 18 hours

Figure 3: GC-MS SIM of the diagnostic *m/z* 518 ion monitored between 14.8 and 15.8 min at t=0 and t=18 hours for subject 1 (left) and 2 (right).

4-androstenediol (4-ADIOL)

GC-C-IRMS again played a pivotal role in the incorporation of androst-2,4-diene and androst-3,5-diene into doping control for the purpose of identifying 4-ADIOL abuse (Figure 4). Detection of the androstdienes in the GC-MS steroid screen will identify samples that require GC-C-IRMS confirmation based on the ^{13}C depleted content of these steroid markers (Figure 5). The integration of GC-C-IRMS as a research and sample analysis tool will – in the future – allow the full potential of this technique to be realised. Similarly for the initial detection of 3α,5-cyclo from DHEA abuse, the identification of the androstdienes following 4-ADIOL administration by GC-C-IRMS analysis presented a most interesting result considering the limitations in molecular sensitivity associated with the technique. The retrospective application of GC-MS analysis to incorporating novel steroid markers that are produced by

metabolic and/or urinary rearrangement mechanisms into the routine screening procedure has provided an analytical improvement for doping control.

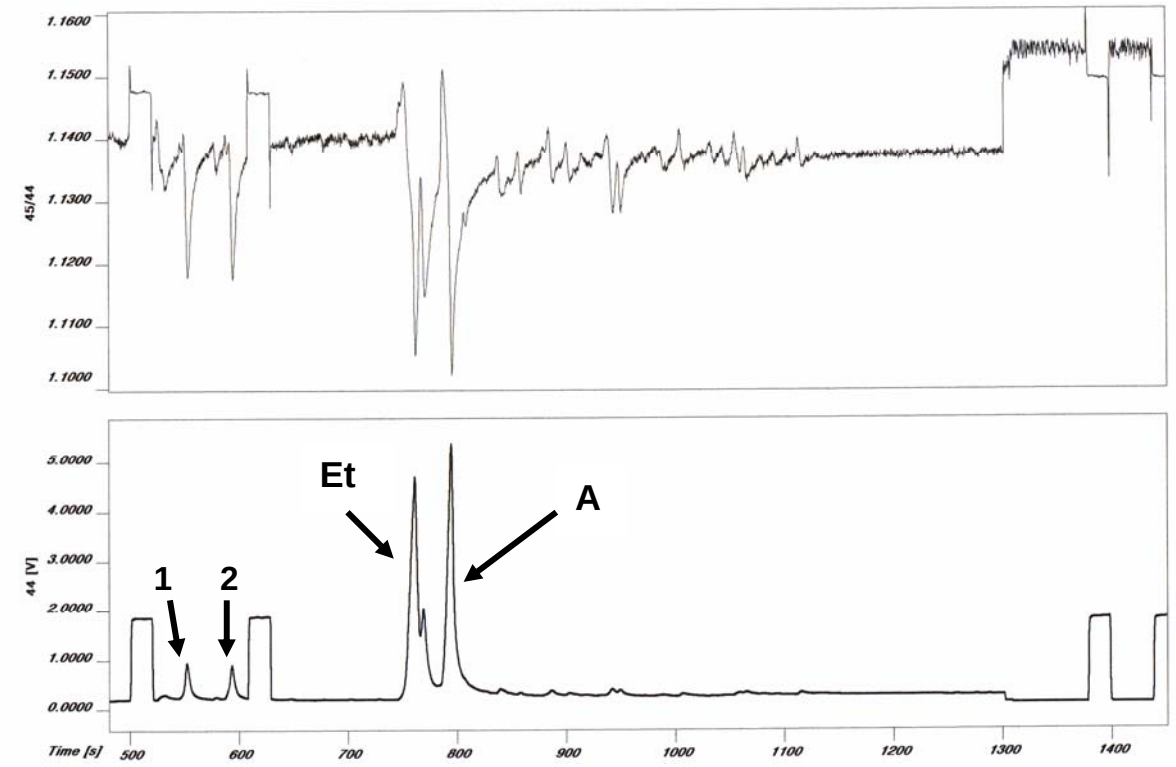


Figure 4: GC-C-IRMS trace showing unknown peaks **1** and **2** at Rt = 549 s and 595 s respectively. Et = 759 s, βαβ-diol is represented by the shoulder at 770 s and A = 800 s.

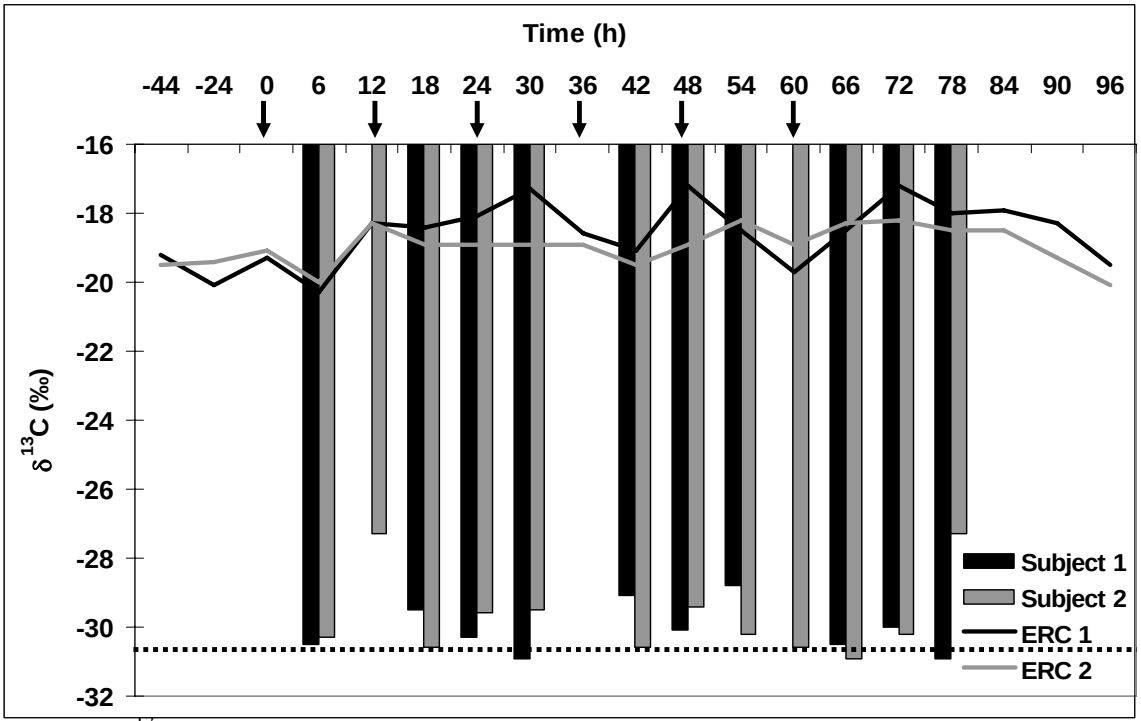


Figure 5: $\delta^{13}C$ values of androst-3,5-diene observed for subjects 1 and 2 from multiple oral (100 mg) doses (marked by ↓) of 4-ADIOL ($\delta^{13}C$ = -30.5‰).

5-androstenediol (5-ADIOL)

A strategy for the detection of 5-ADIOL abuse did not prove to be simple. It was hypothesised, following the detection of DHEA abuse using 3α,5-cyclo, that 5-ADIOL administration could be identified by the presence of its analogous urinary cyclosteroid product. Insufficient excretion of 5-ADIOL-S, however, prohibited this strategy. Similarly, GC-MS screening of 5-ADIOL abuse may be difficult using high excretions of A and/or Et as the primary indicator. Subsequent $\delta^{13}C$ analysis of the sulfoconjugate fraction displayed selective ^{13}C depletion of Et-S in relation to A-S, DHEA-S (used subsequently as an endogenous reference compound (ERC)) and epiA-S, and therefore provided a unique confirmation method for 5-ADIOL abuse (Figure 6). This finding, more than any reported thus far, has demonstrated the potential for GC-C-IRMS to provide a metabonomic dimension for steroid analysis in doping control.

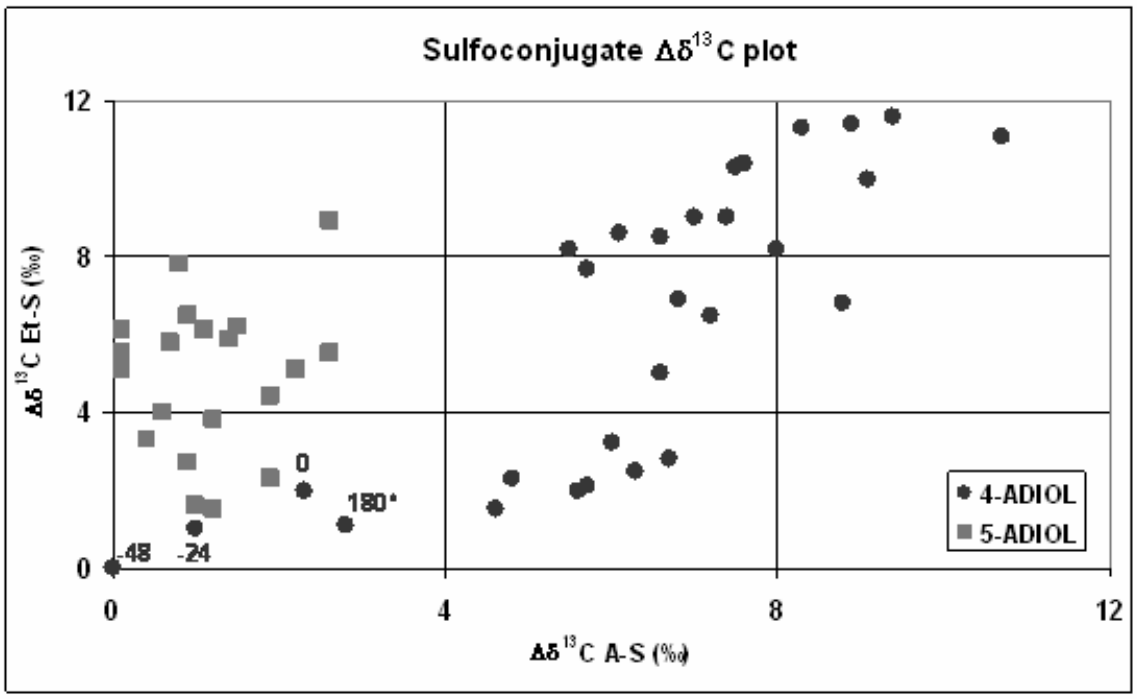


Figure 6: Sulfoconjugate $\Delta\delta^{13}C$ (relative to DHEA-S) plot for subject 2 from multiple ADIOL administrations. Times are provided for 4-ADIOL data points closest to the 5-ADIOL cluster (* indicates time after final administration).

Testosterone (T)

The strategies proposed for specific detection of DHEA, ADIONE, 4-ADIOL and 5-ADIOL abuse are mutually exclusive. This research has shown no overlap of steroid markers or

metabolic distinctions between different administrations. The detection of T abuse, however, requires a strategy based on the $\delta^{13}C$ value of urinary T and a deductive process of elimination. Administration of ^{13}C depleted steroid prohormone substrates will – by definition – influence to varying degrees the $\delta^{13}C$ value of urinary T and afford the individual markers of their abuse. The absence of these markers, together with a low $\delta^{13}C$ T and consideration of the urinary steroid profile is proposed to provide specific detection of T abuse. To explain, high excretion of A and Et have been shown to accompany prohormone administration, while this is not usually found following T abuse. Furthermore, the investigation of longitudinal T/E values for a particular athlete, together with the excretion of A and Et should provide sufficient distinction between T and prohormone abuse.

Conclusion

An improved detection strategy can be proposed in terms of GC-MS screening criteria for the 5 target steroids (Table 1) and the implementation of GC-C-IRMS confirmation according to the criteria outlined in Table 2. Limits associated with $\Delta\delta^{13}C$ values are dependent on the endogenous reference compound used. A limit of 4.0‰ is considered appropriate for the ERC measurement, incorporating 11keto-etiocholanolone (11keto-Et; 5β-androstane-3α-ol-11,17-dione) with relatively high ^{13}C content, to confirm DHEA or 4-ADIOL abuse. Similarly, the confirmation of 5-ADIOL abuse, using DHEA-S as the ERC would require a 4.0‰ limit. The $\Delta\delta^{13}C$ limit can be reduced to 3.0‰ for values derived from pregnanediol (PD; 5β-pregnane-3α,20α-diol) as it more closely reflects the ^{13}C content of the androgen metabolites. Clearly the major limitation of GC-C-IRMS criteria relates to the use of endogenous reference compounds and the subsequent interpretation of $\Delta\delta^{13}C$ values. The dependency of $\delta^{13}C$ and $\Delta\delta^{13}C$ values needs to accompany any proposed criteria. While there are advantages to using $\Delta\delta^{13}C$ values, on the basis of ^{13}C fractionation, it is proposed that any one limit be imposed to identify a doping violation.

The concept of a complementary approach to endogenous steroid analysis, using GC-MS and GC-C-IRMS was crucial to the success of this metabonomic strategy. The basis for this proposal was to use all of the available information relating to an athlete's metabolism to effectively confirm the illegal administration of synthetic steroid copies. It is envisaged that this strategy can be implemented by doping control laboratories that have access to GC-C-

IRMS technology with minimal cost or changes to existing screening and confirmation procedures.

Table 1: GC-MS strategy identifying suspicious samples requiring GC-C-IRMS confirmation.

Target steroid	Marker	Rt (min)	RRt (17α-MeT)	SIM (*m/z*)	Marker Limit (ng/mL)
DHEA	3α,5-cyclo High A, Et T/E	6.7	0.50	432	100 > 5000 T/E > 4
ADIONE	4OH-ADIONE High A, Et T/E	15.4	1.14	518	40 > 5000 T/E > 4
4-ADIOL	Androst-2,4-diene Androst-3,5-diene High A, Et T/E	5.2 5.9	0.39 0.44	342 342	- - > 5000 T/E > 4
5-ADIOL	High A, Et only	-	-	-	> 5000
T	T T/E A/T	11.8	0.87	- - -	> 100 T/E > 4 < 40 Longitudinal profiling

Table 2: GC-C-IRMS confirmation of marker steroids for compound specific detection.

Target steroid	Marker	Rt (sec)	RRt (5α)	Criteria
DHEA	3α,5-cyclo A-G, Et-G	700	1.24	$\delta^{13}C \leq -27.0‰$ $\Delta\delta^{13}C^{ERC} \geq 4.0‰$
ADIONE	4OH-ADIONE A-G, Et-G	986	1.75	$\delta^{13}C \leq -27.0‰$ $\Delta\delta^{13}C^{PD} \geq 3.0‰$ $\delta^{13}C \leq -27.0‰$, $\Delta\delta^{13}C^{ERC} \geq 4.0‰$
4-ADIOL	Androst-2,4-diene Androst-3,5-diene A-G, Et-G	555 595	0.98 1.05	$\delta^{13}C \leq -27.0‰$ $\Delta\delta^{13}C^{ERC} \geq 4.0‰$
5-ADIOL	Et-S Et-G	810	1.43	$(\Delta\delta^{13}C^{DHEA\text{-}S}$Et-S-$\Delta\delta^{13}C^{DHEA\text{-}S}$A-S) $\geq 4.0‰$ $\delta^{13}C \leq -27.0‰$, $\Delta\delta^{13}C^{ERC} \geq 4.0‰$
T	T βαβ-diol	990	1.75	$\delta^{13}C \leq -27.0‰$ $\Delta\delta^{13}C^{PD} \geq 3.0‰$ No presence of marker steroids

ERC = (11keto-etiocholanolone + pregnanediol) PD = pregnanediol

DHEA-S = dehydroepiandrosterone sulfoconjugate

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