RECENT ADVANCES
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Hardware-considerations for Purification of Steroids by Normal-Phase HPLC
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Hardware-considerations for Purification of Steroids by Normal-Phase HPLC for GC-C-IRMS

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Introduction
HPLC is a common technique for purification of steroids for Gas Chromatography Combustion Isotope Ratio Mass Spectrometry (GC-C-IRMS). As there is a $^{13}$C/$^{12}$C-discrimination from the beginning to the end of HPLC-peaks [1], it is mandatory to collect the whole peak and to avoid any significant losses. Steroid-extracts from different matrices can contain significant amounts of further lipids. Due to their strong interaction with reversed-phase material, these might contaminate the column more or less irreversibly. The availability of an alternative cleanup-method therefore is regarded as useful. The objective was to develop a cleanup based on Normal-Phase HPLC (NP-HPLC) for selected steroids.

Experiment 1
Two different HPLC columns from Macherey-Nagel were tested under similar conditions:

Column A: \( \text{EC 250/4.6 Nucleosil 100-5 NH}_2 \)
Column B: \( \text{EC 250/4 Nucleosil 100-5 N(CH}_3)_2 \)

Injection-volume: 50 \( \mu l \)

Analytes: Dehydroepiandrosterone (DHEA), Epiandrosterone (EpiA), Etiocholanolone (Etio), Epitestosterone (EpiT); 100 ng/\( \mu l \) each

Detection: UV/200 nm

Gradient: isocratic 96% n-Hexane / 4% 2-Propanol (IPA) until analytes are eluted; then 50% n-Hexane / 50% IPA (column-washing)

Flow: 2 ml/min (Column A); 1 ml/min (Column B)

Recoveries of the different standards were determined by GC-MS after collection of fractions mentioned in fig. 1-2 (three times for each standard)
Fig. 1: Fraction collection of EpiA (1), DHEA (2), Etio (3) and EpiT (4) with column A ((NH$_2$)-Propyl-column)

Fig. 2: Fraction collection of EpiA (1), DHEA (2), Etio (3) and EpiT (4) with column B ((CH$_3$)$_2$N-Propyl-column)

Fig. 3: Recoveries of keto-steroids after fraction-collection with 2 different NP-columns
**Results of experiment 1**

Under equivalent conditions (50°C; 4% Isopropanol / 96% n-Hexane), the order of elution for both columns is very similar: 1. Epia + DHEA (contemporaneous), 2. Etio, 3. EpiT. The much lower UV-absorption of EpiT on column A (fig. 1) than on column B (fig. 2) indicates, that at least for this compound, column A is not suitable. Recoveries after fraction-collection and GC-MS (fig. 3) indicate, that also for the other keto-steroids, recoveries are worse on the Aminopropyl-column despite the much larger fraction size for column A than for column B.

Also fraction-limits are hard to define for column A: Ghost peaks occur in fraction 1; every keto-steroid is detectable in significant amounts in fraction 3 sometimes in fraction 4.

Using the Dimethylaminopropyl-column, none of these problems could be observed: No ghost peaks in fraction 1, every keto-steroid only detectable in the intended fraction.

**Experiment 1a**

As the objective was to develop a method for GC-C-IRMS, the δ^{13}C_{PDB}-values of the standards and the collected fractions from column A were measured undervatized vs. 5α-Androstan-3β-ol as a reference standard by GC-C-IRMS.

<table>
<thead>
<tr>
<th></th>
<th>mean Δδ^{13}C vs. ref. std. [%]</th>
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<tbody>
<tr>
<td></td>
<td>DHEA</td>
</tr>
<tr>
<td>standards without HPLC</td>
<td>-0,2</td>
</tr>
<tr>
<td>standards after HPLC</td>
<td>-0,2</td>
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</tbody>
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Tab. 1: Δδ^{13}C_{PDB}-values vs. reference std.

**Results of experiment 1a**

Tab. 1 shows, that under given conditions a significant difference in the δ^{13}C_{PDB} vs. reference std. before and after HPLC with column A can be observed for EpiT. This difference is about 1,3 % which means, that ^{13}C-enrichment can be observed after HPLC. Other steroids don’t show this effect.

**Experiment 2**

To explain the results of experiment 1 on column A, 5% n-butyl-amine was added to the different solvents as surrogate. Retention times of the steroids were measured. The respective fraction was collected and measured by GC-MS after silylation with pure MSTFA.
Results of experiment 2
HPLC-retention-times for every compound decreased dramatically. In addition to the TMS-derivatives, four more compounds with m/z = 415 (twice) and m/z = 417 (twice) as molecule ions could be identified by GC-MS.

![Chemical structures]

Fig. 4: Imine-formation exemplified for EpiT

Conclusions
Although very large fractions were collected on column A, recoveries for the tested steroids were unacceptable low (Experiment 1). The $\delta^{13}C_{PDB}$-values are not constant at least for EpiT (Experiment 1a). Experiment 2 results in four derivatives with an additional molecular weight of 55 g/mol which is the mass of n-Butylamine (73g/mol) minus water. In fig. 4 a plausible explanation is shown exemplified for EpiT as most affected analyte. A chemical reaction like the formation of imines (better known as Schiff bases) on the NH$_2$-Propyl-material during HPLC explains losses, ghost peaks and $^{13}C/^12C$-discrimination for EpiT on column A. Fig. 4 also indicates, why EpiT is the most affected analyte: The 3-keto-4-ene-structure facilitates the nucleophilic attack of the primary amine.

Acceptable results were obtained with column B: Tertiary amines ((CH$_3$)$_2$N-Propyl) can not react with keto-groups. Small fractions are promising for cleanup.
References