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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:	EC 250/4.6 Nucleosil 100-5 NH_2
Column B:	EC 250/4 Nucleosil 100-5 $N(CH_3)_2$
Injection-volume:	50 µl
Analytes:	Dehydroepiandrosterone (DHEA), Epiandrosterone (EpiA), Etiocholanolone (Etio), Epitestosterone (EpiT); 100 ng/µl 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)

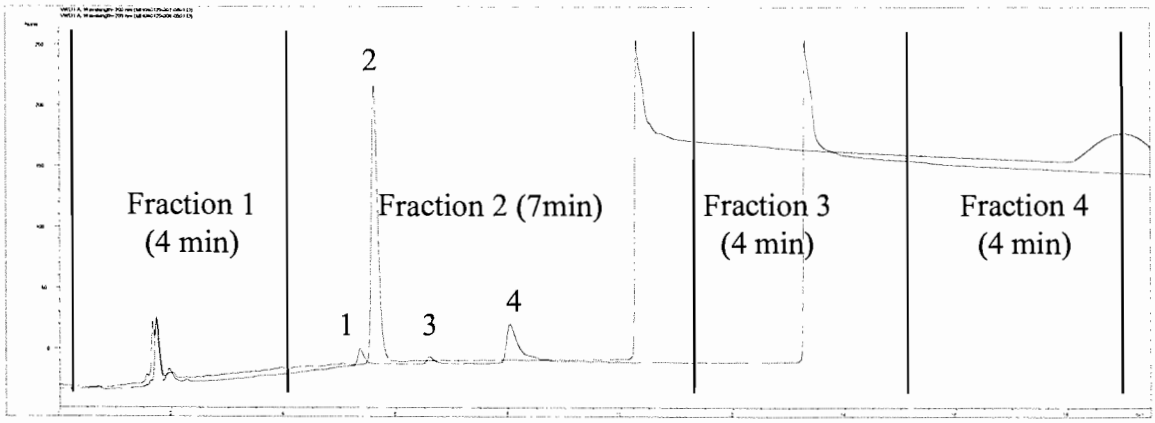

Fig. 1: Fraction collection of EpiA (1), DHEA (2), Etio (3) and EpiT (4) with *column A* ((NH_2)-Propyl-column)

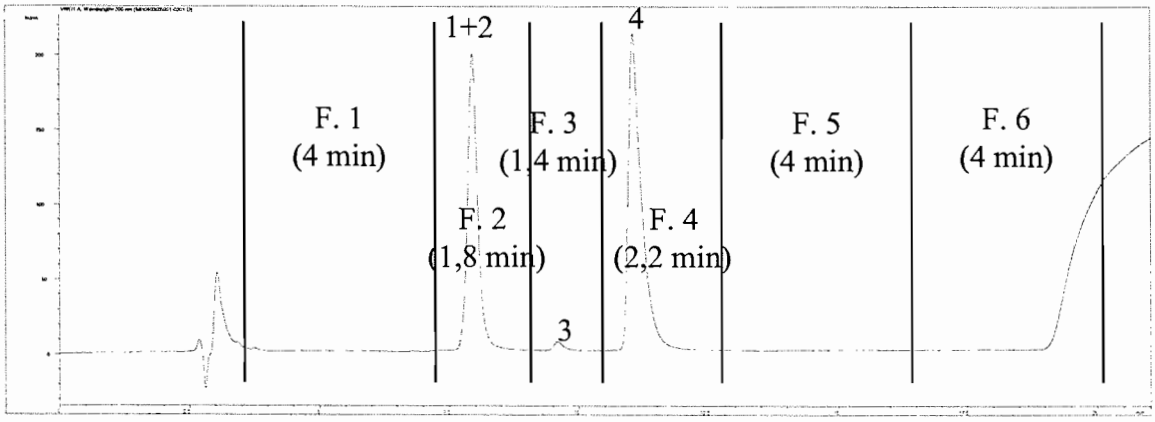

Fig. 2: Fraction collection of EpiA (1), DHEA (2), Etio (3) and EpiT (4) with *column B* ($(CH_3)_2N$-Propyl-column)

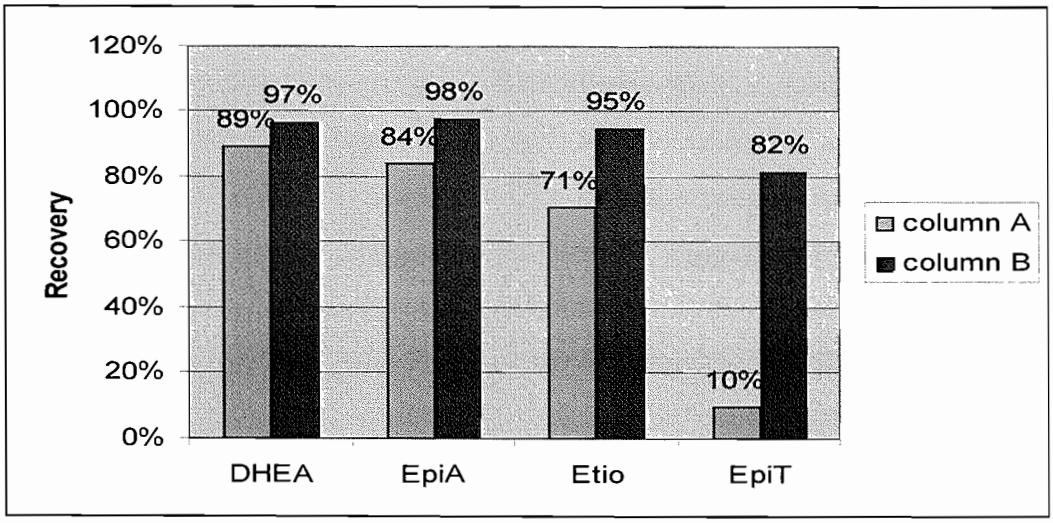

Fig. 3: Recoveries of keto-steroids after fraction-collection with 2 different NP-columns

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 $\delta^{13}C_{PDB}$-values of the standards and the collected fractions from *column A* were measured underivatized vs. 5α-Androstane-3β-ol as a reference standard by GC-C-IRMS.

	mean $\Delta\delta$ ^{13}C vs. ref. std. [‰]			
	DHEA	Etio	EpiA	EpiT
standards without HPLC	-0,2	0,1	0,4	4,1
standards after HPLC	-0,2	0,3	0,5	5,4

Tab. 1: $\Delta\delta^{13}C_{PDB}$-values vs. reference std.

Results of experiment 1a

Tab. 1 shows, that under given conditions a significant difference in the $\delta^{13}C_{PDB}$ 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.

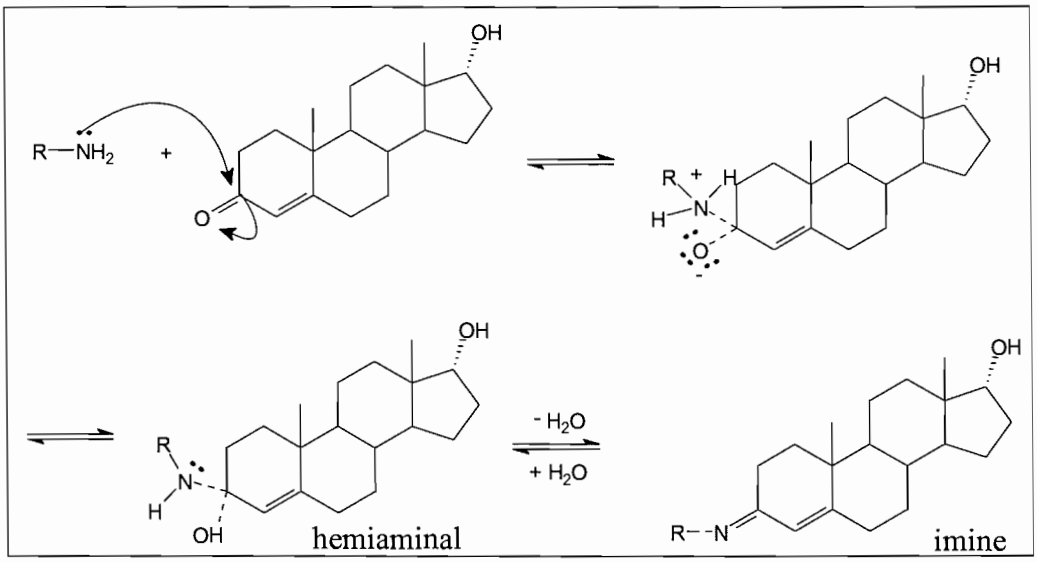

Fig. 4: Imine-formation exemplified for EpiT

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 $^{13}C/^{12}C$-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 ($(CH_3)_2N$-Propyl) can not react with keto-groups. Small fractions are promising for cleanup.

References

[1] Flenker, U.; Horning, S.; Nolteernsting, E.; Geyer, H.; Schänzer W.: *Measurement of* $^{13}C/^{12}C$*-Ratios to confirm Misuse of endogenous Steroids*. In: Schänzer et al. (Eds.) Proceedings of the 16th Cologne Workshop on Dope Analysis, Sport & Buch Strauß, Köln. Recent Advances in Doping Analysis (6), 1999; pp. 243-256