**Ensolisation-silylation reaction study of selected steroids**

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# Foreword

Welcome to CECE 2014. With this 11th CECE in a row we are entering the second decade of the conference. As in the previous year we start with lectures by young scientists (CECE Junior), followed by two days of invited lectures and poster sessions. This book of proceedings includes the program of all three days. This year the meeting is free of charge for all perticipants thanks to the financial support by the European Social Fund and the state budget of the Czech Republic (CZ.1.07/2.3.00/20.0182). Of course our original goal of "bringing together scientists who may not meet at specialized meetings, promote informal communication of researchers from different disciplines and map the current status of the fields shaping the bioanalytical science" remains intact. The organizers want to thank the invited speakers and all the participants and hope that you will enjoy the scientific presentations as well as personal contacts and informal discussions.

Brno, October 18, 2014

# P37 ENOLISATION-SILYLATION REACTION STUDY OF SELECTED STEROIDS

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**Summary**
For the purposes of trace analysis of 1,4-androstadiene-3,17-dione (ADD), 1,4-androstadiene-3-one-17β-ol (Boldenone) and 17-β-estradiol in water, waste water, soil and for the further phytosterols transformation studies the enolisation-silylation reaction was studied using MSTFA and BSTFA with different catalysts as derivatization reagents. The different reaction conditions and stability of the products was also studied using GC-MS technique.

## 1 Introduction

There are increasing numbers of steroid hormones applications in the field of human and animal medicines. Considerable part of the steroid hormones belong to the endocrine disrupting chemicals which bio accumulate in the environment. They are harmful to organisms even at the concentration level less than 1 ng $L^{-1}$. PNEC value (Predicted No Effect Concentration) for 17β-estradiol (E2) is 1 ng $L^{-1}$ (3.67 pM) [1].
Practical trace analysis is in favour of GC-MS but steroid hormones must be transformed into corresponding derivatives, which can be difficult since steroid hormones contain polar keto and hydroxyl functional groups. For the derivatization of keto groups could be the enolization-silylation reaction the right derivatization step [2]. Enolisation-silylation is a complicated reaction by which the keto group of steroid hormones is converted into its enol tautomers. There is no information in the literature regarding the optimal conditions of this reaction, e.g. temperature of silylation, time of reaction and stability of the derivatives during their storage. Therefore, the main goals of our study were to a) test different reagents available on the market possibly suitable for this complicated enolisation-silylation reaction, b) find optimal conditions for the reaction and c) evaluate the stability of the final derivatives during storage. We have selected two compounds containing keto group for our study: the first having one keto group and one hydroxy group (Boldenone) and second one having two keto groups (ADD). 17β-estradiol was chosen for comparison of the hydroxyl group reactivity.

## 2 Experimental

Stock mixed solution of 17-β-estradiol, Boldenone and ADD at concentration 10 µg/mL in toluene was used for all experiments. Quantification was done using

internal standard calibration procedure using 5 calibration levels and cholesterol as internal standard. Prior to derivatization procedure, all glassware was silanized according to [3]. All experiments in this study were conducted in triplicates and samples were measured immediately after preparation.

For optimizing the derivatization process, the following reagents were tested for their ability to silylate the compounds of interest: MSTFA, MSTFA I (activated with ethanethiol and ammonium iodide), MSTFA II (activated with 2-(Trimethylsilyl)ethanethiol), MSTFA III (activated with imidazole), mixture of BSTFA and pyridine (1:1), MSTFA II and pyridine (1:1) (Fluka, Sigma-Aldrich). Derivatization was conducted as follows: 70 µL of sample was evaporated with a stream of nitrogen and afterwards redissolved with 50 µL of each reagent (and 50 µL of pyridine, when used). The mixtures were kept at 65°C for 60 minutes. Subsequently, they were evaporated and redissolved in 70 µL of hexane prior to GC-MS analyses. To optimize the derivatization procedure the set of duration and temperature combinations was tested using the MSTFA II derivatization procedure. The combinations of three temperatures - 55, 65 and 75°C, and three times - 30, 60 and 90 min, were tested. Finally, the in-time stability of the derivatives was examined using the MSTFA II derivatization procedure. The derivatives were stored dissolved in hexane at either -17° C or 4°C for 0, 1, 4, 15 or 40 days until analyses. The unique set of samples was prepared for each storage time.

The GC-MS measurements were conducted on ITQ 1100 mass spectrometer coupled with Trace GC Ultra gas chromatograph (ThermoFisher Scientific, MA, USA). The chromatographic separation was achieved on ZB-5 MS capillary column (Phenomenex®, CA, USA), 30 m × 0.25 mm× 0.25 µm. Helium was used as carrier gas at constant flow of 1 mL/min. The chromatographic conditions were set as follows: inlet temperature 275°C, splitless mode, transfer line temperature 250°C, temperature program - 130°C/0 $min^{-1}$, rate 25 °C $min^{-1}$ to 235°C, then 2°C $min^{-1}$ to 265°C, then 5°C $min^{-1}$ to 290°C, then 10°C $min^{-1}$ to 305°C holding 6 min.

## 3 Results and Discussion

The first part of this study focused on finding an optimum derivatization reagent for the three investigated compounds. The yield of 17-β-estradiol derivatization procedure was comparable among all tested reagents, with the exception of MSTFA III treatment, which yielded lower amounts of TMSi-derivatives ($F = 85.59$; $p<0.001$, Tukey HSD test). Nevertheless, the derivatization procedure was also reproducible (Fig. 1).

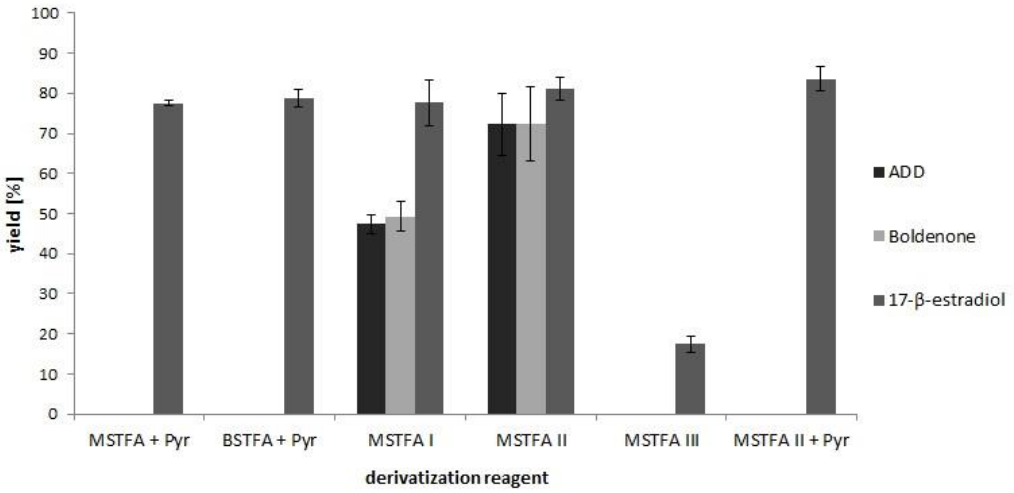


Fig. 1. Comparison of derivatization reagents used for enolisation-silylation.

Derivatization of steroid compounds containing keto groups was successfully achieved only when using MSTFA I or MSTFA II. However, the yields of ADD and Boldenone TMSi-derivatives were significantly higher when the derivatization protocol with MSTFA II was used (ADD: $F = 27.84$, $p<0.01$; Boldenone: $F = 16.1$, $p<0.05$, Tukey HSD test). The absence of derivatives when pyridine with MSTFA II was used (pyrdidine is recommended for routine silylation reactions) can be explained by the suppression of acidity of the hydrogen on the alpha carbon atom, and thus preventing the enolization reaction.

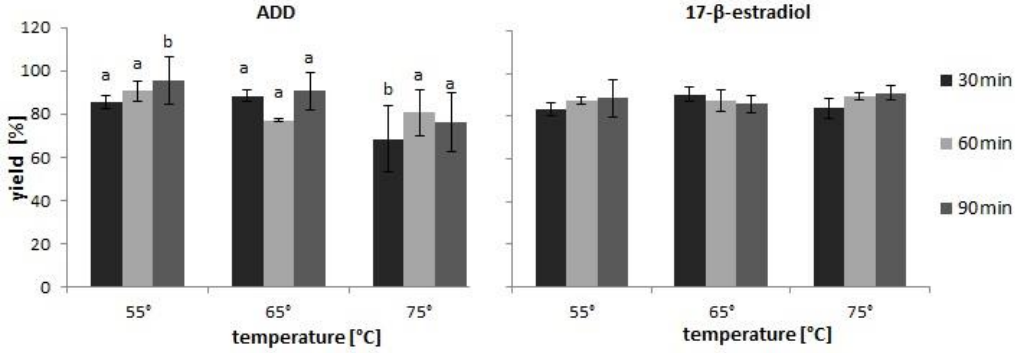


Fig. 2. Optimalization of time-temperature conditions during the derivatization process (only data for ADD and 17-β-estradiol are presented).

Subsequently, the optimum time-temperature combination for the derivatization process was examined. In the case of ADD and Boldenone a significant difference could only be observed between treatment at 55°C for 90 min and treatment at 75°C for 30 min with foremost treatment giving a significantly higher yield (ADD: $F = 2.69$, $p<0.05$; Boldenone: $F = 2.81$, $p<0.05$, Tukey HSD test) (Fig. 2). The range of tested time and temperature combinations during the derivatization process seems to have no effect on the yield of17-β-estradiolTMSi-derivatives.

Finally, the stability of TMSi-derivatives over time was studied. During the whole experiment, we didn´t find any significant difference between the two storage temperatures for none of these steroid compounds. However, a decrease of ADD and

Boldenone derivatives over time was detected. We determined similar degradation rate of these compounds which resulted in decay of approximately 35% of TMSi-derivatives over the 40 day experiment. Our results indicate stability of derivatized steroid compounds containing keto group for at least 4 days when stored in freezer while when stored in the fridge, the stability is maximum 1-2 days.

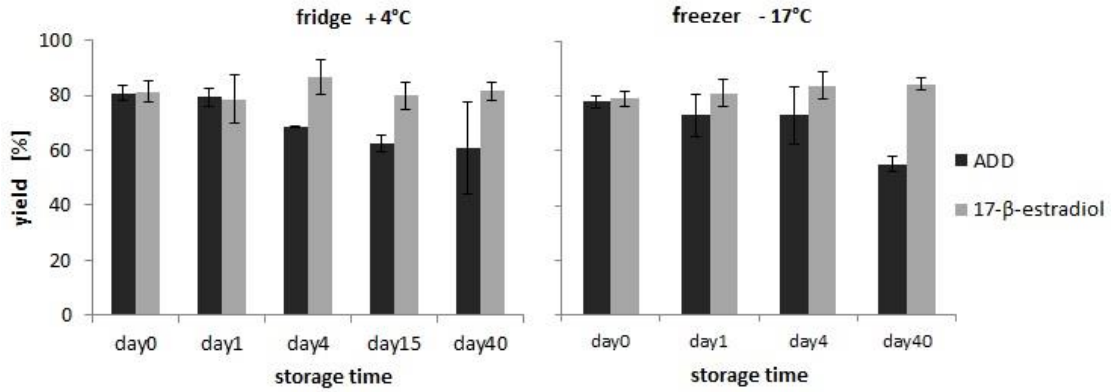


Fig. 3. Time stability of ADD and 17-β-estradiol TMSi-derivatives following storage at -17°C and 4°C.

## 4 Conclusions

Study of enolisation-silylation reaction revealed that it is possible to use this reaction for the determination of ADD and Boldenone and the reaction yield is comparable with estradiol using MSTFA activated II. Since the reduction of time needed for sample preparation is one of the main criterions during method optimization, the time-temperature combination of choice based on our results is at 65°C for 30 min. Due to instability of TMSi-derivatives, the immediate analysis after sample preparation is necessary.


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