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# Multivariate optimization of a derivatisation procedure for the simultaneous determination of nine anabolic steroids by gas chromatography coupled with mass spectrometry

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ABSTRACT

The medical commission of the International Olympic Committee forbids the use of anabolic androgenic steroids to improve sporting performances. Nine anabolic steroids (androsterone (A), nandrolone, estradiol, testosterone propionate, nandrolone-17 propionate, dydrogesterone, testosterone, epitestosterone, boldenone) and α-cholestane as internal standard were studied by gas chromatography coupled with mass spectrometry (GC/MS). The derivatisation reagent employed for the derivatisation of anabolic steroids was a mixture of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA), ammonium iodide and 2-mercaptoethanol (1000:2:6, v/w/v). Trimethylsilyl (TMS) derivatives were obtained. Anabolic steroids can be derivatised into one or two forms, mainly for androsterone into A-monoTMS and A-diTMS. The aim of this study was to research the optimization conditions of the derivatisation process (maximum yield of silylation reaction) of each anabolic steroid into only one form. A two-level factorial Doelhert design was used to determine the influence of different parameters and their interactions on each compound, thanks to response surface methodology. The parameters to be optimized were the reaction time and the temperature. The interaction "temperature–reaction time" is significant and has a positive effect on the improvement of the effectiveness of the derivatisation. Considering the large amount of information, often not convergent, a global desirability function was applied for multi-responses optimization. Thus, the optimized temperature and the reaction time of silylation were 85 °C and 24 min, respectively. Several GC/MS analytical parameters were also studied: linearity (regression coefficient upper than 0.99 for each compound, sensibility (range of concentration 0.05–0.30 μg/ml). Confirmatory experiments were applied to check the predicted values and to validate the model. The confirmatory assay responses are relatively close to the responses predicted. We observed satisfactory resolutions by GC/MS and a run lower than 12 min.



## 1. Introduction

The anabolic steroids are often identified and measured by gas chromatography and mass spectrometry (GC/MS) [1–15]; however, the derivatisation is generally used with an aim of decreasing the polarity, of increasing volatility, of improving separation and of stabilizing the thermolabile substances. The trimethylsilylation is the most usual and various silylation reagents were used alone or in combination with catalysts [16–20].

The presence of various functional groupings favourable to the derivatisation can have, like consequence, the formation of multiple derivatives; moreover, these intermediate derivatives are not stable which leads to confusion in the processes of identification, optimization and quantification [21].

Several reagents of derivatisation were studied with or without catalysts and at various temperatures, the variation in a way independent of the various factors does not make it possible to take into account the interactions between the factors that constitute an important source of inaccuracy and make the determination of the optimal conditions difficult (time, temperature) for a wide range of steroids [22].

The most cited derivatisation techniques are the application of the mixture MSTFA/$NH_4$ I/2-mercaptoethanol [1,2,5,9,11,23].

MSTFA reacts *in situ* with ammonium iodide to give the trimethyliodosilane (TMSI), which is the powerful donor of TMS

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grouping. The 2-mercaptoethanol reduces iodine in hydrogen iodide in order to prevent iodine incorporation into steroid nucleus [24,25].

A quantitative method based on gas chromatography coupled with mass spectrometry was developed with the use of α-cholestane as internal standard (I.S.); the used derivatisation reagent was a mixture of MSTFA/$NH_4$ I/2-mercaptoethanol (1000:2:6, v/w/v).

The use of the experimental designs allows adopting a more economic and more direct strategy. Moreover, the implementation of a procedure of optimization allows the study of the interactions between the various factors. Recently numerous procedures of optimization were used in chromatography [26–35].

In this work, a preliminary study used a factorial design on two levels in order to study the interaction between the two studied factors temperature and reaction time.

The response was then optimized with the two factors by adjusting a quadratic model on the measured responses according to the experimental design presented by Doelhert [36]; this process is called the methodology of response surfaces.

The Doelhert design explores a spherical experimental field with more than two levels to adapt a second-order model. The principal characteristic of the Doelhert designs [36] is to obtain a uniform distribution of the experimental points in an experimental space. The provisions of these points, for a design with two factors, are at the same distance from the centre of the studied field and are located on the trigonometric circle. They form a regular hexagon. Several advantages were reported compared to the composite central. The Doelhert designs minimize the number of experiments and allow an easy displacement in the space of the studied variables [37–42].

The methodology of the experimental designs was applied for the optimization of the reaction with simultaneous derivatisation of nine anabolic steroids by a global desirability function in order to obtain a simple and stable derivate product for each substance.

## 2. Experimental

### 2.1. Chemicals and reagents

Androsterone (A), nandrolone (N), nandrolone-17 propionate (NP), MSTFA and $NH_4I$ were purchased from Riedel-de Haën (Taufkirchen, Germany); estradiol (E), boldenone (BL), testosterone (T), epitestosterone (EP), α-cholestane (CHL) and 2-mercaptoethanol from Sigma (Munich, Germany). Testosterone propionate (TP) and dydrogesterone (DG) in conformity with the current European Pharmacopoeia were gratefully given by Pharmaceutical Laboratories (Brussels, Belgium). Chemical formulas are in Fig. 1. Methanol and hexane with higher purity grade were purchased from Merck (Darmstadt, Germany).

### 2.2. Standard solutions

Separate stock standard solutions (1 mg/ml) of nine anabolic steroids were prepared by dissolving each compound in methanol. Separate working standard solutions (100 μg/ml) were prepared by 1/10 dilution of the stock standard solutions with methanol. A working standard solution of α-cholestane (used as internal standard) was also prepared at a concentration of $3.10^{-2}$ mmol/l by 1/10 dilution of the stock standard solution. All solutions were stored in the dark at −20 °C until analysis.

### 2.3. Derivatisation procedure

MSTFA/$NH_4I$/2-mercaptoethanol stock standard solution was prepared by dissolution of 20 mg of $NH_4I$ in 1 ml of MSTFA and

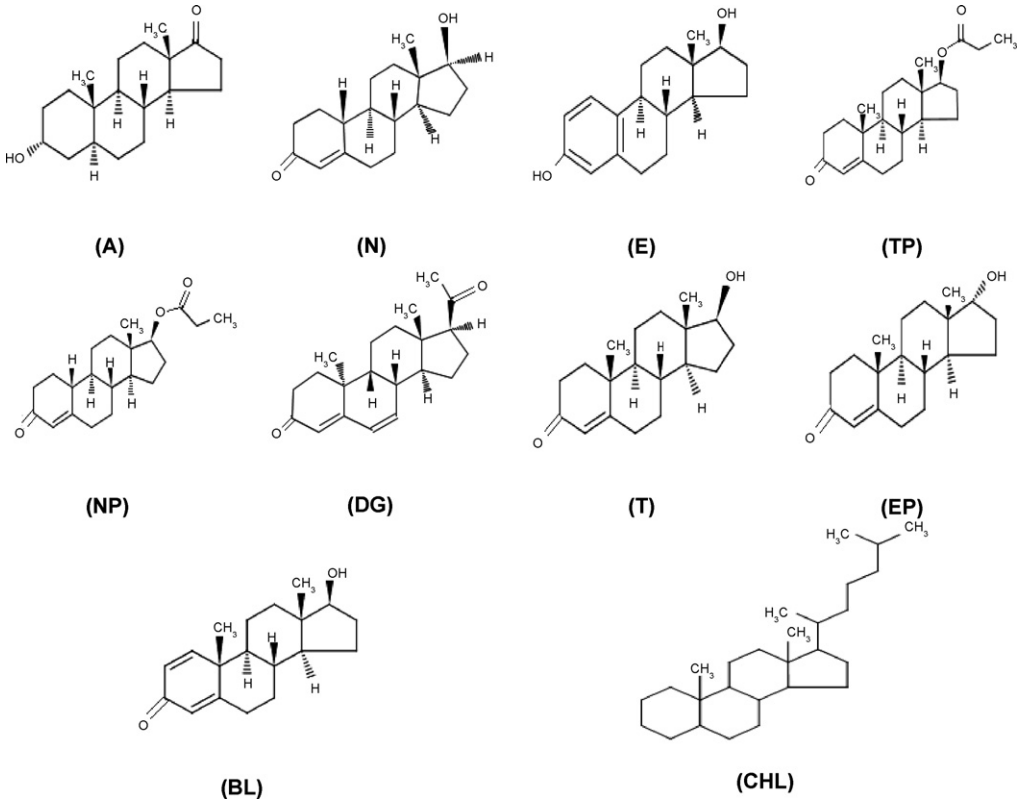


**Fig. 1.** Structure of the anabolic steroids considered in the derivatisation and GC/MS study: (A) androsterone, (N) nandrolone, (E) estradiol, (TP) testosterone propionate, (NP) nandrolone-17 propionate, (DG) dydrogesterone, (T) testosterone, (EP) epitestosterone, (BL) boldenone, (CHL) α-cholestane (internal standard).