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Optimization of the chromatographic conditions for the determination of hormones by gas chromatography with mass spectrometry detection

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Abstract

A gas chromatographic method with mass spectrometry detection (GC/MS) has been developed for the analysis of eight hormones: diethylstilbestrol, hexestrol, dienestrol, nor-testosterone, methyl-testosterone, 17-α-estradiol, 17-β-estradiol and 17-α-ethynylestradiol. As the diastereoisomers, α and β-estradiol, coelute and both have the same mass fragments, the experimental design methodology has been applied for obtaining their chromatographic separation. In this paper, the temperature programme of the column with two ramps has been optimized to improve not only the resolution between the peaks of both isomers but also the quality of the peaks (sharp and symmetric peaks).

Firstly, a 16-experiment screening design was carried out to determine with a reduced number of experiments which factors affect both the resolution and the peak width. The results concluded that the final temperature of the first ramp mainly affects the resolution whereas the final temperature of the second ramp influences both the resolution and the peak width.

A two-factor Doehlert design was subsequently performed to fit a second-order model and jointly optimize the resolution and the peak width through a global desirability function. Thus, the final temperatures of the first and the second ramp are 260 and 300 °C, respectively. The method developed has been validated according to the European Decision 2002/657/EC, including the estimation of the capability of detection (CCβ, $X_0 = 0$, for banned substances) with evaluation of the probability of false positive, α, and of false negative, β.

Keywords: Estrogens; Androgens; α-Estradiol; β-Estradiol; Screening design; Doehlert design; Desirability; CCβ

1. Introduction

The European Union has forbidden the administration of hormonal growth promotants to farm animals destined for human consumption (Directive 96/22/EC amended by Directive 2003/74/EC). Consequently, the laboratories in charge of detecting these residues need to develop techniques allowing not only the analysis in several biological matrices but also the distinction between natural and synthetic hormones.

These compounds are often identified and quantified by gas chromatography with mass spectrometry detection (GC/MS) [1,2] because of the low capability of detection provided by this technique. Derivatization of compounds with polar groups is common in GC in order to decrease their polarity, increase the volatility, improve the chromatographic separation or to stabilize thermolabile substances. Trimethylsilylation is the most habitual reaction for derivatizating hormones and different silylation agents have been used alone [3] or combined with catalysers [1,2,4]. Apart from the increase in the analysis time, some difficulties in derivatization have been observed: hormones have various hydroxyl groups, and consequently, different derivatives might be formed [5]. Besides, few intermediate derivatives are not stable over time, which makes the chromatogram more complex and less specific. In this paper, the problem has been solved by employing a midpolarity column DB-17MS (50% phenyl–50% dimethyl arylene siloxane), which allows the analysis of compounds

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without the prior step of derivatization. Consequently, the formation of multiple derivatives is avoided and the sensitivity and the specificity of the chromatograms are enhanced.

In this paper, eight hormones have been analysed: two steroids with androgenic action, nor-testosterone (nor-TEST) and methyl-testosterone (met-TEST) and six estrogens: the stilbenes diethylstilbestrol (DES), hexestrol (HEX) and dienestrol (DIEN) and the steroids 17-α-estradiol (αE2), 17-β-estradiol (βE2) and 17-α-ethynylestradiol (EE2).

The experimental design methodology has been applied to develop a chromatographic method which allows one to identify and quantify all eight hormones according to the requirements stated in the European Decision 2002/657/EC [6]. Specifically, we will focus on achieving an adequate resolution between the two diastereoisomers, αE2 and βE2, which coelute and have common mass fragments. Preliminary studies showed a resolution smaller than 0.8 so the univariate quantification is not possible because they are not completely separated. On the other hand, both isomers have different biological activity; αE2 is one of the most active estrogens whereas βE2 is almost inactive. An analytical method is consequently necessary to identify each diastereoisomer. Only one paper [7] has been found in bibliography for the simultaneous analysis of αE2 and βE2. High-performance liquid chromatography with diode array detection (HPLC/DAD) was used to separate and detect both isomers. However, either GC/MS or LC/MS is preferred by the European Decision 2002/657/EC for the analysis of forbidden substances. The novelty of this work is the developing of a GC/MS method for the analysis of estrogens, including αE2 and βE2.

The rest of the compounds analysed in this paper have different mass spectra of each other and all the analytes have, at least, one specific mass fragment. Consequently, by selecting a specific ion for a given analyte, the univariate regression, peak area versus concentration, can be performed even if the chromatographic resolution is not completely achieved. In other words, the specificity of the signal can be obtained not only with the resolution of the chromatographic peaks but also with the proper election of the mass fragments. As αE2 and βE2 have the same spectra, the chromatographic resolution is necessary to perform the univariate analysis. That is why this paper deals with the chromatographic resolution of αE2 and βE2. Then the optimized method will be applied for the analysis of all eight hormones.

Two responses are examined: the resolution between αE2 and βE2 to allow univariate quantification and the width of both peaks. The reason for including the study of the peak widths is that, once the chromatograms have been resolved, it is of interest to obtain sharp peaks. The narrower the chromatograms, the less probable the presence of interferences affecting the chromatograms and the less disperse and variable the signals. Hence, the specificity and the quality of the signals will be enhanced. In other words, we will focus on minimizing the peak widths and on guaranteeing acceptable resolutions (at least equal to 1) for the quantification of both isomers.

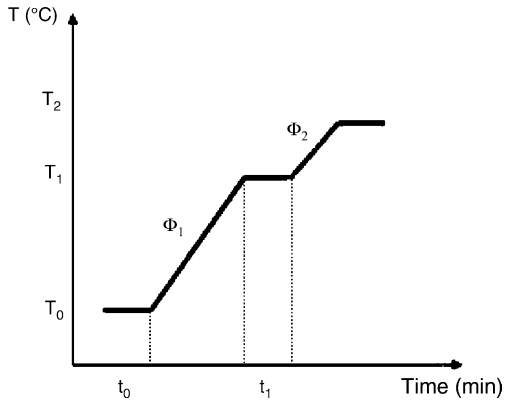

Fig. 1. Temperature programming of the chromatographic column. T_0, initial temperature, t_0, time that the initial temperature is kept, T_1, final temperature of the first ramp, t_1, time that the final temperature of the first ramp is kept, T_2, final temperature of the second ramp, Φ_1 and Φ_2, rate of the column temperature of the first and second ramp.

The elution (the retention time and the resolution between two compounds) as well as the quality of the chromatograms (absence of some artefacts like tailing) are mainly controlled by temperature programming. That is why the effect of seven factors related to the temperature programme of the oven (Fig. 1) will be firstly analysed through a screening design at three levels. This design [8] allows one to know the effect of many factors with few experiments, and consequently, to identify the non-influential factors. In other words, the aim of the screening study is not to obtain complete information about the behaviour of the chromatographic peaks when the factor changes but to get an initial idea of the effects and discriminate the non-active factors. Those factors concluded to be non-significant are fixed at one of the levels and the response is optimized with the active factors by adjusting a quadratic model to the responses measured according to the experimental design introduced by Doehlert [9]. The whole procedure is called the methodology of the response surface.

The Doehlert design explores a spherical experimental domain at more than two levels to fit a second-order model. Specifically for two factors, it consists of six points forming a regular hexagon with a centre point, one of the factors is at five levels and the second one at three levels. Several advantages have been reported [10,11] in comparison with other response surface designs such as the central composite design. (i) The Doehlert design is more efficient because it estimates more coefficients with fewer experiments. For instance, the six coefficients of a quadratic model with two factors are estimated with seven experiments by the Doehlert design (*R*-efficiency 85.71%) and with nine experiments by the central composite design (*R*-efficiency 66.67%). (ii) The Doehlert design is highly sequential [12]. When the optimum is not in the domain experimented it is possible to build a new experimental matrix in the direction of the optimum containing experiments previously performed. (iii) Generalizing, it can be extended in any direction without overlapping. This