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Determination of steroid hormones and their metabolite in several types of meat samples by ultra high performance liquid chromatography—Orbitrap high resolution mass spectrometry

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A new analytical method based on ultra-high performance liquid chromatography (UHPLC) coupled to Orbitrap high resolution mass spectrometry (Orbitrap-HRMS) has been developed for the determination of steroid hormones (hydrocortisone, cortisone, progesterone, prednisone, prednisolone, testosterone, melengesterol acetate, hydrocortisone-21-acetate, cortisone-21-acetate, testosterone propionate, 17α-methyltestosterone, 6α-methylprednisolone and medroxyprogesterone) and their metabolite (17α-hydroxyprogesterone) in three meat samples (chicken, pork and beef). Two different extraction approaches were tested (QuEChERS "quick, easy, cheap, effective, rugged and safe" and "dilute and shoot"), observing that the QuEChERS method provided the best results in terms of recovery. A clean-up step was applied comparing several sorbents, obtaining the best results when florisil and aluminum oxide were used. The optimized method was validated, obtaining suitable results for all validation parameters in the three meat matrices evaluated. Recovery values ranged from 70% to 103% (except for prednisone in beef samples), meanwhile repeatability and reproducibility were obtained at values lower than 18% and 21%, respectively. The limit of quantification (LOQ) was established for most of the compounds at 1.0 μg/kg, except for testosterone in chicken and hydrocortisone-21-acetate and cortisone-21-acetate in pork at 2.0 μg/kg. Decision limit (CCα) and detection capability (CCβ) values ranged from 1.0–2.7 μg/kg and 1.9–5.5 μg/kg, respectively, in the three matrices. Finally, thirty one meat samples were analyzed and two hormones, progesterone and hydrocortisone, were detected in a beef and pork sample at 1.7 and 2.8 μg/kg respectively.

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1. Introduction

Hormones can be produced by the body in endocrine glands, organs and tissues. They are essential for most of life activities, such as to regulate the activity of specific cells or mood control. However, synthetic hormones are used for growth and reproduction in many animals [1] and as pharmaceutical drugs. They can be classified according to their chemical structures, or as natural (cortisone, hydrocortisone, testosterone, 17-α-hydroxyprogesterone and progesterone) and synthetic (prednisone, prednisolone, melengesterol acetate, hydrocortisone-21-acetate, cortisone-21-acetate, testosterone propionate, 17-α-methyltestosterone, 6-α-methylprednisolone and medroxyprogesterone) hormones. Nevertheless the most common classification is between steroids or non-steroids [2].

Steroid hormones are a group of lipophilic and low-molecular-weight compounds and its structure is based on four cycloalkane rings [3]. At first, they are pre-hormones, and then, they can be converted into active paracrine or autocrine by steroid metabolizing enzymes. Some metabolically derived compounds can be more active than their parent compound and they can have different activity. However abnormal hormone exposure can affect some organs and systems, such as cardiovascular system, bone tissue and the central nervous system. In addition, the tissues regulated by hormones may respond to abnormal cell proliferation, leading to hyperplasia, neoplasia and even breast, ovary and prostate cancer [1,4]. In recent years, there have been several concerns about the presence of steroid hormones in food from animal origin because their use as growth promoters in the industry and in farm animals for their anabolic effect [2]. Steroid hormones have

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been analyzed in a wide range of edible matrices, as muscle [1–10], milk [6,8,11,sausage [1], liver [7,9,12] and kidney [7,9] from different type of animals such as pork [1,7,8,13,14], beef [1,2,6,10] and chicken [1,7]. Some studies also analyze these compounds in biological matrices such as serum [15–21], urine [22,23] or feed [24].

Steroids can be divided into different families: mineralocorticoids, glucocorticoids, androgens, estrogens and progestogens [3]. This study is focused on glucocorticoids (cortisone, hydrocortisone [2], hydrocortisone-21-acetate, cortisone-21-acetate, prednisone, 6-α-methylprednisolone and prednisolone [6]), androgens (testosterone propionate [18]), 17-α-methyltestosterone and testosterone [10]), progestogens (progesterone, melengesterol acetate [4] and medroxyprogesterone [10]) and its metabolite (17-α-hydroxyprogesterone [10]). These compounds were selected because glucocorticoids were banned by the EU in 1990 due to their adverse effect [25], as well as the use of progesterone, testosterone and melengesterol acetate [4,26] due to their hormonal action for growth promotion in farm animals and because the androgenic and gestagenic action of some of these compounds that were provisionally prohibited in the European Union (Directive 2003/74/EC) [26].

The European Union's Scientific Committee on Veterinary Measures Relating to Public Health [27] claims that the application of natural and artificial hormones in beef, meat and meat production could provoke human health problems, as well as they have an environmental impact on surface and groundwater.

To ensure consumer's safety, the development of analytical methods capable of determining these compounds at trace levels in complex matrices such as meat is necessary. For that, several extraction procedures have been applied to analyze steroids, such as solid-phase extraction (SPE) [2,6,7,12,18,28], QuEChERS (quick, easy, cheap, effective, rugged and safe) [5], matrix solid-phase dispersion (MSPD) [1,24] or "dilute and shoot" [15]. QuEChERS and "dilute and shoot" methods are faster and easier methods than the others, increasing sample throughput and minimizing errors during extraction stage. These extraction methods provide adequate recoveries and precision, but sometimes a clean-up step is needed in order to minimize the presence of interferences as well as to increase the sensitivity of the method.

For the qualitative and quantitative determination of these compounds, gas chromatography (GC) or liquid chromatography (LC) coupled to mass spectrometry (MS) are the most employed techniques [2]. GC–MS [21] was used for the determination of steroid residues at low levels, but a derivatization step is needed. However, due to the difficulty of such reaction for some steroids, and the absence of appropriate derivatization agents, the use of LC–MS [3] has become more popular for the analysis of these compounds [4]. The application of tandem mass spectrometry (MS-MS) [1–3,5–10,23,24] has become a powerful technique that allows for the analysis of hormone residues in complex matrices, such as meat, at trace levels without the need of derivatization. Moreover, several ionization modes have been used for the identification of steroid hormones such as electrospray ionization (ESI) [1,15] or atmospheric pressure chemical ionization (APCI) [10,13].

High resolution mass spectrometry (Exactive-Orbitrap) [13,28] overcomes the limitations of other techniques, such as the possibility of retrospective data analysis or the selective detection of residues at low concentration levels. LC-Orbitrap operates in the full scan mode and provides accurate mass measurements (<5 ppm). Therefore this technique can be a useful tool for the analysis of hormone residues at low concentration due to its high resolving power [29]. As far as we know only three previous studies employed the Orbitrap analyzer to determine hormone residues in meat [13,30] and biological fluids and eggs [28]. One of them analyze one steroid (androsterone) [13] in porcine meat and meat products meanwhile the other analyze thirty four steroids [30] in meat. In the last study five metabolites were analyzed [28], although they were not steroid hormones. Moreover, only few studies analyzed more steroids than in the present work [10,15,18,30] in bovine meat, serum and plasma samples. Nevertheless, the present method is easy and fast, fulfilling the need of developing analytical methods that allow the quantification of several steroids with high reproducibility and sensitivity. In the current article, steroid hormones and their metabolite have been quantified applying a new analytical method based on ultra-high performance liquid chromatography coupled with Orbitrap mass spectrometry (UHPLC-Orbitrap-MS), which has been developed and validated in meat from different animals (pork, beef and chicken). Two different extraction procedures were compared, QuEChERS and "dilute and shoot", evaluating recoveries and precision.

2. Materials and methods

2.1. Chemicals and reagents

Commercial standards were purchased from different companies with high purity: 17α-hydroxyprogesterone, cortisone-21-acetate, 6α-methylprednisolone, prednisolone, hydrocortisone-21-acetate, testosterone, melengesterol acetate, cortisone, hydrocortisone, prednisone, testosterone propionate, 17α-methyltestosterone and medroxyprogesterone were provided by Sigma Aldrich (St. Louis, MO, USA) with purity ≥ 97%. Progesterone was supplied from Santa Cruz Biotechnology (Dallas, TX, USA) with purity ≥ 99%. Individual stock standard solutions (1000 mg/L, except for 17α-hydroxyprogesterone at 50 mg/L) were prepared in methanol and were stored at ≤5 °C.

Different reagents as primary secondary amine (PSA) and Florisil cartridges were obtained from Scharlab (Barcelona, Spain), C_{18} from Agilent Technologies (Santa Clara, CA, USA), anhydrous magnesium sulfate and sodium chloride from Sigma Aldrich, Z-Sep+ from Supelco (Bellefonte, PA, USA) and aluminum oxide from Bruker (Billerica, MA, USA). All solvents were LC–MS grade. Methanol was purchased from Fluka (Steinheim, Germany) and acetonitrile from Sigma Aldrich. LC–MS water was purchased from J.T. Baker (Deventer, The Netherlands). Formic acid was acquired from Thermo Fisher Scientific (Geel, Belgium). Econofilter Nylon filters (13 mm, 0.20 μm pore size, Agilent Technologies) were used for filtration of extracts.

Different multi-compound working solutions were prepared in methanol by a combination of each individual standard stock solution, ranging from 10 mg/L to 0.01 mg/L. These solutions were kept at ≤5 °C in the darkness.

For accurate mass calibration of the Orbitrap analyzer a mixture of acetic acid, sodium dodecyl sulfate, taurocholic acid sodium salt hydrate and Ultramark 1621 (fluorinated phosphazines) (Proteo Mass LTQ/FT-Hybrid ESI negative mode calibration mix) and a mixture of caffeine, Met-Arg-Phe-Ala acetate salt (MRFA) and Ultramark 1600 (Proteo Mass LTQ/FT-Hybrid ESI positive mode calibration mix) from Thermo Fisher Scientific were used.

2.2. Apparatus

During the extraction procedure several instruments were used: an analytical AB204-S balance (Mettler Toledo, Greinfesee, Switzerland), a rotary agitator from Heidolph (Schwabach, Germany), a Consul 21 high-volume centrifuge from Olto Alresa (Madrid, Spain), and a vortex mixer WX from Velp Scientifica (Usmate, Italy).

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