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Determination of steroid esters in hair of slaughter animals by liquid chromatography with tandem mass spectrometry

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Abstract

Introduction: The use of growth promoters in animal husbandry to increase weight gain and efficiency of feed conversion into muscle has been banned in the European Union since 1988, and under Directive 96/23/EC, surveillance for anabolic steroid hormones is obligatory. The hormones present in animal tissues may be of endogenous origin or may result from illegal administration. Steps have been taken to determine selected steroids in the form of esters in the alternative matrix of animal hair. Their detection in biological material is direct proof of the illegal use of anabolics. **Material and Methods:** The procedure for the determination of steroid esters in animal hair, based on digestion, extraction, purification, and liquid chromatography with tandem mass spectrometry was validated under the current regulations. In total, 348 samples of animal hair were examined using this method. **Results:** Good recoveries and precision values (RSD) were obtained during validation. Decision limits (CCα) and detection capabilities (CCβ) were in the ranges of 2.57–4.18 μg kg^{-1} and 4.38–7.12 μg kg^{-1}, respectively. The method met the criteria for confirmation techniques with respect to Commission Decision 2002/657/EC. **Conclusion:** Testing for steroid esters in animal hair was introduced into the National Residue Control Programme in 2017. Steroid esters were not found in any hair samples above the CCα, which indicates that illegal use of anabolics was not confirmed.

Keywords: bovines, swine, hair, steroid esters, liquid chromatography with tandem mass spectrometry.

Introduction

Consumer safety and health should be a priority for services dealing with the examination of animals and food of animal origin, and there is an obligation upon those services to detect residues of hazardous substances that can be administered to animals for various reasons and could potentially pose a threat. Undoubtedly, one of the sources of consumer exposure to veterinary medicinal products is carelessness at the stage of food production and non-compliance with the prescribed safety measures by the farmers.

The use of hormonal substances for fattening purposes in livestock has been banned in the European Union (EU) since 1988, according to Council Directives 96/22/EC and 2003/74/EC (7, 8). The steroid esters that belong to the A3 group of banned substances should not be present in biological matrices of animal origin, just as other unauthorised substances having anabolic effect included in Annex 1 of Council Directive 96/23/EC. In line with the recommendations of the designated European Union Reference Laboratories (EURLs) which oversee and are responsible for the testing of prohibited substances in the EU, their residues in biological material of animal origin should be monitored (9). The EURLs in Germany, the Netherlands, and France responsible for specific groups of veterinary medicinal products have proposed recommended concentrations (RC) for individual hormones in biological matrices for the purpose of residue monitoring in a joint document referring to analytical techniques used in National Residue Control Programmes (NRCPs) (10). The EURLs in consort, as the institution supervising the range of hormone tests in EU Member States, recommended adopting an RC value of 10 μg kg^{-1} for steroid esters, although the cited legal document does not include these compounds in the hair matrix.

Synthetic steroid hormones are usually administered to animals in the form of ester derivatives, which thanks to the ester chains more easily reach their target sites in the animal's body. This form of administration also allows their easier absorption and transport to the target organs. Due to their chemical structures, steroid esters differ in their properties, *e.g.* volatility. The esters can be administered to animals in the form of pour-on hormone cocktails in places from the neck to the tail and in the form of intramuscular injections. After administration (especially by injection) they are hydrolysed or epimerised and transported with body fluids to peripheral blood. The length of the carbon chain in the steroid ester molecule determines its solubility in water; compounds with short chains are quickly transported from the injection site in the muscles to the blood, resulting in higher concentrations; esters with long side chains, in contrast, are transported slowly, are present in lower concentrations, but last for a long time. Unlike animals, in humans oral administration predominates by a wide margin (28). As compared to administration by other means, *e.g.* orally in non-esterified form, administration of steroid hormones intramuscularly in the form of esters is likely to prolong the effect of their action and improve the bioavailability in the body (16). There are many well-known medicinal preparations available on the market, the main components of which are steroid esters, *e.g.* Omnadren, Sustanon, Testoviron Depot (all with testosterone propionate), Deca-Durabolin (with nandrolone decanoate), Durabolin (with nandrolone phenylpropionate), Boldane, Equipoise, Parenabol, and Vebonol (all with boldenone undecylenate), as well as a wide range of other products.

The hormones present in animal tissues may be of endogeneous origin or may have been illegally administered. The concentration of hormones occurring naturally in living organisms differs from animal to animal depending on the species, sex, age, normality of physiological and individual development, and the state of health and acquired injuries (16). The natural hormones are present in almost all matrices. Some naturally occurring steroid hormones like boldenone, nortestosterone (nandrolone), and testosterone as well as their metabolites may have the same chemical structure as their synthetic analogues (11, 17, 27). A large number of studies in scientific centres were carried out on populations of treated and untreated animals of different species to learn the steroid profile of hormones. Acknowledging the positive results for steroid hormones in EU countries over the years, the reasons for the pseudoendogeneous hormone occurrences were sought. As one of their sources, the involvement was suspected of microorganisms in the course of biochemical reactions taking place in the body and a contribution by natural steroid hormones. In order to clarify the possible origin of these hormones in non-treated animals, the EURLs recommend continuing research on threshold values for specific biomarkers (species and sex dependent) and collecting further data from NRCPs (27).

Although steroid hormones are found in different matrices, of which the most common is urine, it is difficult to distinguish their origin and to prove abuse. Therefore, one alternative way to confirm illegal use is the study and detection of these synthetic steroid hormones in the form in which they are directly administered both to animals and humans, *i.e.* as steroid esters (28). Steroid hormones do not naturally occur in the form of esters; therefore the detection of steroid esters gives incontrovertible proof of the illegal use of anabolics. However, the detection of abuse of synthetic steroid esters in routine urine testing of animals or athletes is practically impossible, because within a few hours of administration hormone esters are quickly hydrolysed to parental synthetic steroid hormones. These are mainly excreted in urine and have a structure analogous to pseudoendogeneous compounds also present in the urine, and the synthetic and pseudoendogeneous are difficult to distinguish from each other by origin. For this reason, steroid esters became attractive to athletes or cyclists as early as the 1980s when it was noticed that they are undetectable in urine (15).

The detection and confirmation of steroid esters in biological samples of animal origin is one of the oldest analytical approaches for proving abuse of natural hormones. However, for many years its applicability was limited to the analyses of alleged injection sites; the tissues for testing were taken from the places where the injection was most likely given while the analyses were carried out using chromatographic techniques less sensitive in relation to more advanced current methods, for example using ultraviolet/diode array detection (UV/DAD) (6, 21, 29, 30, 31). Based on new know-how for the incorporation of steroid ester tests on alternative biological matrices of animal origin and new technical detection capabilities, in recent years steroid esters have become more popular again in the analysis of residues of anabolic hormones. Currently, determining whether the hormone present in the urine is endogeneous or whether it is a result of administration can be carried out through studies of synthetic esters in hair of animal origin. Steroid esters are not metabolised in hair. The residues of different compounds including steroid esters in the hair of animals persist even up to several months after administration, therefore the presence of steroid esters in the hair of animals indicates without ambiguity the illegal administration of anabolics.

Drugs can penetrate into the hair of animals from the blood in systemic circulation (an endogenous vehicle), from skin secretions (absorption from sweat, serum, and sebaceous glands), and even from neighbouring tissues. They can also pervade hair from the external environment (from environmental pollution, an exogenous pathway), and none of these

routes are made impossible even when the hair detaches from the follicle (24). Steroid esters may not only be adsorbed on the surface but also absorbed during hair growth, due to the relatively high lipophilicity (12). In the growing strand of hair, the drug molecules move on the principle of passive diffusion from the blood supply, hence the accumulation of drugs in a given form in this matrix, however, there are no data defining the relationship between the length of the hair and the dose of administration (15). Studies of hair segments indicate that there is no correlation between the time of drug incorporation and hair growth rate. The route by which the drug penetrates the hair and individual metabolic rate differences between specimens has an effect on the drug concentration in a particular part of the hair (28). Many factors can affect the penetration of the substances into the hair: the pH of the substance (neutral, acidic or alkaline), the presence of pigment melanin or the lipophilicity of the substance. The pH of the hair is acidic (typically close to 6), hence its predisposition rather to retain alkaline compounds. Melanin pigment produced in melanocytes in the presence of tyrosine enzyme at an acidic pH of about 3–5 is specialised in the absorption of alkaline substances and helps to transport the substances inside the hair. It is worth adding that dark hair contains more of this pigment and can accumulate a greater number of different substances than fair hair. Scientific research found that basic and hydrophobic compounds are more strongly bound by melanin than others are (15). In connection with this, it has been concluded that black hair has the possibility of accumulating basic compounds, while fair hair more effectively absorbs those which are acidic and neutral. It cannot be ruled out that various beauty and cosmetic treatments can contribute to the degradation of substances contained in the hair.

Hair offers many benefits as a matrix for research, in particular because of the easy non-invasive collection. In addition, animals do not feel stress while hair samples are being collected on the farm. It is a complementary material widely used for research in human medicine in forensic examinations for judicial purposes in cases of suspected possession of drugs, illegal abortion, medical malpractice, rape, kidnapping, attempted murder, or theft. The slow growth of hair allows the detection of chronic exposure to toxic metals, organic poisons, and drugs or the intake of substances over a long time (usually from one to six months), while it is not suitable material for the assessment of acute poisoning. However, there is no correlation between the detected concentration and the administered dose of the substance. In order that reliable results from laboratory analyses are obtained, specific guidelines have been created, applying even at the early stage of taking hair samples for testing. In human medicine it is recommended to collect hair samples for forensic testing between four and five weeks from the time of the suspected introduction of a dangerous substance into the body. For this purpose, hair samples are collected by cutting a bundle with a diameter of about 0.5 cm, close to the skin from the back of the head and tying with white thread in the proximal part of the scalp (1, 19). In the case of animals, an American veterinary medical diagnostic laboratory has recommended collecting samples of hair from the tail and neck of the individual (4). The transport conditions to the laboratory are also important; dry samples should be sent at ambient temperature, in a paper envelope, aluminium foil, and a plastic or glass vial (19).

Recently, hair samples have become a popular matrix of choice for testing hormone abuse in veterinary research in animals. Currently, only one of the available instrumental techniques of detection – the highly specialised gas chromatography combustion isotope ratio mass spectrometry (GC–C-IRMS, based on $^{13}C/^{12}C$ isotope ratio measurement) – gives the possibility to distinguish endogeneous hormones (*e.g.* testosterone, boldenone, nortestosterone (nandrolone), and oestradiol) from their synthetic analogues that may have been administered to animals, and can do this in confirmatory urine tests (23). However, due to the high operating costs, GC–C-IRMS measuring instruments are only available in very few EU laboratories, and they are those performing national official residue surveillance tests. Steroid esters are non-volatile, therefore liquid chromatography with tandem mass spectrometry (LC–MS/MS) methods are preferred for analysis (11, 18), although there are also gas chromatography procedures used for the determination of steroid esters and other hormones in the hair and other matrices like serum (23–26).

The need to develop and implement a method for determination of steroid esters in hair arose from difficulties in the interpretation of animal urine and serum test results where confirmation was sought of the endogeneous or pseudoendogeneous nature of hormones (testosterone, nortestosterone (nandrolone), and boldenone). These difficulties were due to the lack of specific criteria for evaluation. A simple and sensitive method was developed for the detection and confirmation of nortestosterone phenylpropionate, nortestosterone decanoate, boldenone undecylenate, testosterone propionate, and testosterone benzoate as steroid esters in animal hair using LC–MS/MS in accordance with the technical recommendations of the EURLs (10). The procedure for the determination of steroid esters in hair was introduced in 2017 for testing conducted as part of the implementation of the NRCP.

Material and Methods

Reagents and chemicals. Solvents comprising methanol (HPLC, HPLC–MS, and residue-analysed grade), acetonitrile (HPLC–MS grade), and ethyl

acetate (residue-analysed grade) were purchased from J.T. Baker (the Netherlands).

Other chemicals, specifically formic acid, Tris-(2-carboxyethyl) phosphine hydrochloride (TCEP) were obtained from Sigma-Aldrich (Germany); the solid-phase extraction (SPE) Strata-X 33u (3 mL, 200 mg) Polymeric Reversed Phase columns were produced by Phenomenex (USA); purified water was supplied by a Milli-Q apparatus (Millipore, now Merck, USA); and liquid nitrogen 5.0 was delivered by the Linde Group (Poland).

The simplest solutions were 0.1% solution of formic acid and 60% methanol; others were constituted as follows: TCEP (25 mM) was prepared by dissolving the solid substance (7.25 mg) in water (1 mL) each time before analysis; the reconstitution solution for dissolving the dry residue of the sample before LC–MS/MS was an acetonitrile, water, and 0.1% formic acid solution in the proportions 70 + 30 + 2 v/v/v; the LC–MS/MS mobile phase A consisted of water and 0.1% formic acid in the proportions 100 + 2 v/v; and the LC–MS/MS mobile phase B consisted of acetonitrile and 0.1% formic acid in the proportions 100 + 2 v/v.

The standards of steroid esters were testosterone propionate (TP) and testosterone benzoate (TB), purchased from Sigma-Aldrich (Germany); boldenone undecylenate (BU), procured from LGC Standards (Germany); nortestosterone decanoate (NTD), obtained from the Australian Government National Measurement Institute (Australia); and nortestosterone phenylpropionate (NTPhP), bought from Steraloids (UK).

The internal standards (IS) of steroid esters included testosterone-d3 propionate (T-d3P), testosterone-d3 benzoate (T-d3B), and testosterone-d3 decanoate (T-d3D), sold by RIKILT Institute of Food Safety (now Wageningen Food Safety Research – the Netherlands). All standards were stored according to the recommendations of their certificates. Primary standard stock solutions were prepared in methanol at concentrations of 1 mg $mL^{-1}$and 10 µg mL^{-1} and kept in a freezer. Working solutions were obtained by tenfold dilution of primary standard solutions to a concentration of 1 µg mL^{-1} in methanol. Structural formulas of molecules of steroid esters are presented in Fig. 1.

Sample preparation. Hair sample processing was based on the analytical procedure used in the RIKILT EURL (now Wageningen Food Safety Research) in the Netherlands (3), in using which the National Laboratories of the EU Member States were trained prior to the introduction of the procedure for official investigations. After a hair sample's reception at the laboratory, it was initially washed with warm water and dried for 12 h at ambient temperature (when there were visible external contaminants). Subsequently, the hair fibres were cut into smaller pieces of about 0.5 cm with scissors and ground in a mortar in liquid nitrogen to obtain a powder. The hair samples were stored in a frozen state until laboratory analysis, directly before which they were brought to room temperature. The sample preparation process before instrumental analysis was carried out according to the protocol described below.

A total of 200 mg (± 2 mg) of shredded hair was weighed and placed in a 10 mL glass centrifuge tube. Internal standards were added to the sample until a concentration of 50 µg kg^{-1} was obtained, followed by 2 mL of aqueous solution of TCEP. After thorough mixing on a vortex machine, the contents of the tube were shaken for 1 h. Next, 4 mL of methanol was added to the sample and after thorough mixing of the contents of the tube on the vortex machine again, the sample was centrifuged at 2,795 g for 5 min. The upper methanol layer was then collected and placed in a second tube. Water in a 4 mL volume was added to the collected layer of methanol and the entire contents of the tube were thoroughly mixed.

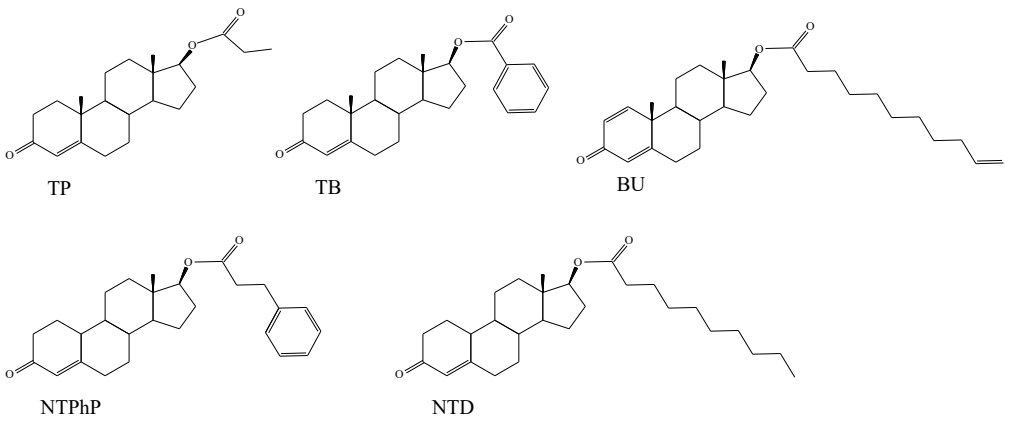

Fig. 1. Structural formulas of molecules of steroid esters

Subsequently, the sample was subjected to a further purification step using solid-phase extraction on Strata-X cartridges. The SPE column was successively activated with 6 mL of methanol and 6 mL of water. The sample extract was then applied onto the cartridge. Next, the cartridge was washed with 3 mL of 60% methanol and dried under a vacuum for 1 min. The steroid esters were eluted from the cartridge with 3 mL of acetonitrile and 3 mL of ethyl acetate. Afterwards, the eluate was evaporated to dryness under a gentle stream of nitrogen at 40 ± 2°C. The dry residue was dissolved in 200 µL of reconstitution solution and placed in an ultrasonic bath for 5 min in order to accurately disperse the sample in the solvent. The content of the tube was centrifuged again at 2,795 g for 5 min. At the final stage the extract was transferred to bottles with glass inserts and placed in an autosampler immediately before the LC–MS/MS analysis was performed.

LC–MS/MS measurement. The chromatographic separation of steroid esters was achieved using an HP 1200 binary pump system (Agilent Technologies, Germany) on a Poroshell 120 EC-C18 analytical column (150 mm × 2.1 mm, 2.7 µm) (Agilent Technologies) with an octadecyl guard cartridge (4 mm × 2 mm) (Phenomenex, USA). Elution solvents were a mixture of water and 0.1% formic acid solution (100:2 v/v) (A) and acetonitrile and 0.1% formic acid solution (100:2 v/v) (B). The mobile phase composition (A:B v/v) was 15:85 from 0 to 8 min, then changed to 2:98 until minute 11, was next maintained at 2:98 from 11 to 18 min, and finally was 15:85 from 18.1 to 20 min for re-equilibration. The mobile phase flow rate was 0.25 mL min^{-1} from 0 to 8 min, and then changed to 0.40 mL min^{-1} until minute 11, and was next maintained at 0.40 mL min^{-1} from 11 to 20 min.

The chromatographic column was maintained at a constant temperature of 45°C, and the injection volume was 25 µL. A triple quadrupole QTRAP 5500 mass spectrometer (Applied Biosystems/MDS SCIEX, Canada) controlled by Analyst Software (version 1.6) was used for MS/MS analysis and for collection and processing of the data. The following detection conditions were set: an electrospray ionisation (ESI) Turbo Spray source operating in positive mode at 250°C with curtain (nitrogen) and nebuliser gas (air) settings of 20 psi, auxiliary gas (air) 25 psi, collision gas (nitrogen) set at the medium position, ionisation voltage 5,500 V, and MRM dwell time 40 ms with a pause between mass ranges of 5 ms.

The LC–MS/MS acquisition parameters used for identification and confirmation of relevant steroid esters are presented in Table 1.

Validation study. The presented LC–MS/MS-based method was validated in accordance with the requirements of Commission Decision 2002/657/EC for confirmatory methods (5). The samples of hair taken from bovines used as reference were tested earlier in the laboratory for the presence of the steroid esters which were the subject of research. The efficacy parameters such as instrumental linearity, specificity, repeatability, reproducibility, apparent recovery, decision limits, and detection capabilities were determined.

Table 1. LC–MS/MS ion acquisition parameters (MRM mode) used for the identification and confirmation of the steroid esters

Compound	MRM transition (m/z)	Collision energy CE (eV)	Declustering potential DP (V)	Entrance potential EP (V)	Collision cell exit potential CXP (eV)	Ion ratio average ± SD	Samples fulfilling the confirmation criteria (%)	
							5.0–50.0 µg kg^{-1}	CCα level
TP	**345.4 > 97.1***	29			27	-	-	-
	345.4 > 109.3	46	200	11	4	0.608 ± 0.113	99.2	100.0
	345.4 > 253.4	25			24	0.272 ± 0.073	100.0	98.1
	345.4 > 271.2	25			20	0.322 ± 0.088	98.1	98.1
TB	393.3 > 97.0	42	70	12	20	0.435 ± 0.070	98.8	94.5
	393.3 > 105.1	20			14	-	-	-
BU	453.4 > 121.3	44	100	10	10	0.466 ± 0.065	97.9	98.1
	453.4 > 135.1	26			16	-	-	-
	453.4 > 149.2	24			17	0.414 ± 0.071	95.3	92.0
	453.4 > 269.1	14			21	0.814 ± 0.097	100.0	100.0
NTD	429.4 > 95.2	30	180	12	12	0.569 ± 0.114	99.1	98.0
	429.4 > 155.1	26			27	-	-	-
	429.4 > 239.0	32			18	0.319 ± 0.063	97.3	94.1
	429.4 > 257.1	24			11	0.833 ± 0.095	99.2	97.8
NTPhP	**407.4 > 105.0**				16	-	-	-
	407.4 > 133.0	48	200	14	23	0.266 ± 0.073	97.2	92.5
	407.4 > 257.2				28	0.301 ± 0.070	98.8	98.2
T-d3P	348.4 > 97.0	28	90	11	15	-	-	-
T-d3B	396.4 > 105.0	28	100	10	15	-	-	-
T-d3D	446.4 > 97.0	51	145	10	22	-	-	-

* transitions shown in bold were used for quantification

TP – testosterone propionate; TB – testosterone benzoate; BU – boldenone undecylenate; NTD – nortestosterone decanoate; NTPhP – nortestosterone phenylpropionate; T-d3P – testosterone-d3 propionate; T-d3B – testosterone-d3 benzoate; T-d3D – testosterone-d3 decanoate

The calculation tool was ResVal software (v. 2.0) shared by the EURL in Wageningen in the Netherlands, and this application assumes the execution of four experiments (2). The instrumental linearity of the method was evaluated on the basis of four calibration curves of standard working solutions of steroid esters, prepared one for each of the four experiments assumed. All the calibration curves had seven cross points for analyte concentrations corresponding to 0, 2.5, 5.0, 7.5, 10, 25, and 50 µg kg^{-1} and for IS solutions at 50 µg kg^{-1} in samples. A validation level (VL) of 10 µg kg^{-1} was adopted in accordance with the EURL suggestions. Based on this level, the software set out specific levels of sample spiking in a validation series. According to that scheme, three series of spiked bovine hair samples were prepared. Each of them contained a blank reference hair sample, six hair samples spiked at concentration levels of 5.0, 10, and 15 µg kg^{-1} and one hair sample spiked to a concentration of 20 and 50 µg kg^{-1} in sequence. The calculations of the concentrations of steroid esters in spiked samples, made based on the standard calibration curves, were performed using the analogous deuterated standards for TP and TB, but for NTPhP T-d3B was used and for BU and NTD T-d3D was taken. Based on these data, matrix-matched calibration curves were plotted. Regression parameters of both types of prepared curves were calculated. Heeding the approach described in the ISO/11843 standard (20), the CCα and CCβ parameters were determined based on the matrix-matched calibration curves of steroid esters, using the mathematical equations from ResVal: $CC\alpha = ((y_a + 2.33 \cdot STD_a) - y_a))/b$ and $CC\beta = ((y_a + 2.33 \cdot STD_a + 1.64 \cdot STD_a) - y_a))/b$, in which y_a defines the intercept of calibration curve, STD_a specifies the standard deviation of y_a, and b means the slope of the calibration curve.

Through these experiments, apparent recovery, precision and uncertainty were also assessed. Specificity was addressed in the fourth experiment which assayed ten different blank bovine hair samples simultaneously with ten identical samples of hair spiked with a 10 µg kg^{-1} mixture of steroid esters in order to check interfering peaks of unknown compounds around the retention time of the steroid esters tested. The expanded uncertainty for relevant steroid esters was calculated by the ResVal software at 10 µg kg^{-1}, as the sum of variances of reproducibility and matrix effect multiplied by a coverage factor of 2. The influence of the matrix on the signal response was also checked. The values of matrix effect (ME) were investigated based on the ratio of the slopes of standard and matrix-matched calibration curves.

In obedience to the guidelines of Decision 2002/657/EC, the confirmation criteria in relation to permitted tolerances for relative ion intensities and the compatibility of the relative retention time for steroid esters in spiked validation samples were checked.

In addition, hair samples spiked at the estimated values of the CCα were appraised for reliability according to SANCO guidelines (13). Fulfilment of the confirmation criteria of Decision 2002/657/EC (at least four identification points) was also ascertained for those hair samples spiked at the estimated CCα level.

A complementary experiment analysing ten samples of blank hair from pigs (previously tested for the presence of steroid esters) fortified to a concentration of 10 µg kg^{-1} was additionally performed in order to demonstrate the veracity of the assumption that the results of the validation process for bovine hair sample testing are versatile. The values of average apparent recovery and the standard deviation obtained for pig hair were compared statistically to the corresponding values received in the fourth validation experiment for the samples of bovine hair spiked at 10 µg kg^{-1}. For result comparison purposes, two statistical tests were used: a *t*-test ($t = (x_1 - x_2)/s\sqrt{(1/n_1 + 1/n_2)}$) to compare the means and a *F*-test ($F = s_1^2/s_2^2$) to compare the standard deviations (22).

Samples. The research material was samples of hair which were taken during the years 2017–2018 by authorised Veterinary Sanitary Inspectors throughout Poland in accordance with the specifications and in the ambit of NRCPs. Within the entire pool of samples, targeted samples and suspect samples reported as follow-ups of non-compliant targeted samples were collected. The samples for testing were taken from live animals on the farm and from slaughterhouses, in accordance with the requirements of national and EU legislation on levels of sampling (9). The hair samples for research were drawn from two species of animals: bovines and swine. In the period 2017–2018, 348 samples of animal hair (155 from cattle and 193 from pigs) were tested for the presence of steroid esters. Among all the samples, 336 were taken from targeted animals and additionally 12 (5 from bovines and 7 from pigs) were taken from suspect animals following non-compliant results found in urine for nortestosterone (nandrolone) and/or boldenone. In the case of cattle, 80 samples came from the farm and 75 from the slaughterhouse. Males provided 64 of these samples and females 91. Most of them, that is 124 samples, were dark and only 31 were light. In the case of pigs, 34 samples came from the farm and 159 from the slaughterhouse. Of them, 56 were taken from males and 137 from females. Only 13 swine hair samples were dark and 180 were light. All samples from official monitoring were tested using the described validated confirmatory method.

Results

A summary of the results of the validation of the confirmatory method for the analysed steroid esters is presented in Table 2. The linear regression parameters

for the standard and matrix-matched calibration curves were correct for all examined steroid esters over the whole range of the tested concentrations. The calculated regression coefficients for plotted curves were greater than 0.98 as presented in Table 2. An overview of the calibration parameters is summarised in the lower part of the table. The chromatographic analysis of blank matrix samples for interferences did not show any accompanying peaks in the retention time ranges of steroid esters, proving the specificity for the tested compounds.

In total, the apparent recovery of steroid esters from the hair matrix at all validation spiking levels ranged from 84.3% for TP to 109.7% for NTPhP with repeatability expressed as relative standard deviation not exceeding 30% (8.6–29.6%) and coefficient of variations (CV) under within-laboratory reproducibility conditions less than 40% (13.9–39.9%). For all tested steroid esters, the calculated values of CCα and CCβ presented in detail in Table 2 were below the 10 µg kg^{-1} adopted as the RC and the level of validation. The apparent recoveries of steroid esters from the hair samples spiked to the estimated values of CCα were correct and ranged from 89.8% for NTPhP to 95.7% for BU. Additionally, the designated values of expanded uncertainty ranged from 3.02 µg kg^{-1} for TB to 4.94 µg kg^{-1} for BU (30.2–49.4%). The matrix effect presented as a percentage did not exceed 25% and was in the range of −23.6% for TB to 22.3% for BU, which is shown in Table 2. Representative LC–MS/MS (MRM) chromatograms of the hair sample spiked with steroid esters at the validation level of 10 µg kg^{-1} are presented in Fig. 2.

Table 2. Parameters of analytical efficiency of the LC-MS/MS method for the determination of steroid esters in animal hair

Compound		TP	TB	BU	NTD	NTPhP
Apparent recovery (%)	5*	96.4	104.7	93.1	96.0	109.7
	10	84.3	108.5	92.7	98.1	89.7
	15	92.3	96.5	89.1	95.8	92.6
	20	97.3	107.2	102.5	109.3	103.8
	50	93.4	91.8	90.1	97.9	88.8
	CCα	90.4	92.0	95.7	92.1	89.8
Repeatability (RSD, %)	5	11.8	14.6	10.1	25.4	8.6
	10	19.7	12.7	20.4	11.2	21.7
	15	26.1	9.2	17.9	19.0	29.6
	CCα	11.8	10.5	11.3	13.6	12.9
Within-lab reproducibility (RSD, %)	5	29.9	39.8	39.9	32.1	17.4
	10	22.6	13.9	26.7	17.6	21.7
	15	37.2	34.4	39.7	30.6	37.0
Decision limit (CCα, µg kg^{-1})		3.51	2.57	3.51	3.98	4.18
Detection capability (CCβ, µg kg^{-1})		5.98	4.38	5.99	6.78	7.12
Measurement uncertainty at validation level of 10 µg kg^{-1} (U, k = 2, %)		38.0	30.2	49.4	37.8	39.0
Matrix effect (%)		−5.2	−23.6	22.3	−16.9	−18.2
Comparison of accuracy and repeatability in bovine (n = 10) and pig (n = 10) hair samples spiked at 10 µg kg^{-1}						
Apparent recovery (%)		95.2	109.2	84.6	101.2	82.5
Repeatability (RSD, %)		19.6	14.1	22.6	13.1	16.9
t $test_{exp.}$		1.358	1.448	0.947	0.368	1.239
F $test_{exp.}$		1.915	0.925	0.487	2.721	2.787
Standard curve						
Slope ± s_b		0.0116 ± 0.0001	0.0245 ± 0.0032	0.2404 ± 0.0103	0.0171 ± 0.0038	0.0308 ± 0.0005
y-Intercept ± s_a		−0.0009 ± 0.0021	0.0408 ± 0.0182	−0.1264 ± 0.2725	−0.0073 ± 0.0173	0.0059 ± 0.0301
Correlation coefficient		0.9996	0.9983	0.9965	0.9873	0.9990
Standard error		0.0062	0.0264	0.3919	0.0536	0.0255
Matrix matched calibration curve						
Slope ± s_b		0.0110 ± 0.0004	0.0187 ± 0.0020	0.2939 ± 0.1603	0.0142 ± 0.0327	0.0252 ± 0.0048
y–Intercept ± s_a		−0.0080 ± 0.0236	0.0693 ± 0.1065	−0.1453 ± 1.1592	0.0728 ± 0.6266	0.0642 ± 0.0682
Correlation coefficient		0.9961	0.9943	0.9928	0.9812	0.9812
Standard error		0.0187	0.0389	0.6812	0.0536	0.0947

* spiking level (µg kg^{-1}), 1.734 – the value of *t* $test_{crit.}$ (eighteen degrees of freedom), 3.178 – the value of *F* $test_{crit.}$ (nine degrees of freedom)

RSD – relative standard deviation

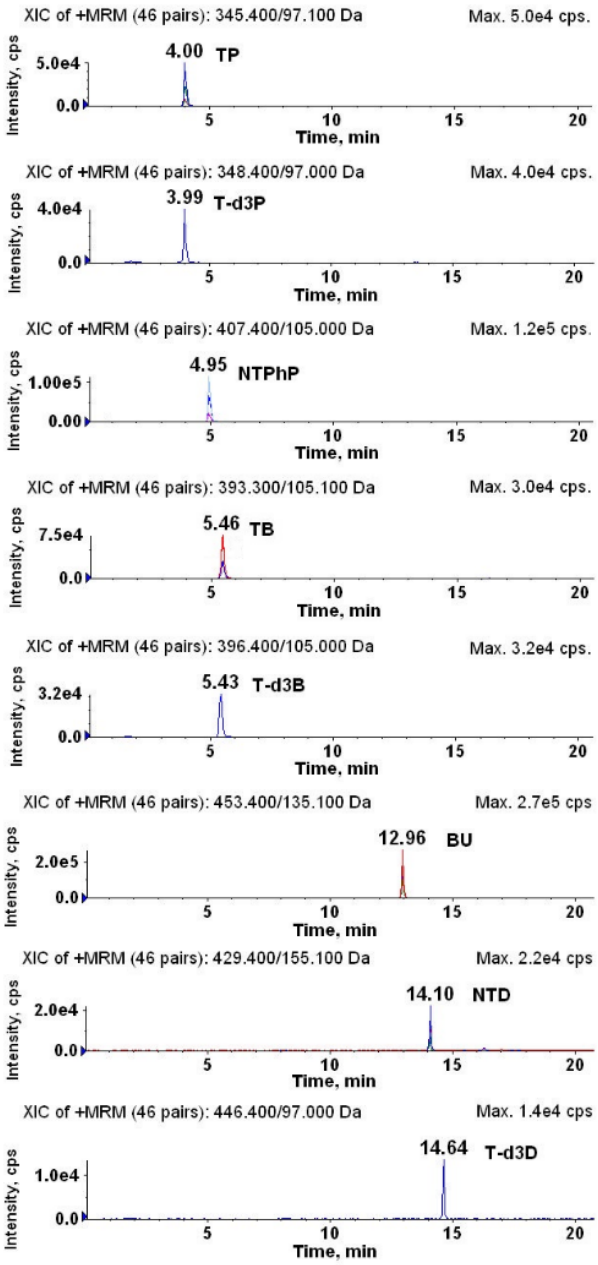

Fig. 2. LC–MS/MS MRM chromatograms of steroid esters (testosterone propionate, nortestosterone phenylpropionate, testosterone benzoate, boldenone undecylenate, and nortestosterone decanoate) in bovine hair samples spiked at 10 μg kg^{-1}

Regarding the criteria for confirmatory methods, average ion ratios for relevant steroid esters and the percentage of samples fulfilling those criteria are provided in Table 1. The results obtained indicate that both for samples spiked at 5–50 μg kg^{-1} and for samples spiked at the designated CCα, the criteria relating to the relative intensities of the transitions and to the retention time were within the specified tolerance ranges. The percentage of samples meeting the criteria for individual steroid esters and appropriate MRM transitions was in the range of 92.0–100%.

For ten pig hair samples fortified with steroid esters at 10 μg kg^{-1}, prepared in order to compare the recovery and CV to those obtained for cattle hair, all values of apparent recovery were in the range of 82.5–109.2% with the relative standard deviations not exceeding 25%, as shown in Table 2. For all steroid esters the experimental values of t-test$_{exp.}$ and F-test$_{exp.}$ did not exceed the critical values of t-test$_{crit.}$ or F-test$_{crit.}$ from statistical tables, assuming the appropriate numbers of degrees of freedom as presented in Table 2.

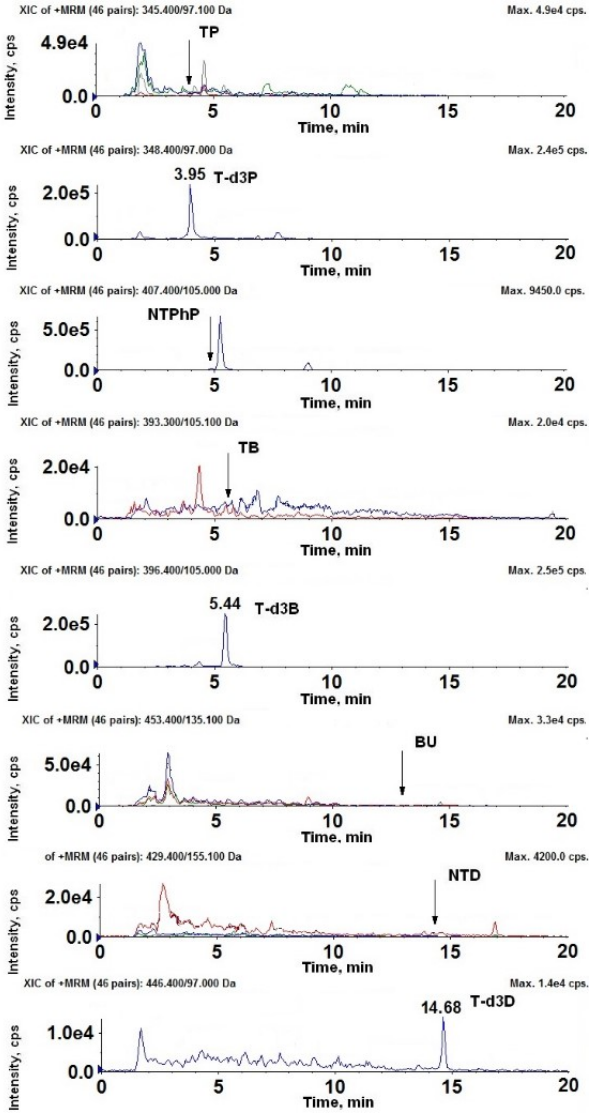

Fig. 3. LC–MS/MS MRM chromatograms of steroid esters in a blank bovine hair sample in which no testosterone propionate, nortestosterone phenylpropionate, testosterone benzoate, boldenone undecylenate, or nortestosterone decanoate were found in official monitoring studies

The presence of TP, TB, BU, NTD, or NTPhP was not detected in any of the samples taken as part of the official monitoring studies nor confirmed above the decision limits determined for the method used. Representative LC–MS/MS (MRM) chromatograms of

an official bovine hair sample in which no steroid esters were found are presented in Fig. 3.

Discussion

The first step in the development of this analytical method was optimisation of the detection conditions of the steroid esters (TP, TB, BU, NTD, and NTPhP) and relevant internal standards (T-d3P, T-d3B, and T-d3D). Based on the literature, a positive ionisation mode was used for these compounds (3, 12, 16). Manual tuning was carried out to the infusion of the steroid ester standard solutions, each separately, into the MS apparatus. The MRM registration mode was selected that provides complete structural specificity of the analyte and the relative or absolute concentration measurement. In the first stage, a specific precursor ion of interest was pre-selected in the first quadrupole (Q1) and the fragmentation was induced by collision excitation with a neutral gas in a pressurised collision cell (the second quadrupole (Q2)). In the second stage, fragments (product ions) generated in Q2 were analysed in the third quadrupole (Q3). For all compounds tested, the minimum two diagnostic MRM signals necessary to confirm the presence of an analyte per Commission Decision 2002/657/EC were obtained, as presented in Table 1. For all MRM transitions, the identified conditions of declustering potential (DP), entrance potential (EP), collision energy (CE), and cell exit potential (CXP) were optimised. The transitions with the most intensity for particular steroid esters were used for quantification purposes.

To obtain the correct chromatographic separation of TP, TB, BU, NTD, and NTPhP two C18 columns were tested which differed in their size and grain properties. They were the Restek Ultra C18 (150 mm × 2.1 mm, 5 μm) and Poroshell 120-EC C18 (150 mm × 2.1 mm, 2.7 μm). Better results were obtained regarding chromatographic separation of compounds as well as signal intensities and geometry of the peaks from the second column. Therefore the Poroshell column was selected for further study. An example of the LC–MS/MS chromatogram registered during the validation process and presenting the separation of tested steroid esters in a spiked hair sample is in Fig. 2.

For the prepared test procedure intended for the determination of steroid esters in animal hair, two specific laboratory stages should be distinguished for their potentially significant impact on the final result of the analysis. However, pre-laboratory events can also have impact. Before the hair sample is processed in the laboratory, it is recommended to transport it there at ambient temperature, putting the tested material in a paper envelope or aluminium foil (plastic film packaging is not recommended) so as to eliminate external sources of chemical contamination. In the laboratory the hair samples were pre-treated. This process included the removal of external pollution and was not complicated if hair samples were taken from live animals on the farm in the recommended way. In such cases, it was sufficient to just wash the hair with warm water and dry it. However, in the case of hair samples taken from animals (mainly pigs) in the slaughterhouse, there were situations in which the samples contained fragments of adipose tissue. The reason for this was probably inaccurate sampling or collection at the wrong time during slaughter. This circumstance necessitated washing the hair sample with an organic solvent (*e.g.* dichloromethane) to remove lipophilic impurities like wax and then washing with warm water and drying.

Nevertheless the first impactful stage in the laboratory that concerned the pre-treatment of the hair sample prior to the purification step was grinding, which aimed for the formation of a homogeneous material. For this purpose, under the conditions of our laboratory, hair was shredded manually in liquid nitrogen. It naturally required a greater amount of force than mechanical processing, but obtained appropriately homogeneous material for the research. Although it is recommended to use a special grinding mill, our technique without such did not cause analytical difficulties. Furthermore the correct values of RSD for repeatability in the range of 4.4–29.6% as shown in Table 2 were obtained, which indicate that the material for the tests was appropriately prepared.

The most critical stage of the analysis is undoubtedly the hair digestion reaction, which may be influenced by how well the sample was ground. Hair is a complicated matrix that can cause analytical problems. As a filamentous, callous formation, made of the hard, cohesive, fibrillar protein keratin, it exhibits high resistance to physical and chemical factors as well as the action of proteolytic enzymes.

Breaking long chains of keratin proteins in the hair structure to release steroid esters for further extraction in the next stage requires the use of strong reducing compounds. Adopting suggestions in the available literature, the suitability of various compounds for this purpose was tested in preliminary studies. Basic hydrolysis was carried out with sodium hydroxide, acid hydrolysis with hydrochloric acid, and hydrolysis with phosphate buffer as well as with sodium methylate, as other researchers have described in their papers (15, 16, 25). In addition, dithiothreitol (DTT) was also used for this purpose as a weak reducer, capable of reducing disulphide bonds, but operating in a limited pH range and unstable due to its easy oxidation under the influence of atmospheric oxygen. In our laboratory the mixture for derivatisation of other hormones tested included DTT as a constituent. The effectiveness of the most commonly used reagent, TCEP, has also been tested (12, 17, 18, 23, 28). This compound is one of the stronger, non-regenerating reducers, used in biochemistry and molecular biology. It is able to break disulphide bonds inside and between proteins, reacts in a wide pH range, is stable, and does not oxidise under

the influence of atmospheric oxygen. The recovery and repeatability obtained for individual steroid esters (TP, TB, BU, NTD, and NTPhP) were compared after using specific reducers. Because the best results were obtained for TCEP, it was selected for further research stages.

During the optimisation of the sample purification step, the use of two types of reversed-phase cartridges was evaluated in the solid-phase extraction process. One was a C18 column with hydrophobic silica sorbent retaining non polar compounds powerfully, and the other was a Strata-x column with polymeric sorbent, relying on the three mechanisms of retention pi-pi bonding, hydrogen bonding, and hydrophobic interaction, retaining neutral, acidic or basic compounds strongly under aggressive, highly organic wash conditions. The Strata-x columns gave comparable recovery to the C18, but better repeatability, and more specific chromatograms were obtained; therefore these columns were chosen for further stages of the research.

The confirmatory method's performance was appraised on various parameters required by legislation for the validation of quantitative confirmatory methods (5). It was found that the method has sufficient selectivity and specificity, because no interfering unidentified compounds were noticed in the range of retention time of target steroid esters, which is confirmed by the chromatogram of the negative sample in Fig. 3. The linear regression parameters determined for the calibration curves of standards and matrix-matched curves fitted the curve well and meant a linear response over the range tested, in line with statistical modelling theory. From the statistical point of view, the convergence factor, one of the basic measures of quality and an indication of the closeness of match, should be greater than 0.9, proving an excellent fit (only a score of 1 qualifies as a perfect fit). Satisfactory apparent recoveries above 84% and below 110% were obtained for a concentration range from 5 to 50 μg kg^{-1}. The recovery values were in the reference range defined for minimum trueness of quantitative methods spanning −30% to +10% according to Commission Decision 2002/657/EC for concentrations from 1 to 10 μg L^{-1} (kg^{-1}) in a sample (5). The method was also characterised by good precision when expressed as RSD under repeatability (less than 30%) and within-lab reproducibility (less than 40%). In accordance with current legislation requirements for analyses carried out under repeatability conditions, the intra-laboratory CV would typically be between one half and two thirds of the above values, while for analyses carried out under within-laboratory reproducibility conditions, the within-laboratory CV cannot be greater than the reproducibility CV. In general for spiking levels lower than 100 μg kg^{-1}, CVs should be as low as possible (5), in which case calculations using the Horwitz equation give unacceptably high values. With regard to our procedure, the values can be considered correct; in some cases the RSD of within-lab reproducibility is higher than 30%, but in general it does not exceed 40%. The values resulting from our validation especially in reference to apparent recovery and RSD were comparable with those obtained by other researchers (12, 15, 16). Moreover, the apparent recoveries of TP, TB, BU, NTD, and NTPhP in hair samples spiked at the estimated CCα were correct because they fell in the required range of 70–110% stipulated by Commission Decision 2002/657/EC for concentrations below 10 μg L^{-1} (kg^{-1}) in a sample (5). At the same time the RSD of repeatability at the CCα level had acceptably low values not exceeding 15%. The decision limit values of CCα and the detection capability values of CCβ calculated during the validation process were below the recommended level RC of 10 μg kg^{-1}. Because prohibited compounds and their residues should not be present in tissues of animal origin, the SANCO guide for implementation of 2002/657/EC for banned and unauthorised compounds rests on the assumption that where no minimum required performance limit (MRPL) has been established, detection capability should be as low as reasonably achievable (ALARA) (13). The obtained values for the selected compounds are comparable with those presented by other authors who have applied methods using LC (12, 18) and higher than values resulting from GC techniques coupled with tandem mass spectrometry (12, 15).

All concentration calculations were made based on the calibration curves of standard solutions, because generally a weak matrix effect of about ± 20% for all analytes was found. The slope of the matrix-matched calibration curve was only higher than the slope of the standard calibration curve for BU, which indicates a suppressing effect of the matrix. For TP, TB, NTD, and NTPhP the opposite situation was observed, which indicates an enhancing effect of the matrix for the final result. The obtained percentages describing the size of the ME were a tolerable phenomenon, opinions expressed in the literature on this matter concurring that ME of approximately ± 20% does not indicate suppression or enhancement of the chromatographic signal. Moreover it is worth adding that the slight increase in recovery (over 100%) was within the required reference range.

Introduction of a new matrix to the method usually necessitates additional validation of the research procedure. Our attempt was made to directly compare the analytical performance characteristics on pig hair with those for the original validated matrix of bovine hair. Based on the results achieved after application of the statistics, it was concluded that there are no significant differences between the compared parameters for bovine and pig hair for all steroid esters tested. It was decided to adopt the CCα and CCβ limits estimated for steroid esters in bovine hair for steroid esters in pig hair.

The elaborated method was to be designated for confirmation purposes, and so the identification criteria

(identification points, relative intensities of ions, and relative retention time) required by CD 2002/657/EC were inspected (5). For testosterone benzoate, one precursor ion with two corresponding daughter ions was obtained, which yielded four identification points (IPs). For nortestosterone phenylpropionate, one precursor ion and three corresponding daughter ions were obtained, whereby 5.5 IPs were achieved. For the remaining three steroid esters testosterone propionate, boldenone undecylenate, and nortestosterone decanoate, one precursor and four corresponding daughter ions were received, to provide 7.5 IPs. The minimum required number of IPs called for by the current legislation is 4 and therefore the confirmatory criteria for the MRM relating to the number of IPs obtained for all analytes were met. As is apparent from Table 1, the relative ion intensities for TP, TB, BU, NTD, and NTPhP were compliant with the permitted tolerances established on the basis of the standard solutions. Also the criteria for compatibility of the relative retention time of steroid esters in spiked samples and standards within the specified 2.5% tolerance range were confirmed as met. It should also be noted that for all samples, signal to noise ratio was greater than 3 for the characteristic transitions and the presence of five steroid esters was confirmed in more than 92% of the tested samples, in accordance with applicable requirements.

The prepared confirmatory LC–MS/MS method for the determination of selected steroid esters in animal hair has been evaluated twice in proficiency tests organised by the RIKILT EURL in the Netherlands: in 2011 (on testosterone cypionate) and in 2016 (on nortestosterone phenylpropionate) (14). In both cases, satisfactory performance for the compound of interest was achieved, as evidenced by the z-scores in the region of < -2 and $2 >$, which prove the efficiency of the method for confirmatory purposes.

Its suitability thus established, the method was included in the official anabolic hormone residue surveillance in Poland in 2017; however, so far the presence of steroid esters above the decision limits of CCα has not been detected in any of the official samples. According to the legislation in force, each sample in which concentration of a prohibited substance above the CCα is found should be considered non-compliant. Taking into account the research results obtained over two years, all tested samples were adjudged compliant (negative). It is not possible to assess the influence of the sex of animals or the colour of hair on the accumulation of steroid esters from these results. Analysing the results of research obtained over many years, it can be stated that hormones were not used for fattening purposes, and if the industry is currently similarly non-transgressive, products and manufactured food of animal origin are safe for consumers. Cases of hormone residue in the samples are probably due to their pseudoendogeneous nature.

Conflict of Interest Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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