Food Anal. Methods (2017) 10:727–739
DOI 10.1007/s12161-016-0620-5

Determination of Hormones Residues in Milk by Gas Chromatography-Mass Spectrometry

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Received: 31 March 2016 / Accepted: 18 July 2016 / Published online: 19 August 2016
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Abstract The article presents the use of gas chromatography-mass spectrometry (GC-MS) technique in a method for the determination of 18 anabolic hormones from synthetic stilbenes, steroids and resorcylic acid lactones (RALs) groups in raw milk and milk powder. Sample preparation consisted of liquid-liquid extraction with diethyl ether and purification by solid phase extraction (SPE). Prior to instrumental analysis, the reaction of derivatisation with the heptafluorobutyric anhydride or *N*-methyl-*N*-trimethylsilyltrifluoroacetamide was performed. Method validation was carried out according to the required performance criteria of the Commission Decision 2002/657/EC. The apparent recovery of all analytes at 1 μg L^{-1} (kg^{-1}) level was ranged between 70.4 and 119.4 % with the coefficients of variation values less than 30 %. The decision limits (CCα) and the detection capabilities (CCβ) were in the range of from 0.11 to 0.44 μg L^{-1} (kg^{-1}) and from 0.19 to 0.75 μg L^{-1} (kg^{-1}), respectively. The procedure has been accredited and successfully applied as a screening method for the presence of hormone residues in the study of commercial samples of milk.

Keywords Hormones · Gas chromatography-mass spectrometry · Raw milk · Milk powder

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Introduction

In order to ensure the food safety and health of consumers, a large-scale research on the presence of prohibited substances and residues of chemical, biological and veterinary medicines products in animals as well as in biological material and food of animal origin is conducted. Milk as a raw material is in a group of edible tissues selected and recommended in the specified range of substances for these kinds of research.

Milk is a complex biological fluid which contains a variety of macromolecules: proteins, lipids, large amount of polar substances such as amino acids, carbohydrates, mineral salts and vitamins, and hormones both steroidal (estrogens, androgens, gestagens) and peptide origin which posses bioactive properties (Fritsche and Steinhart 1999; Hartmann et al. 1998; Jouan et al. 2006; Malekinejad et al. 2006). Both hormones and non-hormonal biologically active compounds can be synthesized in the mammary gland and moving from the mother's circulation. Bioactive compounds, which are transported in the blood, can be detected in the milk, since the milk, mammary secretion consists of components or precursors from blood plasma. Hormones and growth factors contained in milk can be present in concentrations exceeding levels in maternal plasma (Jouan et al. 2006). Some of them, in particular those having lipophilic properties and the capacity to penetrate the blood plasma, are quickly transported through the diffusion to the milk or in its original structure or can be modified during the complicated biochemical processes and chemical reactions directly in the mammary gland or the ultimately in milk (Daxenberger et al. 2001). The presence of high concentrations of growth factors and inhibitors in milk is important from the point of view of excretion of these compounds, the mammary functions, immune system development, and circulation of nutrients. Hormones in milk also temporarily regulate the activity of a variety of tissues

including endocrine organs. However, the presence of the hormones in the milk can result not only from the endogenous origin.

Hormones as well as other veterinary drugs can be administered to live animals in order to hasten production important from the viewpoint of industrial sector or for therapeutic purposes mostly for the prevention of animal diseases. The result of the incorrect administration of such compounds may be the presence of undesirable residues in tissues and biological material from animals undergoing the therapy. Among the hazardous compounds, both natural and synthetic hormones should be mentioned (Buiarelli et al. 2010). By referring to the milk, it must be stated that it is the important component of the human diet. However, accumulation of some drug residues in milk reducing its quality may pose a risk to the human population of different groups especially for infants and children (Cirkva and Šťastný 2013; Courant et al. 2008; Gamba et al. 2009). Contaminated milk from the epidemiological point of view can be the cause of allergic reactions, hormone-dependent diseases and potential risk factor for cancer in humans (Azzouz et al. 2011; Ganmaa and Sato 2005; Khaniki 2007).

Taking the above into consideration, the use of anabolic hormones in animal production is prohibited in the European Union by Directive 96/22/EC and Directive 2003/74/EC (96/22/EC 1996; 2003/74/EC 2003). Therefore, hormones should not be present in animal tissues and no MRL is established. Proposed by European Union Reference Laboratory, the recommended concentration (RC) value of 1 μg kg^{-1} is established only for muscle tissue for the control of hormone residues (CRL Guidance Paper 2007). We decided to adopt this value for milk in our research. The control of compliance is regulated by Directive 96/23/EC by applying the National Residue Control Plans (96/23/EC 1996). According to Annex II of Council Directive 96/23/EC, the milk is not a target, obligatory matrix of choice indicated for the residues of anabolic hormones those belonging to groups A1–A4, and therefore was not included in the official control of the residues of hormones. Milk testing, however, is carried out for the reason of export requirements concerning the quality of that raw material from which in the production line, the products of dairy origin are finally prepared.

For an effective control research, specific and sensitive methods of high analytical performance, which allow the determination of residues of compounds mentioned above at low concentration levels, are required. A few reports relate to the analytical methods based on gas chromatography (Azzouz et al. 2011; Choi et al. 2002) or liquid chromatography (Chen et al. 2011; Kaklamanos and Theodoridis 2013; Malone et al. 2010; Ortelli et al. 2009) techniques were developed and used for the determination of residues of the selected hormones in milk. The aim of the study was to develop a reliable multiresidue, screening, GC-MS-based method for the determination of hormones from three different, in terms of structures and properties, groups: synthetic stilbenes (diethylstilbestrol, dienestrol, hexestrol), steroids (17α/17β-19-nortestosterone, 17α/17β-trenbolone, methyltestosterone, 17β-boldenone, methylboldenone, 17β-testosterone, 17β-estradiol, medroxyprogesterone 17-acetate) and RALs (zeranol, taleranol, α-zearalenol, β-zearalenol, zearalanone) in milk samples. The presented method was validated in accordance with the current legislation and applied as a screening in research of commercial samples of raw milk and milk powder.

Material and Methods

Reagents and Chemicals

Solvents, namely diethyl ether and methanol (analytical grade), were obtained from POCh (Poland); methanol (analytical, HPLC, resi grade), ethanol, ethyl acetate (HPLC grade) and acetone (resi grade) were purchased from J.T. Baker (The Netherlands); isooctane (GC grade) was obtained from Merck (Germany) and n-pentane (Picograde® for residue analysis) was received from LGC Standards (Germany). Other chemicals, namely concentrated acetic acid, anhydrous sodium sulphate, sodium acetate, sodium bicarbonate and sodium carbonate, were obtained from POCh (Poland); TRIS buffer substance was obtained from Merck (Germany); SPE C_{18} 500 mg/3 mL and NH_2 500 mg/3 mL cartridges were obtained from Mall Baker (The Netherlands) and purified water was obtained with a Milli-Q apparatus (USA). Buffers, namely acetate buffers (0.04 M, pH 5.2) and (0.05 M, pH 4.8), carbonate buffer (mixture of 100 mL of 10 % sodium hydrogen carbonate solution with 500 mL of 10 % sodium carbonate solution, pH 10.25) and TRIS buffer (0.02 M, pH 8.5), were prepared in the laboratory. Derivatisation reagents, namely heptafluorobutyric anhydride (HFBA), *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (MSTFA) (GC grade), ammonium iodide (NH_4I), and DL-dithiothreitol (DTT), were all purchased from Sigma-Aldrich (Germany) and iodine (I_2, resublimed GR for analysis) was obtained from Merck (Germany). For derivatisation of RALs, the mixture by dissolving 2 mg of NH_4I and 5 mg of DTT in 1000 μL of MSTFA was prepared. For derivatisation of 17α/17β-trenbolone, a solution of 10 mg of I_2 in 1000 μL of MSTFA has been drawn up. The derivatisation solutions were stored at the temperature <−18 °C for no longer than a month from the date of preparation. After the expiry date, fresh solutions were prepared.

Standards of diethylstilbestrol (DES), dienestrol (DIE), hexestrol (HEX), 17β-19-nortestosterone (17β-19-NT), methyltestosterone (MT), 17β-boldenone (17β-BOL), methylboldenone (MBOL), 17β-testosterone (T), 17β-estradiol (17β-E2), medroxyprogesterone 17-acetate (MPA),

α-zearalenol (α-ZOL), β-zearalenol (β-ZOL) and zearalanone (ZAN) were all obtained from Sigma-Aldrich (Germany). Furthermore, standards of 17α-19-nortestosterone (17α-19-NT), 17α-trenbolone (17α-TBOH), 17β-trenbolone (17β-TBOH), α-zearalanol (ZER), β-zearalanol (taleranol-TAL) and internal standards (IS) of 17β-19-nortestosterone-d3 (17β-19-NT-d3), methyltestosterone-d3 (MT-d3), 17β-boldenone-d3 (17β-BOL-d3), methylboldenone-d3 (MBOL-d3), 17β-testosterone-d2 (T-d2), 17β-trenbolone-d3 (17β-TBOH-d3), 17β-estradiol-d3 (17β-E2-d3), medroxyprogesterone 17-acetate-d3 (MPA-d3), α-zearalanol-d4/β-zearalanol-d4 (ZER-d4/TAL-d4) (50:50, *w*/*w*), diethylstilbestrol-d6 (DES-d6), dienestrol-d2 (DIE-d2) and hexestrol-d4 (HEX-d4) were purchased from the Institute of Food Safety-RIKILT (The Netherlands). The IS of α-zearalenol-d7 (α-ZOL-d7) and β-zearalenol-d7 (β-ZOL-d7) were obtained from Toronto Research Chemicals Incorporation (Canada). The standard ampoules were stored as recommended in their certificates (at 18–28 °C or at 2–8 °C except 17α/17β-TBOH and ZAN which were kept in a freezer). Structural formulas of molecules of hormones included in the procedure are presented in Fig. 1. Primary stock standard solutions of the individual hormones at concentrations of 1000, 100 or 10 μg mL^{-1} were prepared in methanol HPLC grade and stored below −18 °C. Working standard solutions at a concentration of 1 or 0.1 μg mL^{-1} were obtained by tenfold dilution of the stock solutions with methanol HPLC and were held at 2–8 °C (except standard solutions of 17α/17β-TBOH which were stored in a freezer even as ampoules) for not longer than 6 months.

Sample Preparation

Five milliliters of raw cow's milk was measured (optionally, 2 g of milk powder was weighted and dissolved in 10 mL of water), and 10 μL of working IS solutions of all hormones with a concentration of 1 μg mL^{-1} was added. The raw milk samples and samples of the aqueous solution of milk powder were processed in the same way according to the following procedure. Hormones were extracted from milk twice successively with 30 and 20 mL of diethyl ether. Then, the collected organic layers were washed with 20 mL of carbonate buffer, next with 20 mL of distilled water, afterwards dried on anhydrous sodium sulphate layer and evaporated under the gentle stream of nitrogen at 60 °C (±2 °C). The residue was dissolved in 3 mL of acetate buffer (0.05 M, pH 4.8) and applied onto C_{18} SPE column previously conditioned with 3 mL of methanol and 3 mL of TRIS buffer/methanol mixture (80:20, *v*/*v*). Then, column was washed with 3 mL of TRIS buffer/methanol mixture (80:20, *v*/*v*) and 3 mL of methanol/water mixture (40:60, *v*/*v*) and dried under vacuum for 2 min. The elution of hormones was performed using 3 mL of acetone, and the eluate was directly loaded on NH_2 SPE column previously conditioned with 5 mL of methanol/water mixture (40:60, *v*/*v*). The eluate was collected in glass tube and evaporated to dryness, under the gentle stream of nitrogen at 60 °C (±2 °C). Next, the residue was reconstituted in 900 μL of ethanol; the sample was divided into three equal portions and transferred to derivatisation vials, subsequently evaporated to dryness again, and derivatised using the suitable derivatisation reagent depending on the group of compounds tested.

Stilbenes and steroids except 17α/17β-TBOH were derivatised with 30 μL of HFBA during incubation at 60 °C for 1 h. After the incubation and evaporation of an excess of derivatisation reagent, the dry residue was finally dissolved in 50 μL of isooctane and injected into GC-MS. For 17α/17β-TBOH, two-step derivatisation initially with 30 μL of MSTFA/I_2 solution (3 min at 18–28 °C; evaporation at 45 °C) before addition of 30 μL of MSTFA (40 min at 60 °C) was performed. The derivative was injected into GC-MS without evaporation, with the possibility of dilution with isooctane in justified cases also. For the RALs, the reaction of dry residue with 50 μL of the derivatisation mixture of MSTFA/NH_4I/DTT (1000:2:5, *v*/*w*/*w*) for 20 min at 60 °C (±2 °C) was carried out. The derivative without evaporation was injected into GC-MS (if necessary, in analogy to TBOH, the sample was diluted with isooctane).

In the case of obtaining insufficiently pure extract after purification on NH_2 column (visual evaluation), double extraction with n-pentane prior to derivatisation process may be carried out. For this purpose, before the dissolution of the sample in ethanol and the division into three portions, 200 μL of methanol was added to the dry residue and the whole was

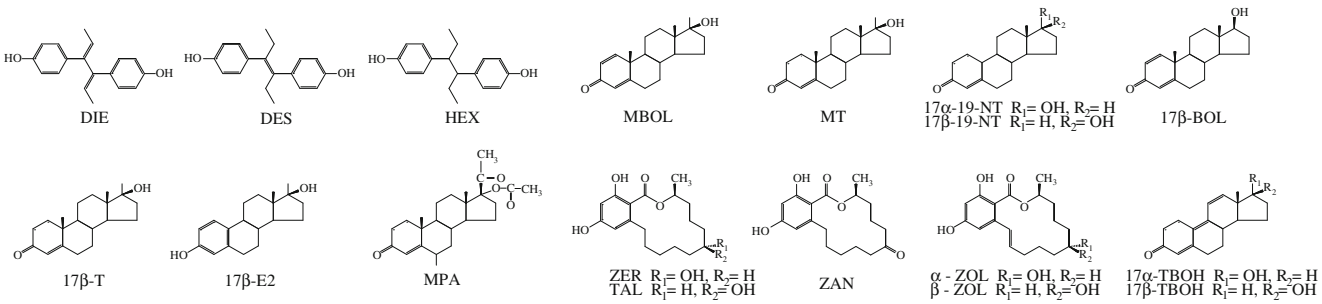


Fig. 1 Structural formulas of molecules of hormones

mixed. Then, 2 mL of TRIS buffer (pH 8.5) was added and the whole was mixed again. Subsequently, double extraction with 6 mL of n-pentane was carried out. After each single extraction step, centrifugation at 4830×*g* for 10 min was performed, to make it easier to separate the layer of n-pentane. Collected fractions of n-pentane were evaporating to dryness under the stream of nitrogen and the dry residue was dissolved in 900 μL of ethanol. Thereafter, the procedure stated above was continued.

GC-MS Analysis

For GC-MS analyses, an Agilent 6890N Gas Chromatograph interfaced to a single quadrupole Mass Spectrometer 5973 MSD controlled by Chemstation software was used (Agilent Technologies, Germany). The machine was operated in positive electron ionisation (EI) mode. Data acquisition was performed in selected ion monitoring (SIM) mode at 70 eV of electron energy applied to the system. Injection was done in the pulsed splitless mode. The injector, MS source and Quadrupole were heated to 250, 230 and 150 °C, respectively. Chromatographic separation of anabolic hormones was carried out on a non-polar HP-5MS capillary column (30 m, i.d. 0.25 m, 0.25 μm film thickness) (Agilent Technologies, Germany). Helium was used as carrier gas at a constant flow of 0.9 mL min^{-1}. The column oven temperature programmes applied were as follows: for stilbenes and steroids except 17α/17β-TBOH, the initial temperature of 120 °C was kept constant for 2 min, then was increased by 8 °C per min to 240 °C and again by 20 °C per min to 300 °C and in fine kept for 9 min. For 17α/17β-TBOH, the initial temperature of 120 °C was kept constant for 2 min, then was increased by 20 °C per min to 200 °C and again by 4 °C per min to 280 °C and finally kept for 3 min. For RALs group compounds, the initial temperature was kept constant at 120 °C for 2 min, then was increased by 14 °C per min to 270 °C and kept for 7 min, further was increased by 15 °C per min to 280 °C and was maintained for 2 min. A 10-μL dosing syringe was washed with isooctane and ethyl acetate before and after each injection. The volume of 2 μL of the sample was applied directly to the capillary GC column. The ions monitored used for detection of hormones are presented in Table 1.

Validation Study

The presented method was validated in accordance with the general guidelines of the Commission Decision 2002/657/EC (Commission Decision 2002). The samples of bovine raw milk that were prepared earlier in the laboratory with a pot of milk, beforehand tested for the presence of residues of anabolic hormones, were used as a reference. The whole process of validation globally included 111 of raw milk samples. The parameters such as instrumental linearity, specificity, repeatability, reproducibility, apparent recovery, decision limits, and detection capabilities were determined. For the factorial effect analysis, the software "ResVal" (v 2.0) (CRL Laboratory, The Netherlands) assumes the execution of four experiments was used (ARO SOP 475B 2004; Jonker et al. 1999). The instrumental linearity of the method was evaluated on the basis of four calibration curves of standard working solutions of hormones, drawing in seven points with analyte concentrations corresponding to 0, 0.2, 0.5, 1.0, 2.0, 4.0, and 6.0 μg L^{-1} and also containing an amount of each IS corresponding to 1 μg L^{-1} in a sample. Three series of raw milk samples were prepared. Each of them contained a blank reference milk sample, exactly along six samples spiked at concentration levels of 0.5, 1.0, and 1.5 μg L^{-1} and exactly one sample spiked to a concentration of 2 and 5 μg L^{-1}. Based on the acquisition data obtained from these experiments, matrix-matched calibration curves were plotted. The regression parameters of the standard calibration curves in the range 0–6 μg L^{-1} as well as of the matrix-matched calibration curves

Table 1 GC-MS diagnostic ions used for the identification of hormones

Compound	Mass monitored [m/z][b]	Additional diagnostic ions [m/z]	Compound	Mass monitored [m/z][b]	Additional diagnostic ions [m/z]
DIE/DIE-d2[a]	658/660[a]	461-445-430	17β-E2/17β-E2-d3[a]	664/667[a]	451-409-356
DES/DES-d6	660/666	631-447-417-341	MPA/MPA-d3	582/585	479-439-383
HEX/HEX-d4	331/333	303	ZER/ZER-d4	433/437	538-523-335-307
MBOL/MBOL-d3	478/481	463-435-367	TAL/TAL-d4	433/437	538-523-335-307
MT/MT-d3	480/483	465-369-355	ZAN/ZER-d4	521/437	536-479-446
17α-19NT/17β-19NT-d3	666/669	453-306-239-133	α-ZOL/α-ZOL-d7	536/543	446-431-333-305
17β-BOL/17β-BOL-d3	678/681	464-369-251	β-ZOL/β-ZOL-d7	536/543	446-431-333-305
17β-19NT/17β-19NT-d3	666/669	453-306-239-133	17α-TBOH/17β-TBOH-d3	380/445	449-442-323
T/T-d2	680/682	467-451-320	17β-TBOH/17β-TBOH-d3	442/445	524-380-323

[a] Internal standard

[b] The ratio of the most abundant diagnostic ions used for quantification

in the range of from CCα to 5.0 μg L^{-1} were calculated. The matrix-matched calibration curves were used to establish the CCα and CCβ according to the approach described in the ISO standard (ISO/11843 2000). The CCα and CCβ limits were calculated by ResVal software from the mathematical Eqs. (1) and (2) presented below, in which the y_a defines the intercept of calibration curve, the STD_a specifies the standard deviation of the y_a and b mean slope of calibration curve.

$$CCa = ((y_a + 2.33 \cdot STD_a) - y_a))/b \quad (1)$$

$$CC\beta = ((y_a + 2.33 \cdot STD_a + 1.64 \cdot STD_a) - y_a))/b \quad (2)$$

In addition, through these experiments, apparent recovery, precision and uncertainty were assessed. Furthermore, the fourth experiment which concerned the specificity study was demonstrated to verify the absence of interfering peaks of unknown compounds around the retention time of hormones tested. Therefore, ten different blank bovine raw milk samples with different fat contents simultaneously with the same ten samples of raw milk spiked with a mixture of hormones tested at 1 μg L^{-1} were analysed. Moreover, ten raw milk samples spiked at the estimated values of CCα were checked for reliability according to SANCO guideline (European Commission SANCO/2004/2726-rev 2008).

Furthermore, complementary experiment relies on the analysis of ten samples of blank milk powder (previously tested for the presence of hormones) fortified to a concentration of 1 μg kg^{-1} and was performed in order to demonstrate the veracity of the assumption that the results of the validation process of raw milk are versatile. The values of average apparent recovery and the standard deviation obtained for milk powder were compared statistically to the corresponding values received in fourth validation experiment for the samples of raw milk spiked at 1 μg L^{-1}. For the purpose of the comparison of the results, two statistical tests were used. T test statistics ($t = (x_1 - x_2)/s\sqrt{(1/n_1 + 1/n_2)}$) was performed to compare the means and F test ($F = s_1^2/s_2^2$) to compare the standard deviations (Miller and Miller 1988).

For each compound, the diagnostic ion characteristic for decay of molecules of hormones derivatives was selected from the corresponding spectrum. Calculations of concentration of the hormones were made on the basis of the most intense diagnostic ions of standards and internal standards using standard calibration curves. For the calculation of the concentration of hormones, deuterated analogues have been applied, except ZAN, for which ZER-d4 was used as IS.

Results

The linear regression parameters for the standard and matrix-matched calibration curves were correct for all examined hormones in the whole range of the tested concentration. The calculated regression coefficients for plotted curves were greater than 0.98. Overview of the calibration parameters is summarized in Table 2.

The chromatographic analysis of respective blank samples in the experiment concerning the term of specificity study showed no interfering peaks of endogenous origin compounds in the retention time ranges of hormones being tested as shown in Fig. 2.

The results of the validation performance of the method of hormones from the group of stilbenes, steroids and RALs in milk are summarized in Tables 3 and 4. In total, the apparent recovery of the tested compounds from raw milk matrix at all levels of spiking ranged from 62.7 % for MT to 119.9 % for MBOL with coefficients of variations (CV) under within-laboratory reproducibility conditions less than 35 % (6.0–34.4 %). For stilbenes, the mean apparent recovery was in the range of from 75.3 % for DIE to 115.8 % also for DIE, with CV less than 34 %; for steroids was in the range of from 62.7 % for MT to 119.9 % for MBOL with CV less than 30 %; and for RAL compounds was in the range of from 78.9 % for TAL to 118.2 % for β-ZOL with CV less than 35 %. For all tested anabolic hormones, the calculated values of CCα and CCβ presented in Table 3 and Table 4 were below the RC level of 1 μg L^{-1} (kg^{-1}).

The expanded uncertainty of the method for relevant hormones was calculated by ResVal software at 1 μg L^{-1} validation level, as the sum of variances of reproducibility and matrix effect, multiplied by the coverage factor of 2 and finally ranges from 26 to 63 %.

For ten milk powder samples fortified with all hormones at 1 μg kg^{-1}, prepared in order to compare the recovery and CV to those obtained for raw milk, all values of apparent recovery were in the range of 70.4–115.9 % with the relative standard deviations did not exceed 30 % as shown in Table 3 and Table 4. For all hormones, the experimental values of t $test_{exp.}$ and F $test_{exp.}$ (except for the value of t $test_{exp.}$ for β-ZOL) did not exceed the critical values of t $test_{crit.}$ and F $test_{crit.}$ from statistical tables, assuming the appropriate numbers of degrees of freedom as presented in Table 5.

Discussion

The GC-based analytical methods in combination with MS are frequently used to identify volatile substances, which due to the step of derivatisation make it easy for reducing the polarity of the molecules, and increasing the thermal stability of the compounds is expectedly required (Azzouz et al. 2011; Choi et al. 2002).

An important step in GC methods is the choice of the way of ionisation. Taking into account previous experience (Matraszek-Żuchowska et al. 2012; Woźniak et al. 2011, 2013), in the presented study in order to choose the ions

Table 2 Calibration parameters

Compound	Linear range ($\mu g\ L^{-1}$ (kg^{-1}))		Slope ± s_b	y-intercept ± s_a	Correlation coefficient	Standard error
DIE	0.1–6[a]	0.44–5[b]	1.87[a]/1.05[b]	0.50[a]/0.47[b]	0.9967[a]/0.9827[b]	0.384[a]/0.111[b]
DES		0.28–5	1.29/0.30	−0.10/0.07	0.9952/0.9957	0.315/0.163
HEX		0.30–5	0.91/0.64	0.14/0.16	0.9981/0.9923	0.141/0.165
MBOL		0.27–5	2.87/1.84	0.45/1.50	0.9854/0.9873	1.224/0.605
MT		0.21–5	0.55/0.45	0.18/0.08	0.9927/0.9969	0.163/0.043
17α-19NT		0.22–5	0.70/0.59	−0.06/0.03	0.9993/0.9979	0.065/0.085
17β-BOL		0.16–5	1.32/1.22	−0.28/−0.20	0.9948/0.9981	0.334/0.141
17β-19NT		0.29–5	0.83/0.59	−0.11/0.08	0.9980/0.9959	0.129/0.118
17β-T		0.16–5	0.66/0.83	0.03/−0.02	0.9938/0.9993	0.184/0.062
17β-E2		0.19–5	0.52/0.59	−0.12/0.02	0.9944/0.9904	0.138/0.181
MPA		0.19–5	0.84/0.71	−0.08/0.04	0.9967/0.9949	0.168/0.156
ZER		0.23–5	1.66/1.07	−0.11/0.29	0.9985/0.9956	0.227/0.130
TAL		0.16–5	1.45/1.47	0.20/0.21	0.9997/0.9997	0.094/0.019
ZAN		0.22–5	0.15/0.11	−0.01/0.03	0.9991/0.9929	0.015/0.025
α-ZOL		0.19–5	0.34/0.27	−0.04/0.01	0.9957/0.9973	0.077/0.045
β-ZOL		0.30–5	0.40/0.23	−0.07/0.14	0.9911/0.9903	0.133/0.077
17α-TBOH		0.11–5	0.36/0.36	0.01/−0.01	0.9996/0.9993	0.026/0.030
17β-TBOH		0.17–5	0.41/0.04	−0.35/0.01	0.9975/0.9870	0.072/0.142

[a] Standard calibration curve

[b] Matrix-matched calibration curve

characteristic for the decomposition of the hormones, derivatives electron ionization was used, which is by far seen as the most common and standard form. EI allows getting satisfactory fragmentation of derivative, as well as the presence of the molecular ions [$M^{+\bullet}$] (m/z) in the spectrum. Before moving to a selected ion monitoring instrument method, first, the data in the full scan mode in the broad range of 100–750 m/z for the derivatives of 30 ng of hormones standards were collected. Based on the analyses of obtained full scan spectra of the standard derivatives, retention times have been established and the most characteristic mass fragments of derivatives were selected. Afterwards, the mass spectra were registered in SIM MS scanning mode, which advantage is the possibility of gaining lower detection limits, when instrument is only looking at a small number of fragments during the scan, thereby increasing the sensitivity and minimizes the noise. This is important for the study of residues of various substances at low concentration levels in the samples. The diagnostic mass fragments of all hormones studied taken into account during detection of them are presented in Table 1. For all diagnostic ions obtained as a result of fragmentography of derivatives of all analytes, signal-to-noise ratio was ≥3 according to general guidelines of Commission Decision 2002/657/EC (Commission Decision 2002).

Undoubtedly, one of the important elements during the proceedings of the sample in the GC method having an impact on the performance is the derivatisation step. In the case of hormone-like compounds that are the subjects of this work, their structures incorporate polar and chemically reactive hydroxyl and keto groups. Thanks to this fact, it permits effective conversion of them into volatile derivatives and determination by GC-MS.

Crucial is also the choice of derivatisation reagent that is suitable for chemical modification of the compound in order to enable detection of that newly formed during the instrumental analysis. The reagent should also produce the derivative that will not interact with the GC column.

For derivatisation of RALs compounds in the milk samples, the mixture of MSTFA/NH_4I/DTT (1000:2:5, *v*/*w*/*w*) contains NH_4I as a catalyst and DTT as a stabilizer in its composition was used. That derivatisation reagent was chosen as the most selective and appropriate based on optimization study which was presented in the publication issued in 2012 (Matraszek-Żuchowska et al. 2012). Consecutively, the derivatisation reaction of 17α/17β-TBOH in milk samples was carried out in a two-stage process: at first with the mixture of MSTFA/I_2 and then with MSTFA powerful silylating agent. The best effectiveness of applied way of derivatisation was previously demonstrated in a paper published in 2013, even though transferring the TBOH into a derivative form as well as obtaining reproducible analyses can therefore be a challenge (Woźniak et al. 2013). For the derivatisation of 17β-T and 17β-E2 HFBA, reagent was used based on optimization studies published before (Woźniak et al. 2011).

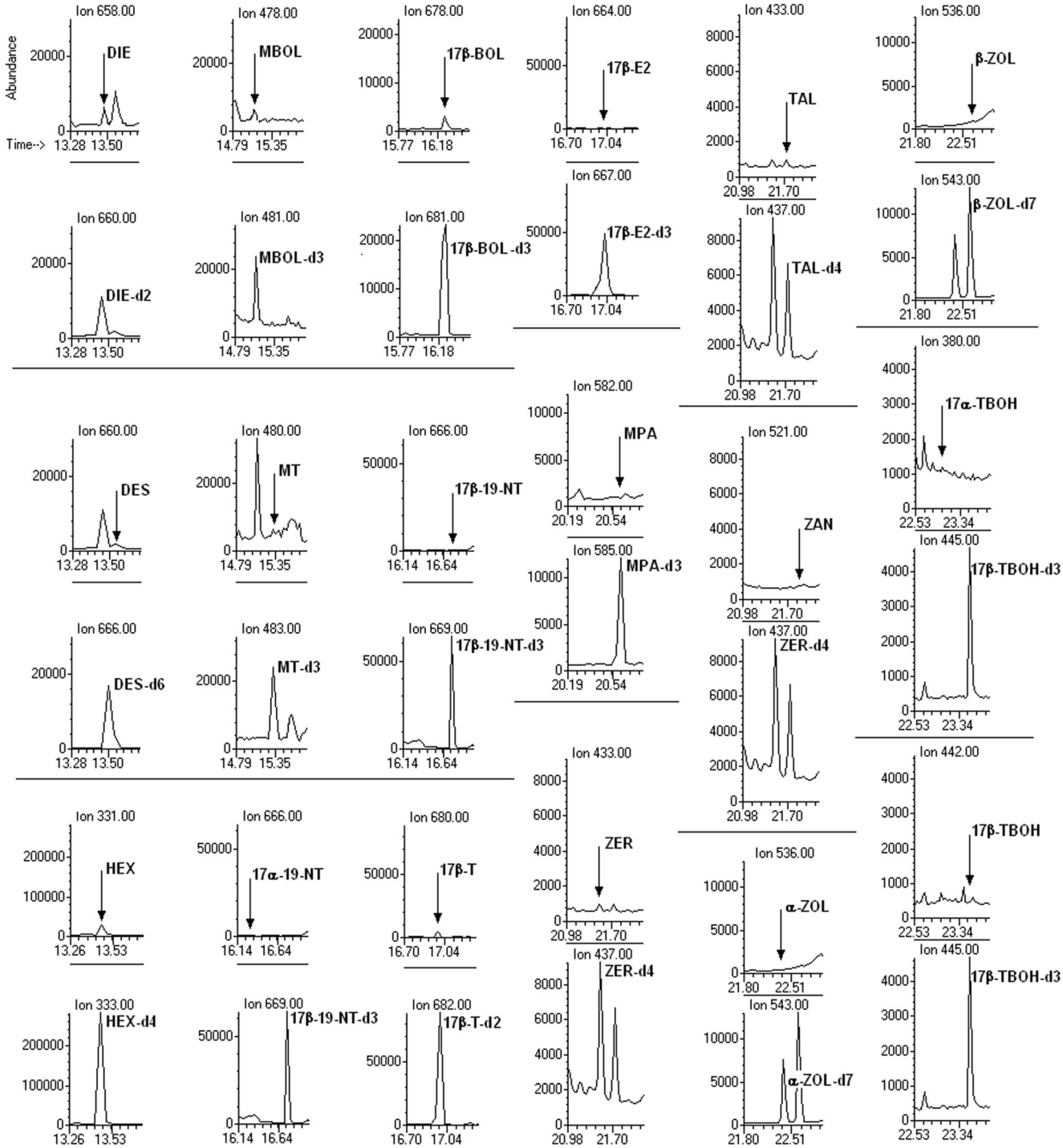


Fig. 2 GC-EI-MS chromatograms of blank raw milk samples

For DES, DIE, HEX, 17α/17β-19-NT, MT, 17β-BOL, MBOL and MPA, selection of an appropriate reagent for the derivatisation was performed in the same manner as for other hormones. For this purpose, several approaches with different analytical reagents, namely HFBA, HFBA/acetone (1:4, *v*/*v*) mixture, bistrimethylsilyltrifluoroacetamide (BSTFA), MSTFA and the composition of MSTFA with NH_4I or iodine (I_2), have been used. Both in the case of trimethylsilyl (-TMS) and (-HFB) derivatives, narrow and symmetrical peaks were obtained on the chromatograms. Referring to the (-TMS) derivatives, obtained after reaction with BSTFA, MSTFA and mixtures of MSTFA with modifiers, it was generally possible to distinguish one the most intense bandwidth from full mass spectra. Moreover, the fragmentation of these derivatives was

Table 3 Analytical performance characteristics of determination of DIE, DES, HEX, MBOL, MT, 17α-19-NT, 17β-BOL, 17β-19-NT and 17β-T in milk

Compound		DIE	DES	HEX	MBOL	MT	17α-19NT	17β-BOL	17β-19NT	17β-T
Recovery (%)	0.5[a]	115.8[b]	100.4	111.1	88.1	62.7	92.2	107.6	96.8	97.9
	1	106.6[b]/95.7[c]	88.7/102.3	103.6/91.2	92.1/78.3	78.2/85.3	87.6/70.4	93.5/115.9	94.4/90.1	98.1/102.6
	1.5	75.3	100.0	87.0	87.1	75.9	96.2	81.5	83.3	97.7
	CCα	68.4	106.8	88.3	119.9	79.8	106.1	96.7	92.6	110.4
Repeatability (R.S.D., %)	0.5	22.4	13.4	17.0	17.7	23.8	11.7	10.5	13.5	15.0
	1[a]	20.4[b]/16.0[c]	14.7/21.6	20.0/16.5	21.2/7.3	19.4/23.5	20.7/12.9	12.1/15.3	21.3/16.4	12.2/12.6
	1.5	29.9	21.0	27.9	19.6	24.7	16.1	16.0	22.7	13.8
	CCα	16.3	17.1	27.2	0.5	26.3	19.9	16.3	20.6	16.0
Within-lab reproducibility (R.S.D., %)	0.5	22.4	26.1	18.6	20.0	23.8	16.3	17.9	17.6	17.3
	1	20.4	27.9	20.0	22.4	19.4	25.5	23.0	26.6	17.8
	1.5	33.4	27.7	27.9	26.1	24.7	29.1	17.4	29.3	22.0
Decision limit (CCα, μg L^{-1} (kg^{-1}))		0.44[b,c]	0.28	0.30	0.27	0.21	0.22	0.16	0.29	0.16
Detection capability (CCβ, μg L^{-1} (kg^{-1}))		0.75[b,c]	0.47	0.51	0.45	0.35	0.38	0.27	0.49	0.27
Measurement uncertainty at 1 μg L^{-1} (U, $k = 2$)		0.63	0.50	0.42	0.42	0.51	0.45	0.43	0.50	0.50

[a] Spiking level (μg L^{-1} (kg^{-1}))

[b] Raw milk ($n = 18$)

[c] Milk powder ($n = 10$)

weak simultaneously with negligible share of bandwidth derived from the molecular ion. It would seem sufficient from the point of view of purpose of a method for screening, although better fragmentation provides greater certainty to evaluate the performance in a situation of equivocal cases of test samples results. With respect to (-HFB) derivatives obtained as a result of reaction with HFBA or a mixture of HFBA/acetone, in each case of full mass spectra, the presence of intense bandwidth derived from the molecular ion as well as those from product ions has been found. The results obtained for (-HFB) derivatives evidenced that in comparison with (-TMS) derivatives, the fragmentation achieved is significantly better. However, in the case of a BSTFA and HFBA/acetone mixture, a decrease in repeatability of mass spectra was observed. The cause in loss of the sample during the derivatisation process could be reaction medium. It is very important to be careful to prevent water from entering the sample as it will lead to hydrolysis and deactivation of derivatizing reagent. The results of undertaken experiments indicated that the best repeatability of spectra and satisfactory fragmentation of hormones derivatives were obtained after the application of HFBA only. Therefore, the HFBA reagent was used for derivatisation and selection of characteristic ions of DES, DIE, HEX, 17α/17β-19-NT, MT, 17β-BOL, MBOL and MPA in further studies. The schemes of probable fragmentation of the derivatives of hormones as referred to above are presented in Fig. 3.

Satisfactory separation of compounds tested was achieved on a non-polar HP-5MS column, commonly used and recommended for hormones analyses.

The starting point for the selection process for the purification of the milk samples prior to instrumental GC-MS analysis was the method for urine samples published previously (Matraszek-Żuchowska et al. 2013). The optimal way of sample pre-treatment was then evaluated for non-steroidal compounds of the RALs group. The usefulness of the method of purification was examined and checked for steroid hormones and stilbenes, especially considering the recovery of analytes. In view of the fact that the results obtained were satisfactory, the sample preparation previously published for urine samples was adopted for the determination of steroid hormones, synthetic stilbenes and RALs in milk in the further stage of research.

The analytical performance of the method was evaluated with respect to the various parameters. Examination of specificity has shown that the method had sufficient selectivity for the entire range of tested compounds. A significant increase of the analyte peaks on chromatograms of spiked raw milk samples can argue about the specificity of the method. The linear regression parameters determined for the calibration curves of

Table 4 Analytical performance characteristics of determination of 17β-E2, MPA, ZER, TAL, ZAN, α-ZOL, β-ZOL, 17α-TBOH and 17β-TBOH in milk

Compound		17β-E2	MPA	ZER	TAL	ZAN	α-ZOL	β-ZOL	17α-TBOH	17β-TBOH
Recovery (%)	0.5[a]	106.0[b]	98.6	102.1	98.2	107.8	103.0	105.3	100.2	118.4
	1	96.4[b]/94.7[c]	91.7/110.0	89.8/88.5	104.2/97.6	95.0/85.0	86.7/102.6	106.9/82.5	101.5/103.8	111.3/115.8
	1.5	107.4	86.8	88.0	100.6	109.8	100.8	110.1	105.1	104.8
	CCα	99.0	89.0	96.8	78.9	96.4	103.3	118.2	90.0	116.1
Repeatability (R.S.D., %)	0.5	12.7	13.3	17.4	15.6	18.8	12.1	14.1	18.8	12.2
	1[a]	16.3[b]/27.1[c]	17.4/17.0	17.0/17.1	12.2/18.8	18.0/25.5	20.4/19.5	22.3/19.0	15.6/17.6	8.0/11.2
	1.5	11.5	15.7	8.2	14.3	4.7	17.5	14.7	10.0	16.3
	CCα	26.0	19.1	25.2	20.4	28.9	14.4	19.1	6.5	4.9
Within-lab reproducibility (R.S.D., %)	0.5	12.7	18.8	21.3	28.0	18.8	31.8	14.1	18.8	12.2
	1	16.8	20.3	19.8	12.0	18.0	27.5	22.3	18.2	9.4
	1.5	19.8	16.2	34.4	18.5	6.0	21.3	14.7	10.0	16.3
Decision limit (CCα, μg L^{-1} (kg^{-1}))		0.19[bc]	0.19	0.23	0.16	0.22	0.19	0.30	0.11	0.17
Detection capability (CCβ, μg L^{-1} (kg^{-1}))		0.33[bc]	0.32	0.39	0.28	0.38	0.33	0.51	0.19	0.30
Measurement uncertainty at 1 μg L^{-1} (U, k = 2)		0.45	0.38	0.36	0.26	0.36	0.48	0.48	0.37	0.27

[a] Spiking level (μg L^{-1} (kg^{-1}))

[b] Raw milk (n = 18)

[c] Milk powder (n = 10)

standards and matrix-matched indicated a good curve-fit and provide a linear response over the range tested.

Satisfactory recoveries above 62.7 % as well as good precision (CV) which was less than 35 % under within-laboratory reproducibility conditions obtained were acceptable. The application of stable deuterium-labelled analogues of hormones as internal standards compensated the loss of analytes during sample preparation. Comparable values of recovery to those obtained were presented for different hormones in milk by other authors (Azzouz et al. 2011; Chen et al. 2011; Kaklamanos and Theodoridis 2013; Shao et al. 2005).

The detection limits values of CCα determined during validation process were below RC of 1 μg L^{-1}. The obtained values for the selected compounds are comparable with those presented by other authors who have applied methods using GC (Azzouz et al. 2011) and slightly higher relative to the values resulting using LC techniques coupled to tandem mass spectrometry (Chen et al. 2011; Kaklamanos and Theodoridis 2013; Malone et al. 2010; Yan et al. 2009).

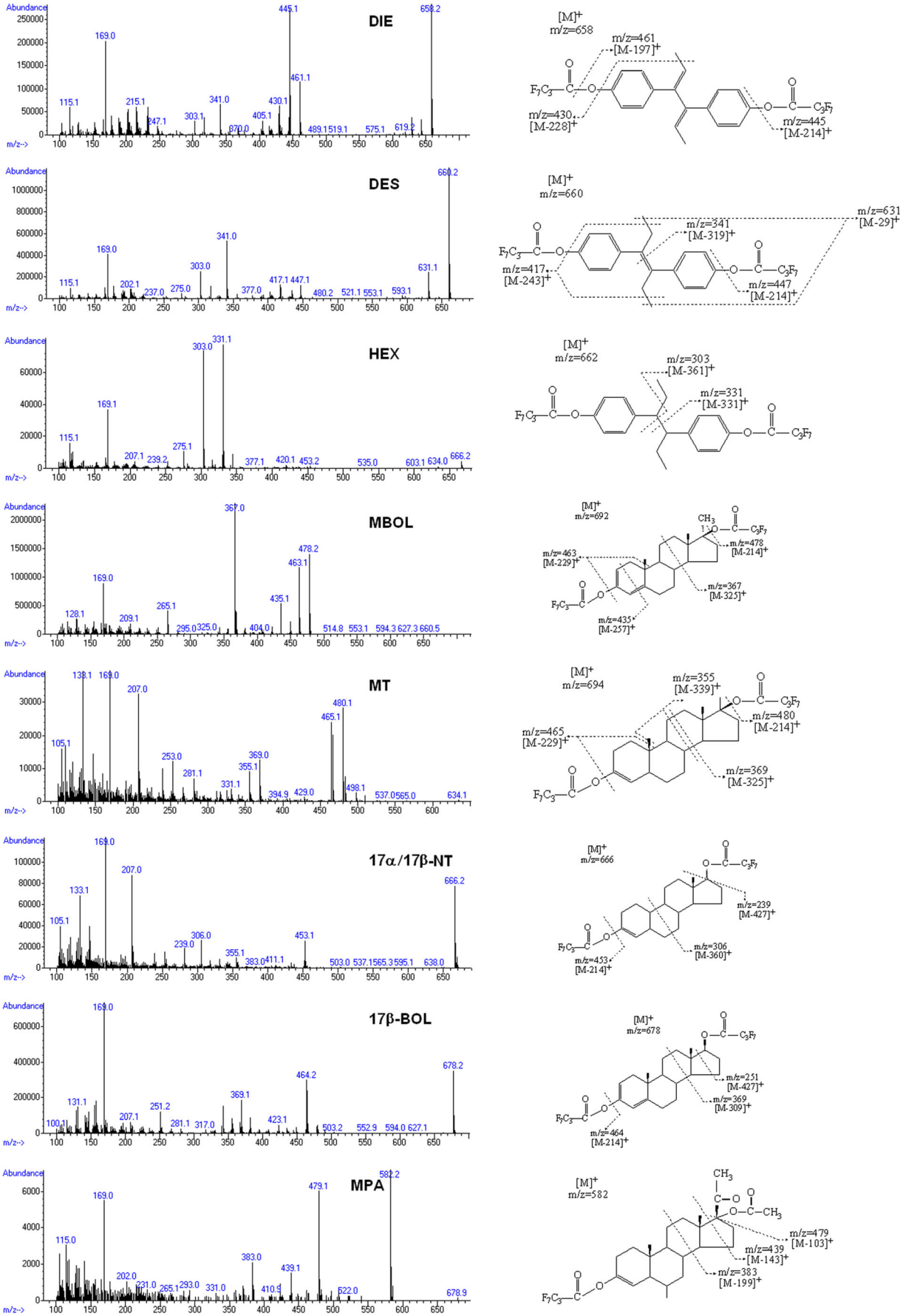
The representative GC-EI(SIM)-MS chromatograms of milk samples spiked at 1 μg L^{-1} are presented in Fig. 4.

The aim of the study was to dedicate the elaborated method for screening purposes. According to the assumption in the SANCO guide for implementation of 2002/657/EC (European Commission SANCO/2004/2726-rev 2008) for banned and unauthorised compounds where no minimum required performance limit (MRPL) has been established detection capability should be as low as reasonably achievable (ALARA). Methods for screening purposes should be properly able to detect the analyte at the CCβ level in 95 % of the cases. The confidence and credibility of detection of tested hormones were checked at estimated CCα levels. In 100 % of the raw milk samples spiked at individual CCα level, the presence of the tested compounds (stilbenes, steroids and RALs) was noted. The average apparent recovery ranged from 68.4 % for DIE to 119.9 % for MBOL, with CV from 4.9 % for 17β-TBOH to 28.9 % for ZAN.

Table 5 Comparison of accuracy and repeatability in raw (n = 10) and powdered (n = 10) milk samples spiked at 1 μg L^{-1} (kg^{-1})

Compound	t $test_{exp.}$	F $test_{exp.}$	Compound	t $test_{exp.}$	F $test_{exp.}$
DIE	1.377	2.112	17β-E2	1.062	1.673
DES	0.495	0.488	MPA	0.206	0.351
HEX	1.693	2.107	ZER	1.641	0.122
MBOL	1.360	2.863	TAL	1.078	0.293
MT	1.251	1.231	ZAN	1.021	0.517
17α-19NT	0.135	1.371	α-ZOL	0.983	0.353
17β-BOL	0.913	0.234	β-ZOL	3.219	0.215
17β-19NT	0.127	1.650	17α-TBOH	0.459	0.197
T	0.336	2.841	17β-TBOH	1.432	0.786

1.734—the value of t $test_{crit.}$ (18 degrees of freedom); 3.178—the value of F $test_{crit.}$ (nine degrees of freedom)


DIE
DES
HEX
MBOL
MT
17α/17β-NT
17β-BOL
MPA
[M]+ m/z=658
m/z=461 [M-197]+
m/z=430 [M-228]+
m/z=445 [M-214]+
[M]+ m/z=660
m/z=631 [M-29]+
m/z=341 [M-319]+
m/z=417 [M-243]+
m/z=447 [M-214]+
[M]+ m/z=662
m/z=303 [M-361]+
m/z=331 [M-331]+
[M]+ m/z=692
m/z=463 [M-229]+
m/z=478 [M-214]+
m/z=367 [M-325]+
m/z=435 [M-257]+
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m/z=465 [M-229]+
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m/z=369 [M-309]+
m/z=464 [M-214]+
[M]+ m/z=582
m/z=479 [M-103]+
m/z=439 [M-143]+
m/z=383 [M-199]+

◀ **Fig. 3** GC-MS full mass spectra and the proposed schemes of (-HFB) derivatives fragmentation of DIE, DES, HEX, MBOL, MT, 17α/17β-19-NT, 17β-BOL and MPA

Whereas on the basis of quality control charts carried out for the milk samples spiked at a concentration of CCβ level that were carried out in each series of routine samples, the recovery and repeatability were calculated. Similarly, as in the case of samples spiked at CCα, the presence of compounds over the entire range was observed in 100 % of the cases, while the apparent recoveries were in the range from 87.9 % for 17β-E2 to

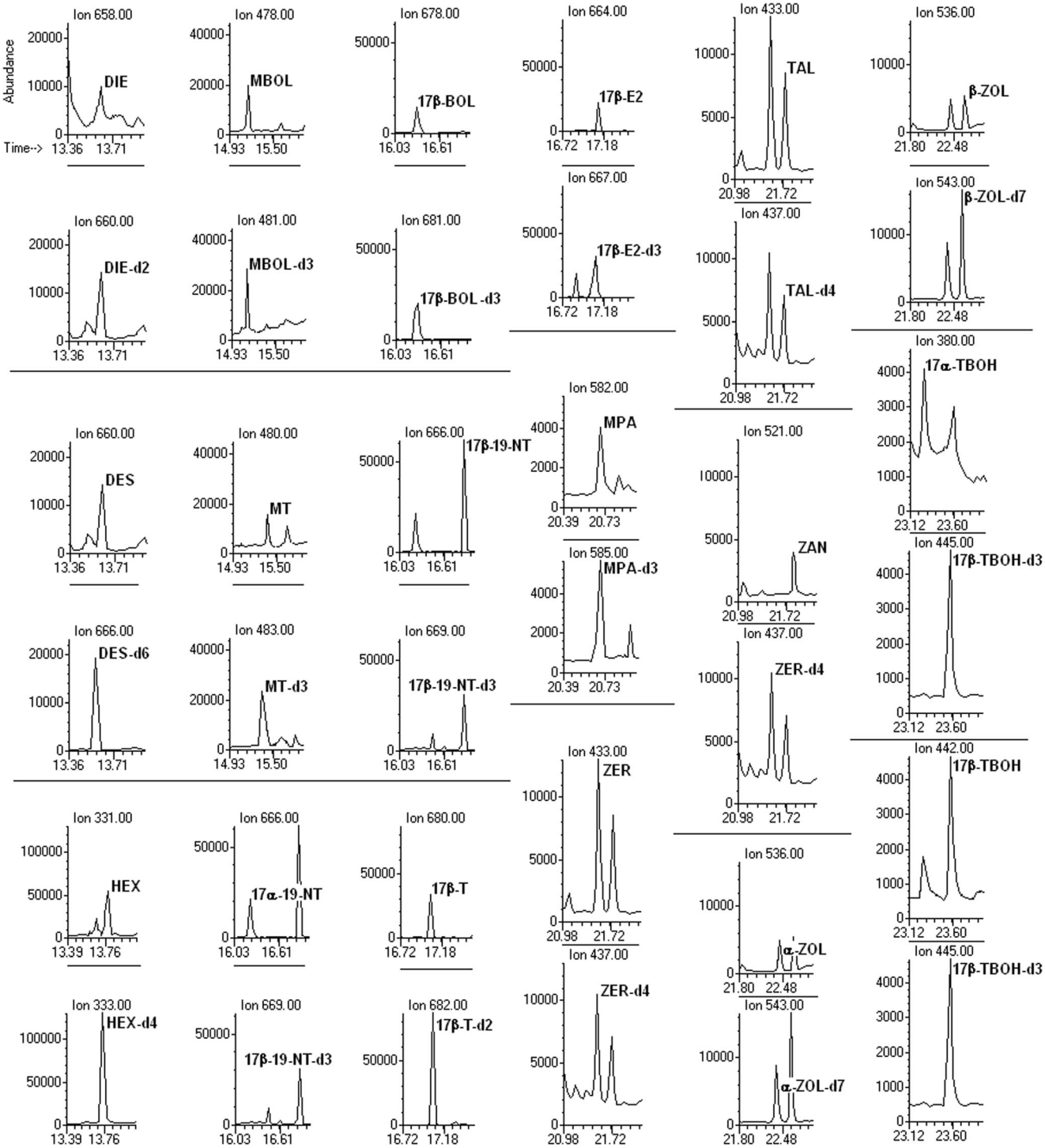

Fig. 4 GC-EI-MS chromatograms of spiked raw milk samples at 1 μg L^{-1} level with DIE, DES, HEX, MBOL, MT, 17α-19-NT, 17β-BOL, 17β-19-NT, 17β-T, 17β-E2, MPA, ZER, TAL, ZAN, α-ZOL, β-ZOL, 17α-TBOH and 17β-TBOH

113.5 % for 17α-TBOH with a CV in the range from 17.5 % for α-ZOL to 29.3 % for 17β-T.

However, the attempt was made to verify the suitability of the method for confirmatory purposes taking into account official criteria. According to the SANCO guide for implementation of 2002/657/EC (European Commission SANCO/2004/2726-rev 2008), methods for confirmatory purposes should be able to detect the analyte at the CCβ level in 95 % of the cases and in 50 % at the CCα level.

Decision Commission 2002/657/EC defines additional requirements for confirmatory methods by introducing the concept of identification points (IPs) and defining criteria for ion intensities. A specific number of IPs depending on the technique used have to be collected in order to evaluate the designation of the method. For A group-banned substances, four IPs are required. The criteria for confirmation were checked during the validation process for all analytes in spiked milk samples. Because compliance of the criteria for confirmation with the relevant requirements were obtained for less than 80 % of the number of samples, the method applied was intended to screening purposes.

Introduction of a new matrix to the method usually requires and force additional validation of the research procedure (Commission Decision 2002). Nevertheless, the attempt was made to directly compare the analytical performance characteristics obtained for the new matrix of milk powder with those of for the original validated matrix of raw milk. The purpose of this proceeding was to demonstrate the veracity of the application of the decision parameters (CCα, CCβ) determined during validation process with the assumption of a great similarity in physicochemical properties between the raw milk and milk powder matrices. Based on the results achieved after application of the statistics, it was concluded that there are no significant differences between the compared parameters for the two matrix of raw milk and milk powder for all hormones except β-ZOL. However, since for all ten spiked milk powder samples, the apparent recovery of β-ZOL was correct (58–118 %) according to the requirements of Commission Decision 2002/657/EC (Commission Decision 2002) and was in the reference range 50–120 %, it was decided to not perform a separate validation and to include milk powder matrix over the whole profile of compounds to the scope of the analytical procedure. Simultaneously, in the method, the CCα and CCβ limits estimated for hormones in raw milk were adopted for hormones in powder milk, respectively.

Application of the Method in the Study of Real Samples

The developed method has been verified in the studies of real fresh raw milk samples and milk powder. Sixty-six commercial samples of milk including 36 raw milk samples and 30 samples of milk powder were tested towards residues of anabolic hormones included in the scope of the procedure. In all samples tested, no residues of hormones above the determined CCα concentrations were found. It is important to note that both synthetic and endogenous origin hormones have not been identified. Based on the results of screening research performed, it can be concluded that the milk as a basic material is safe for the consumers.

Conclusions

Due to the increasing production, farmers tend to use more intensive integrated manufacturing systems and legal or illegal veterinary medicinal products, including hormones. The usage of these compounds is strictly controlled within national and international legislative frameworks. It is therefore necessary to have availability of a considerable number of analytical approaches for the detection of hormones in edible tissues because of food safety and consumers protection. A sensitive GC-MS screening method for the detection of 18 anabolic hormones differing in chemical structure and properties in milk has been developed. Satisfactory method performance characteristics including detection limits for all anabolic hormones below the RC of 1 μg L^{-1} (kg^{-1}) were obtained. The proposed method was successfully applied for testing the presence of hormones residues in various commercial raw milk and milk powder samples.

Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical Approval This article does not contain any studies with human participants or animals performed by any of the authors.

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