Analysis of eight free progestogens in eggs by matrix solid-phase dispersion extraction and very high pressure liquid chromatography with tandem mass spectrometry

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ABSTRACT

A rapid method to identify and quantify unconjugated progestogens in eggs is presented. Samples were prepared based on matrix solid-phase dispersion (MSPD) using C18 as dispersant, followed by a clean-up step with graphitized carbon black (GCB) solid-phase extraction (SPE) cartridges. The analytes were separated by very high pressure LC on a BEH C18 column for a period of 5 min. Electrospray ionization tandem mass spectrometry (ESI-MS/MS) was operated in the positive time-scheduled multi-reaction monitoring mode. Recovery studies were performed at two fortification levels. Average recoveries of the target compounds varied from 83.8% to 111.2% and relative standard deviations ranged from 10.5% to 23.7%. The limits of detection (LODs) and limits of quantitation (LOQs) were in the range of 0.2–2.0 $\mu g\,kg^{-1}$ and 0.6–5.0 $\mu g\,kg^{-1}$, respectively. Investigation of real samples indicated that the range of progesterone in eggs was 9.9–40.0 $\mu g\,kg^{-1}$.

1. Introduction

Progestogens including naturally occurring and synthetic compounds are anabolic hormonal-type growth promoters. Previously, progesterone together with estradiol or its esters was used as a growth promoter to increase the growth rate and improve feed efficiency and carcass composition in cattle [1]. Progestogens sometimes are used for therapeutic purposes to treat fatty liver hemorrhagic syndrome (FLHS) and egg binding [2,3]. However, these drugs also give rise to health risks in humans. The high incidence of mammary cancer and cervical cancer has been confirmed by epidemiology studies to be related with imbalances in progestogens levels [4,5]. According to the International Agency for Research on Cancer (IARC), medroxyprogesterone and medroxyprogesterone acetate were classified as being possibly carcinogenic to humans. Therefore, the use of progestogens as growth promoters in poultry breeding has been forbidden by regulations of both China and European Union. Nevertheless, illegal use and failure to follow the withdrawal period may lead to high levels of progestogens residues in eggs, which may be potentially harmful to consumers.

Because of the low levels ($\mu g\,kg^{-1}$–$ng\,kg^{-1}$) in samples and the complexity of biomatrices, the analysis of residual progestogens is a challenging task. Research has been mainly focused on sample preparation and sensitive instrumental methods. Presently, the liquid–liquid extraction (LLE) [6] and SPE methods [7–11] are often used to prepare biosamples for progestogens assay. LLE is a traditional technology with some disadvantages including the use of large volumes of organic solvent, and relatively high cost [12]. SPE methods are often used to concentrate and purify progestogens in tissue, serum and water. The immunoaffinity technique has also been used to purify the 19-nortestosterone in biosamples; however, it cannot be used for all of other compounds due to its high specificity [13].

As for assaying techniques, HPLC, GC, GC–MS and LC–MS are usually used for analysis of progestogens in biosamples [11,14–16]. LC and GC coupled with mass spectrometric detectors are prevailing techniques for screening and confirmation of trace residues in foods of animal origin according to criterion of Commission Decision 2002/657/EC [17]. However, GC–MS involves a tedious derivatization process and molecular information may be lost during derivatization [18–20]. LC coupled with tandem mass spectrometry is a promising alternative for analysis of residual

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progestogens by direct analysis without derivatization procedure [10,21].

Matrix solid-phase dispersion (MSPD), first introduced by Barker et al. in 1989 [22], is a sample preparation technique for solid and semi-solid samples using bonded-phase solid supports as an abrasive to produce disruption of sample architecture and a bound solvent to aid complete sample disruption during the sample blending process. MSPD techniques have been successfully applied to isolate a wide range of compounds such as antibiotics, pesticides, β-agonists and tetracyclines from a variety of complex plant and animal samples [23-26]. Application of MSPD in food analysis can greatly reduce the analysis time; it requires less solvent, which decreases the cost of purchasing and disposing of solvent [12,27]. However, to the best of our knowledge, only few papers have been published using MSPD to analyze steroid hormones in biosamples: Gentili et al. analyzed melengestrol acetate, progesterone and other anabolic steroids by MSPD in crude meat and infant foods [26]; an automated extraction method of acetylgestagens from kidney fat by MSPD was established by Rosen et al. [28]. On the other hand, reports focused on detection of steroid hormones in egg samples are relatively scarce, though eggs are a popular food throughout the world. Natural occurrence of steroid hormones in many kinds of food (including eggs) was investigated by Hartmann et al. [29] using the EU method [30], which is relatively time-consuming and solvent-consuming. Recently, Courant et al. [31] detected oestrogens and androgens in milk and eggs using a modified EU method. In this paper, an MSPD method followed by very high pressure LC–MS/MS was developed to assay trace levels of unconjugated progestogens in eggs.

2. Experimental

2.1. Chemicals and reagents

Standards of both natural and synthetic progestogen, including 19-norethindron, 21α-hydroxyprogesterone, 17α-hydroxyprogesterone, D(−)-norgestrel, progesterone, medroxyprogesterone, megestrol acetate and medroxyprogesterone acetate were purchased from Sigma (St. Louis, MO, USA). Internal standard, progesterone-d_9, was obtained from Cambridge Isotope Laboratories (MA, USA). HPLC grade methanol and dichloromethane were purchased from Scharlau Chemic S.A. (Barcelona, Spain). Formic acid (99%) was from Acros Organics (New Jersey, USA). Deionized water was from a Milli-Q system (Millipore, Bedford, MA, USA).

Stock solutions (1 mg ml^{-1}) were prepared by dissolving 10 mg individual target compound in 10 ml methanol and stored at 4 °C. Working solutions at serial concentrations were obtained by combining aliquots of stock solutions followed by subsequent dilution with methanol.

2.2. Apparatus and materials

The solid phase material used for MSPD was Discovery DSC-18 (Supelco Co., Bellefonte, PA, USA), which is a polymerically bonded trifunctional C18 silica. Supelclean ENVI-Carb graphitized carbon black (GCB) cartridges (500 mg, 6 ml) were purchased from Supleco Co. (Bellefonte, PA, USA). Sep-Pak C18 (500 mg, 6 ml) and Oasis HLB (500 mg, 6 ml) were purchased from Waters Co. (Milford, MA, USA). Identification and quantification of analytes were carried out using a Waters ACQUITY UPLC (Waters, Milford, MA, USA) liquid chromatograph system equipped with a Quattro premier XE tandem mass spectrometer (Micromass, UK). LC separation was achieved on an ACQUITY UPLC BEH C18 column (100 mm × 2.1 mm, 1.7 μm particle).

2.3. Sample preparation

Shelled eggs were homogenized using an electric blender for 10 min at low speed. For method validation, eight eggs were mixed together and homogenized. For real sample analysis, each egg was homogenized individually. 0.5 g of homogenized egg sample fortified with 10 ng internal standards was placed in a glass mortar containing 2 g C18 powder. The solid support and sample were ground using a pestle until the pulverized material was incorporated into the packing material. After being dried at room temperature, the MSPD blend was packed into an empty cartridge (6 ml, id 10 mm) with a polyethylene filter and a top filter being used to condense the blend and eliminate voids or channeling. Then the cartridge was rinsed with 10 ml methanol–water (10:90, v/v) and eluted with 8 ml methanol. The extract was collected and evaporated to near dryness under a gentle stream of nitrogen at 40 °C. The residue was redissolved with 1 ml methanol and 3 ml water and mixed using a vortex stirrer.

The resulting solution was loaded onto the GCB cartridge, which had been conditioned sequentially with 6 ml dichloromethane–methanol (70:30, v/v), 6 ml methanol, and 6 ml water, at a flow rate of 3–4 ml/min. After washing with 2 ml × 4 ml water and 1 ml methanol, the crude analytes were eluted with 6 ml dichloromethane–methanol (70:30, v/v). Then the eluate was dried under a gentle nitrogen stream, and the residue was redissolved with 1 ml methanol for LC–MS/MS analysis.

The empty SPE column, filter, mortar and pestle were prewashed with ultra pure water, acetone, dichloromethane and methanol. All glassware were cleaned and then heated for 3 h at 400 °C. In addition, procedural blanks were conducted for each batch of samples to ensure minimal contamination.

2.4. LC–MS/MS analysis

For the analysis procedure, methanol and water containing 0.1% formic acid were used as mobile phases with a total flow of 0.3 ml/min. Gradient elution was performed as follows: methanol was linearly increased from 68% to 100% in 4.5 min and held for 2 min, finally brought back to 68% and held for 3 min to the next injection. The column oven was maintained at 40 °C and the injection volume was 10 μl.

The mass spectrometer was operated in positive electrospray ionization mode with multiple-reaction monitoring (MRM). The capillary voltage was maintained at 3.3 kV. The multiplier voltage was 650 V. The flow rate of the desolvation and cone gas was set to 700 l/h and 50 l/h, respectively. The source temperature and desolvation gas temperature were held at 100 °C and 450 °C, respectively. The RF lens was set at 5 V. The ion energy 1 and ion energy 2 were both 1.0. The entrance and exit were 0 and 2. During tandem mass spectrometric analysis, ultra high purity argon was used as the collision gas and the pressure of the collision chamber was kept at 3.2 × 10^{-3} mbar. For each analyte, two parent-product ion transitions were chosen and the corresponding cone voltage and collision energy were optimized for maximum intensity. The relevant parameters are given in Table 1.

2.5. Method validation

In order to compensate for the matrix signal suppression and loss during processing, matrix-fortified calibration curves were obtained by spiking a mixture of standards into eggs at a series of concentrations between 1.0 and 100 μg kg^{-1} (5.0–500 μg kg^{-1}
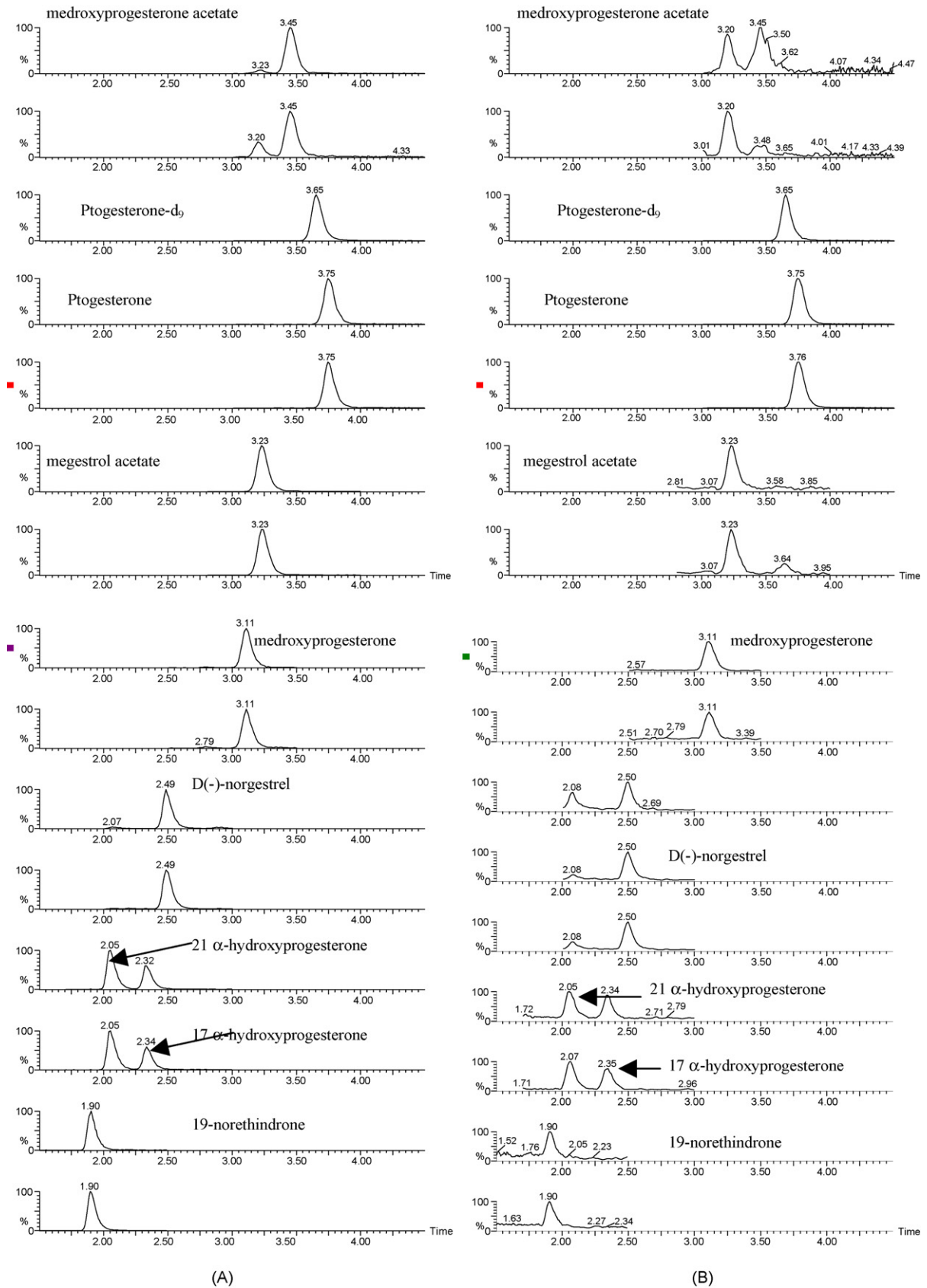
Fig. 1. (A) Chromatograms of standards in methanol (10 μg/l, internal standard 10 μg/l); (B) Chromatograms of spiking sample (2 μg/kg, D(−)-norgestrel 10 μg/kg, internal standard 20 μg/kg).

Table 1
LC–MS/MS acquisition parameters for the 8 compounds

Compound	Retention time (min)	Precursor ion (m/z)	Cone voltage (V)	Product ion (m/z)	Collision energy (eV)
19-Norethindrone	1.90	299.3	35	<u>109.1</u>[a], 231.4	27, 17
21α-Hydroxyprogesterone	2.05	331.5	35	<u>96.9</u>, 108.9	21, 21
17α-Hydroxyprogesterone	2.33	331.5	35	<u>96.9</u>, 108.9	22, 22
D(−)-Norgestrel	2.49	313.4	38	<u>108.9</u>, 245.4	26, 20
Medroxyprogesterone	3.11	345.5	35	<u>123.0</u>, 97.0	24, 24
Megestrol acetate	3.23	385.5	30	<u>267.3</u>, 325.6	16, 16
Progesterone	3.74	315.5	35	<u>97.0</u>, 297.5	20, 35
Medroxyprogesterone acetate	3.47	387.5	30	<u>327.3</u>, 285.4	16, 16
Ptogesterone-d_9	3.65	324.6	35	<u>100.0</u>	24

[a] The underlined product ion was used for quantitative analysis.

for D(−)-norgestrel) except for progesterone, for which, an isotopic internal standard was used for quantification. Calibration curves of progesterone were obtained by performing a linear regression analysis using the ratio of the peak area progesterone to that of progesterone-d_9 against analyte concentrations ranging from 0.5 to 50 μg l^{-1} containing 10 μg l^{-1} internal standard. The recovery of each analyte using this method was evaluated by spiking standards and internal standard to egg samples at two levels in six replicates. Fig. 1(A) and (B) shows the MRM chromatograms of a standard sample and a spiked egg sample at level of 2 μg kg^{-1} (10 μg kg^{-1} for D(−)-norgestrel), respectively.

3. Results and discussion

3.1. Selection of mobile phase

Different mobile compositions (methanol–water and acetronitrile–water) were used to investigate the effect on the sensitivity and chromatographic behavior. The results suggested that good sensitivity and chromatographic behavior can be achieved upon using methanol–water as mobile phase in positive mode, although the maxium backpressure (about 11,000 psi) is higher than that when using acetonitrile–water (about 8000 psi). Considering that the pressure tolerance of the very high pressure LC instrument was 15,000 psi, we selected methanol–water as mobile phase.

3.2. Sample preparation

The dispersant agent and wash solvent are the most critical factors during MSPD extraction. As the octanol–water partition coefficients (log K_{ow}) for the target compounds were from 2.97 to 3.87, nonpolar solid phase C18 powder was chosen as the dispersant sorbent for the extraction of these relatively hydrophobic chemicals. A previous paper indicated that the disruption of the sample structures and the dispersal of the sample over the surface of C18 sorbent depend on the mechanical shearing forces [1]. Therefore, we have compared recoveries of the analyts obtained by mechanical grinding with that obtained by vortex mixing. The results suggested that the recoveries obtained by vortex mixing were obviously lower than using mechanical grinding. Especially for 19-norethindrone, the absolute recovery by vortex mixing was less than 50%, while it was about 80% by mechanical shearing forces. Additionally, the reproducibility obtained by mechanical shearing was also higher than by vortex mixing.

Because of the high protein content in eggs, a washing step for the MSPD cartridge is necessary. Various washing solvent compositions were tested using 10 ml methanol–water solutions with different ratios (0:100, 5:95, 10:90, 20:80, 30:70, 40:60, v/v). As a result, methanol–water (10:90, v/v,) turned out to be preferable. Washing solutions with lower proportions of methanol (0:100, 5:95) could not provide a satisfactory protein removal efficiency, while higher proportions of methanol in the washing solution induced partial elution of some target compounds, such as 19-norethindrone. Therefore, methanol–water (10:90, v/v) was selected as the washing solvent. The volume of the eluting solvent (methanol) was also studied and 8 ml was found to be optimal.

Significant mass signal suppression was observed for MSPD processed samples, which was attributed to the complicated egg matrix. For most of the analytes, the matrix effects, calculated by comparing the peak areas of matrix-matched standards with those of the pure standards at a concentration of 10 μg l^{-1}, were higher than 30% (Table 2), especially for 19-norethindrone (65.5%) and progesterone (56.5%). In order to reduce the matrix effect, a further clean-up step was performed by solid-phase extraction. Several commercial cartridges including Sep-Pak C18, Oasis HLB and GCB solid phase extraction cartridges were evaluated by determining the absolute recoveries of the analytes at a spiking level of 50 ng l^{-1} in 10 ml water, and results were 80–105% for all of these three cartridges, which indicated that three cartridges were all potentially suitable for further processing. However, quite different matrix effects were found when using these three different cartridges in egg samples purification. As shown in Table 2, high matrix effects, for several compounds, e.g. 21α-hydroxyprogesterone, still existed even being purified by C18 or HLB; while after GCB purification, the signal suppression effects for all the analytes were

Table 2
Matrix effect of target compounds in blank egg samples

Compound	Matrix suppression ratio (%)			
	MSPD only	MSPD followed HLB	MSPD followed C18	MSPD followed GCB
19-Norethindrone	65.5	35.5	26.8	20.7
21α-Hydroxyprogesterone	55.8	52.3	51.2	26.4
17α-Hydroxyprogesterone	32.9	38.4	29.7	13.7
D(−)-Norgestrel	33.1	27.8	38.6	20.6
Medroxyprogesterone	0	11.7	10.3	0
Megestrol acetate	13.9	0	0	0
Progesterone	56.5	10.2	22.8	12.8
Medroxyprogesterone acetate	0	0	0	0

Table 3
Linearity for each component ($n = 5$)

Compound	Mean slope ± standard deviation	Mean intercept ± standard deviation	Mean r^2	Linear range (µg/kg)
19-Norethindrone	64.6 ± 9.7	27.1 ± 4.4	0.9889	1–100
21α-Hydroxyprogesterone	376.4 ± 43.6	94.6 ± 14.2	0.9839	1–100
17α-Hydroxyprogesterone	376.4 ± 48.5	94.6 ± 13.1	0.9831	1–100
D(−)-Norgestrel	267.3 ± 37.4	23.9 ± 4.2	0.9906	5–500
Medroxyprogesterone	279.6 ± 29.3	76.9 ± 10.9	0.9902	1–100
Megestrol acetate	285.3 ± 17.9	24.7 ± 2.9	0.9958	1–100
Progesterone	0.6 ± 0.05	0.12 ± 0.01	0.9937	1–100
Medroxyprogesterone acetate	609.8 ± 15.1	69.7 ± 8.2	0.9932	1–100

Table 4
Spiked recoveries, relative standard deviations (RSD), LOD and LOQ of target compounds in eggs ($n = 6$)

Compound	Spiked level (2 µg/kg)		Spiked level (4 µg/kg)		LOD (µg/kg)	LOQ (µg/kg)
	Recovery (%)	RSD (%)	Recovery (%)	RSD (%)		
19-Norethindrone	92.3	23.7	100.2	22.7	0.6	2
21α-Hydroxyprogesterone	94.4	16.4	95.3	15.3	0.2	0.8
17α-Hydroxyprogesterone	89.4	14.4	83.8	20.9	0.3	1
D(−)-Norgestrel[a]	109.6	19.7	95.9	13.9	2.0	5.0
Medroxyprogesterone	111.2	13.9	97.5	15.6	0.2	0.6
Megestrol acetate	106.2	14.7	110.7	18.7	0.4	1.0
Progesterone	90.6	14.1	102.3	10.5	0.2	0.6
Medroxyprogesterone acetate	95.8	16.8	100.2	19.7	0.4	1.0

[a] Spiking level was 10 and 20 µg/kg.

lower than 30%, indicating that GCB cartridge is more effective and selective.

3.3. Calibration curves and method validation

For each analyte, good linearity was obtained with acceptable correlation coefficients of $r^2 > 0.98$. The results are listed in Table 3. Average recoveries of each compound ranged from 83.6% to 111.2%. The within-day reproducibility was represented by the percent relative standard deviation (RSD), which ranged from 10.5% to 23.7% at two levels for each compound on a day. The between-day repeatability, ranged from 11.4% to 26.2% for each compound at two spiked levels during five consecutives days. The results indicated that the RSD of 19-norethindrone (spiking level $2 \mu g \, kg^{-1}$, $4 \mu g \, kg^{-1}$) and the RSD of the D(−)-norgestrel (spiking level $10 \mu g \, kg^{-1}$) were higher than 20%, which might be due to the relatively low sensitivity of this chemical. Nevertheless, this procedure is suitable for screening purposes. The limits of detection (LODs), defined as the concentration that yields an S/N = 3 for the diagnostic transition ions, were $0.2–2.0 \mu g \, kg^{-1}$ for eight compounds (Table 4). The limits of quantitation (LOQs), defined as the concentrations that yield an S/N = 10 for the diagnostic transition ions, were $0.6–1.0 \mu g \, kg^{-1}$ except for 19-norethindrone and D(−)-norgestrel, the LOQ of which were 2.0 and $5.0 \mu g \, kg^{-1}$, respectively. This result is similar to LOQs of the EU method. However, it should be pointed out that those LOQs were based on about 50 g sample.

3.4. Application to real samples

In this study, retention time and the chromatographic area ratio of two transition ions are used as identification criteria. If the retention time and area ratio are the same as those of standards, the positive samples can be identified. Ten eggs samples commercially available from the market were assayed using the procedure described above. Only progesterone was found, at levels varying from 9.9 to $40.0 \mu g \, kg^{-1}$, which was consistent with a previous investigation ($12.5–43.6 \mu g \, kg^{-1}$) by Hartmann et al. [29].

In addition, we detected egg white and yolk separately for six eggs using the method described above. Progesterone was detected both in egg white and yolk. Results for six egg white were 4.3, 4.2, 4.2, 3.9, 4.3, and $4.0 \mu g \, kg^{-1}$, and results for yolk were 37.1, 45.7, 39.9, 38.5, 43.7, and $36.9 \mu g \, kg^{-1}$. Ratios of progesterone in yolk to that in white for these six eggs were calculated as 8.6, 10.9, 9.5, 9.9, 10.2, and 9.2, respectively, with a RSD of 8.1%.

4. Conclusion

MSPD followed by GCB cartridge purification and very high pressure LC–MS/MS as described in this study allow the simultaneous screening and determination of eight unconjugated progestogens in eggs. Comparing with the EU method [30], this method is faster, more workable and saves organic solvent. The LOQs of the MSPD / very high pressure LC method is approximately same as the EU method. This method was successfully applied to analyze the residual progesterone in commercially available eggs with occurrence of $9.9–40.0 \mu g \, kg^{-1}$.

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