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Determination of naturally occurring estrogens and androgens in retail samples of milk and eggs

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4 **Determination of naturally occurring estrogens and androgens in retail**
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7 **samples of milk and eggs**
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Abstract

The occurrence of the main steroid hormones (estrone, 17 α -estradiol, 17 β -estradiol, 17 α -testosterone, 17 β -testosterone, dehydroepiandrosterone, 4-androstenedione), especially in milk and eggs, was investigated. An analytical method based on GC-MS/MS was developed for steroid measurement at ultra-trace level in food products. The limits of detection for estrogens were found to be about 5 and 30 ng kg⁻¹ in milk and eggs respectively. For androgens, the limits of detection were around 10 and 50 ng kg⁻¹ in milk and eggs respectively. The method was applied to milk and egg samples collected in a French supermarket. In milk, estrone was found at levels between 100 and 300 ng L⁻¹, while 17 β -estradiol levels were estimated near 20 ng L⁻¹. 17 α -testosterone was found from 50 ng L⁻¹ in skim milk to 85 ng L⁻¹ in whole milk. In egg samples, estrone and 17 β -estradiol were found at 1.5 and 0.9 μ g kg⁻¹, respectively, while 17 α -estradiol was found to be in lower concentrations (i.e. around 0.55 μ g kg⁻¹). Regarding androgens, 17 α and 17 β -testosterone were estimated at 1.9 and 1.3 μ g kg⁻¹, respectively. These results represent a first attempt to estimate the food exposure to steroid hormones. In the future, the collection of additional data should permit the comparison between this exogenous dietary intake and the daily endogenous production in pre-pubertal children, as a basis of risk assessment regarding endocrine disruption linked to these molecules for this critical population.

Keywords: children, egg, milk, GC-MS/MS, estrogens, androgens

Introduction

Global concern has been raised in recent years over the adverse effects that may result from exposure to chemicals that may interfere with the endocrine system. Among the different classes of substances that have been pointed out (dioxins, phytosanitary products, phytoestrogens, hormones...), steroid hormones largely remain the most biologically active compounds. A specific concern is related to low-dose effects and long-term exposure consequences, especially for specific populations at critical stage of development (foetus, new born, prepubertal children).

In the field of toxicological substances used in food-producing animals, the US Food and Drug Administration ruled that ‘... no physiological effect will occur in individuals chronically ingesting animal tissues that contain an increase of endogenous steroid equal to 1% or less of the amount in micrograms produced by daily synthesis in the segment of the population with the lowest daily production’ (FDA, 1999). Regarding naturally occurring hormones such as estradiol, daily production rate (PR) and exogenous intake are the crucial elements of a risk assessment. The main measurable parameter linked to the daily production rate is the plasmatic circulating hormone concentration. The first reports on estradiol concentrations in serum from pre-pubertal children, which were obtained using radioimmunoassay, were published in the 1970s (Jenner et al. 1972, Bidlingmaier et al. 1973, Angsusingha et al. 1974). According to these reports, the levels of estradiol in this population were ranging from 22 to 41 pmol L⁻¹. However, the plasma levels of estradiol in these studies appeared in most cases below or close to the detection limit of those assays. More recent comparisons of these radioimmunoassay data with results obtained by more specific techniques seems

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4 to indicate a tendency of the earlier methods to overestimate the estradiol concentrations
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6 (Ikegami et al. 2001, Juul 2001, Nelson et al. 2004). Consequently, a re-evaluation of
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8 these plasmatic hormonal levels using a very sensitive and specific confirmatory
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10 measurement technique (GC-MS/MS and /or GC-HRMS) appeared today relevant. The
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12 authors already initiated this work and the first results confirmed that estrogens
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14 endogenous level in prepubertal children is unsurprisingly extremely low (Courant et al.
15
16 2007) and significantly lower than the commonly admitted values. Consequently, even a
17
18 small exogenous intake would account for a major change in the total activity of the
19
20 involved hormone, which is reflected in phenotypic effects in the child (Aksghlaede et al.
21
22 2006). Thus, food intake is presumed to contribute significantly to the daily exposure.
23
24 There is no doubt about the presence of steroid hormones in food of animal origin
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26 (Hartmann et al. 1998, Andersson et al. 1999, Daxenberger et al. 2001). These different
27
28 authors reviewed most of articles dealing with natural occurrence of sex steroid
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30 hormones in food products. They noticed that pork, meat products, fish and poultry
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32 contain similar amounts of steroids as cattle (in the ng kg^{-1} range). They observed that
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34 milk products and eggs are an important source of steroids. Nevertheless, it must be
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36 noticed that concentrations reported for various animal tissues were mainly determined
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38 by the same methods suspected to over-estimate the very weak concentrations of steroid
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40 hormones in biological fluids. Indeed, problems in measuring low levels of steroids
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42 precisely have been recognized by studies showing high variation in the concentrations
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44 obtained between different assay methods and different laboratories (Potischman et al.
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46 1994, Carlström 1996). Thus, a new accurate evaluation of steroids levels present in the
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48 most contributing food, e.g. milk and eggs, appears relevant. Our main purpose was to
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50 investigate to what extent steroid hormones in food may represent a risk of endocrine
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4 disruption for pre-pubertal children. We developed an analytical method for steroid
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6 measurement at ultra-trace level in food products and for further application to steroid
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8 occurrence in milk and egg samples.
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10 11 12 13 14 15 16 **Materials and methods**

17 18 19 *Reagents and chemicals*

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21 All solvents and reagents were of analytical or HPLC grade quality (SDS, Peypin,
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23 France). All SPE (ChromP, SiOH) were single use cartridges (SDS, Peypin, France).
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25 Purified *Helix pomatia* preparation was used for steroid deconjugation (Sigma, St Louis,
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27 MO, USA). N-methyl-N-(trimethylsilyl)-trifluoroacetamide (MSTFA), N,O-Bis
28
29 (trimethylsilyl)-trifluoroacetamide (BSTFA), and pentafluorobenzylbromide (PFBBr)
30
31 were purchased from Fluka (Buchs, Switzerland). Dithiothreitol (DTE) and
32
33 trimethyliodosilane (TMIS) were purchased from Sigma-Aldrich (St. Quentin Fallavier,
34
35 France). Standard reference steroids were purchased from Sigma (St Louis, MO, USA)
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37 and deuterated internal standards were provided by Steraloids (Wilton, NY, USA).
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43 44 *Samples*

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46 Milk (whole, half-skim, and skim) samples ($n=12$) and egg samples ($n = 8$) were
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48 commercial products collected in a French supermarket.
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50 51 52 *Samples preparation*

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56 *Milk samples* (3 mL) were spiked with 300 pg of internal standard (17β -estradiol- d_3 and
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58 methyltestosterone- d_3 for estrogens and androgens, respectively). The first steps in the
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4 sample preparation procedure (enzymatic hydrolysis of phase II metabolites, liquid-
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6 liquid extraction with ether and purification onto SPE ChromP cartridge) have been
7
8 previously described (Courant et al., 2007). The extracts were then evaporated to
9
10 dryness (N₂, 45°C), and resuspended in 0,5 mL of 1 M sodium hydroxide. Thus, a
11
12 liquid-liquid extraction was performed at pH 14 with 2x4 mL hexane:diethylether
13
14 (70:30, v/v), permitting the specific extraction of androgens. The pH of the aqueous
15
16 layer was then adjusted to 5.2 with glacial acetic acid (150 µL) and a second liquid-
17
18 liquid extraction was carried out using 2x4 mL diethyl ether in order to extract
19
20 estrogens. Both fractions (A and E) were evaporated to dryness and resuspended in
21
22 dichloromethane (200 µL) and n-hexane (300 µL). They were then applied onto a SiOH
23
24 SPE column (1 g) previously conditioned with 18 mL n-hexane. The cartridge was
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26 washed with n-hexane:ethyl acetate (85:15, v/v) (5 mL for estrogens, 10 mL for
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28 androgens) before elution of target analytes with 13 mL n-hexane:ethyl acetate (60:40,
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30 v/v). The collected fractions were evaporated to dryness (45°C, N₂). Dry residues were
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32 finally resuspended in 50 µL n-hexane:isopropanol (90:10, v/v) or 50 µL n-
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34 hexane:isopropanol (85:15, v/v) for androgens and estrogens, respectively. As an
35
36 ultimate purification step, the fractions were injected onto a HPLC system. Collected
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38 target fractions were evaporated to dryness (45°C, N₂) and 300 pg of external standard
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40 (estriol and norgestrel for estrogens and androgens respectively) were added.
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50 *Egg samples*- Freeze-dried egg samples (500 mg) were dissolved in 2 mL water and
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52 incubated with 500 pg of internal standard (17β-estradiol-d₃ and methyltestosterone-d₃
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54 for estrogens and androgens, respectively). Liquid/liquid extraction (LLE) with
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56 2x10 mL methanol/acetone (1:1, v/v) was then performed for extraction of steroids (free
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58 + conjugates). Organic layers were combined and evaporated to 2 mL at 45°C under a
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60

gentle stream of nitrogen. 2 mL of 2M acetate buffer (pH 5.2) and 200 μ L of β -glucuronidase from *Helix pomatia* were added to the samples. The enzymatic hydrolysis was carried out overnight at 52°C. The rest of the procedure was identical as the one described for milk samples. After HPLC fractionation, the collected extracts were evaporated to dryness (45°C, N₂) and 500 pg of external standard (estriol and norgestrel for estrogens and androgens respectively) were added.

Semi preparative HPLC

The HPLC system used was a Hewlett-Packard HP-1100 pump equipped with a fraction collector and UV-detector (DAD, diode array detector) operating from 200 to 280 nm. A 3-(dimethylamino)propyl-functionalized silica gel column (EC-Nucleosil 100-5 N(CH₃)₂-propyl, 250 x 4 mm, 5 μ m) from Macherey-Nagel (Hoerd, France) held at 50°C was used for semi-preparative purification. Mobile phase was a mixture of n-hexane (solvent A) and isopropanol (solvent B). Mobile phase composition (A:B; v/v) was 96:4 at 0 min. Two different gradients were applied depending on the analytes. For androgens, the initial mobile phase composition was kept during 15 min, then linearly changed to reach 20:80 at 19 min and maintained until 28 min. For estrogens, the mobile phase composition A:B was linearly changed from 0 to 15 min from 96:4 to 85:15, then modified so that the mobile phase reached 20:80 at 19 min, and finally kept unchanged until 9 min. Flow rate was set at 1 mL/min and injected volume was 50 μ L.

Derivatisation reaction

Derivatisation of the androgen fraction was carried out with 20 μ L of a mixture MSTFA/TMIS/DTE (1000:5:5; v/v/w) during 40 min at 60 °C. Derivation of the

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4 estrogen fraction with PFBBBr / BSTFA has been previously described (Courant et al.,
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6
7 2007). Both final extracts (2 μL) were injected onto the GC-MS/MS system.
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10 11 *GC-MS/MS measurement*

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13 Estrogens measurements were carried out by GC-MS/MS with negative chemical
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15 ionisation (NCI) using methane as reagent gas. A HP-6890 gas chromatograph was
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17 coupled to a VG-QuattroII® or QuattroMicro GC® (Waters-Micromass, Manchester,
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19 UK) triple quadrupole device. Injector and transfer line temperatures were set at 250°C
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21 and 280°C, respectively. Source and analyser temperatures were set to 280°C and
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23 100 °C, respectively. GC column was a 30 m x 0.25-mm id., film thickness 0.25 μm ,
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25 ZB-5MS (Zebron). Temperature programme was set as follows: 120°C (2 min),
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27 15°C min^{-1} until 280°C (0 min), 5°C min^{-1} until 320°C (6 min). Helium (N55) was used
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29 as carrier gas at 1 $\text{mL}\cdot\text{min}^{-1}$. Electron energy was set at 40 eV.
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38 Androgens measurements were carried out using GC-MS technique. However, positive
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40 electronic ionisation was preferred as ionisation for this steroid group. Electron energy
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42 was set at 70 eV. Temperature programme was set as follows: 120°C (2 min),
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44 15°C min^{-1} until 250°C (0 min), 5°C min^{-1} until 300°C (5 min). Injection volume was 2
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46 μL (purge splitless 1,5 min). The mass spectrometer was operated in the multiple
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48 reaction monitoring (MRM) acquisition mode. Argon was used as collision gas at
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50 $4.5\cdot 10^{-4}$ mbar. Table I summarizes the retention times (RT values), transitions and
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52 collision energies used for each monitored molecule.
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59 [Insert table I about here]
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Quantification procedure

Standard extracted calibration curves were established by adding a fixed amount of internal standard (i.e. 300 pg for milk samples and 500 pg for egg samples) to 3 mL water and increasing quantities of the target analytes ranging from 0.03 to 1.5 ng for milk and from 0.05 to 25 ng for eggs. Samples were then extracted and derivatised as described here before.

Results

Validation data

Linearity. The response linearity was found to be satisfactory with R^2 values better than 0.99 for all the target analytes. Calibration curves obtained in egg samples for estrogens and androgens are represented in figure 1a and 1b, respectively.

Performance limits. Limit of detection (LOD) and limit of quantification (LOQ) for each analyte were classically determined on the basis of concentration inducing signal/noise ratio of 3 and 6, respectively. LOD for estrogens were found to be near 5 and 30 ng kg⁻¹ in milk and egg sample respectively. For androgens, LOD were around 10 and 50 ng kg⁻¹ in milk and egg samples respectively. LOQ were found to be twice than LOD.

[Insert figure 1 about here]

Specificity. Figure 2a shows an example of a typical ion chromatogram obtained for a milk sample in which 17 α -testosterone and 17 β -testosterone were quantified at 87 and

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4 13 ng kg⁻¹, respectively. Androstenedione (lower trace) was quantified at 536 ng kg⁻¹.
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7 The complexity and final selectivity of the purification strategy (4 steps) produced a
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9 clean extract with no interference detected at ions monitored for androgens by GC-(EI)-
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11 MS/MS. Figure 2b shows an example of a typical ion chromatogram obtained for an
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13 egg sample in which 17 α -estradiol and 17 β -estradiol were quantified at 0.85 and 0.87
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15 μ g kg⁻¹, respectively. The specificity in term of absence of interfering compounds
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17 appearing on the ion chromatograms is greatly improved by the use of GC-(NCI)-
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19 MS/MS but clearly also by the efficient purification process.
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26 [Insert figure 2 about here]
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31 *Repeatability.* The repeatability was calculated on the basis of the 17 β -estradiol-d₃ and
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33 Methyltestosterone-d₃ internal standard signals. Indeed, the presence and variability of
34
35 natural estrogens in milk and egg made difficult this evaluation on the native target
36
37 analytes. 17 β -estradiol-d₃ and Methyltestosterone-d₃ were added to 3 mL milk sample
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39 (300 pg equivalent to a 100 ng L⁻¹ concentration in milk) and to 0.5 g egg sample (500
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41 pg equivalent to 1 μ g kg⁻¹ concentration in egg). These fortified samples were treated as
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43 described above, with a final GC-(NCI)-MS/MS measurement and GC-(EI)-MS/MS
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45 measurement for estrogens and androgens, respectively. Ten replicates of these
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47 experiments were carried out. The repeatability was found to be satisfactory with an
48
49 RSD of around 20 % for the target compounds in the two matrices. Considering the
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51 actual current EC regulation regarding analytical criteria for steroid residues analysis in
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53 food (2002/657/CE), these values appears in accordance with the maximal variability
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4 allowed at these very low concentration levels (i.e. RSD < 45 % when $C < 1 \text{ ng g}^{-1}$
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6 according to the Horwitz equation).
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10 11 *Quantification*

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13 *Milk samples.* For all analysed milk samples, the sum of conjugated and free hormones
14 was determined. The results obtained for the estrogens are presented in Table IIa.
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21 [Insert Table IIa about here]
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26 Estrone (E1) was quantified at levels in-between 100 and 300 ng L^{-1} . 17α -estradiol and
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28 17β -estradiol levels (17α -E2 and 17β -E2) were estimated near 40 ng L^{-1} . A comparison
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30 between hydrolysed and non-hydrolysed samples demonstrated that estrogens were
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32 found mainly as phase II conjugated metabolites (>90%). As a consequence, estrogens
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34 seem to be present essentially in the aqueous fraction of milk, explaining that no major
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36 relation was found between the observed concentrations and the fat content of the
37
38 samples. Results obtained for androgens (for whole, half-skim milk and skim milk) are
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40 presented in table IIb. In opposition with estrogens, α -testosterone, 4-androstenedione
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42 (AED) and free dehydroepiandrosterone (DHEA) contents appeared linked to the milk
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44 fat content. Total 17α -testosterone (free + phase II conjugated metabolites) was
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46 quantified from 50 ng L^{-1} in skim milk (25 - 30 % of conjugated forms) to 85 ng L^{-1} in
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48 whole milk (< 10 % of conjugated forms). Considering the actual LOD of the method
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50 used, only total 17β -testosterone (average around 10 ng L^{-1}) was quantified in these
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52 samples.
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[Insert Table IIb about here]

Egg samples. Results obtained for estrogens and androgens are presented in Table III. On the basis of these preliminary measurements, estrone and 17 β -estradiol were quantified at 1.5 and 0.9 $\mu\text{g kg}^{-1}$ respectively. 17 α -estradiol was found to be in lower concentrations than 17 β -estradiol (i.e. around 0.55 $\mu\text{g kg}^{-1}$). Regarding androgens, 17 α and 17 β -testosterone were estimated at 1.9 and 1.3 $\mu\text{g kg}^{-1}$, respectively. Free DHEA was quantified at 1.5 $\mu\text{g kg}^{-1}$. 4-androstenedione was found to be in higher concentrations compared to the other measured compounds (near 85 $\mu\text{g kg}^{-1}$).

[Insert Table III about here]

Discussion

In milk

Due to the relative permeability of the blood/milk barrier, lipophilic hormones circulating in blood plasma may transfer in milk. Therefore, the estrogen content of milk is supposed to reflect the physiological state of the lactating animal. Narendran et al. studied estrogen concentrations in milk during the estrous cycle by radioimmunoassay. During most of the estrous cycle, estrogen concentrations remained at approximately 200 ng L⁻¹ and reached a proestrous peak of 360 ng L⁻¹. Considering that commercial milks are in fact mixtures of milk collected on different animals, the great variability observed in term of hormone concentrations is not really surprising. Additional analyses are on going in the laboratory in order to continuously improve the statistical representativeness of this data set, especially by taking into account the

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4 influence of the physiological state, alimentation and age of lactating animals. A second
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6 observation was that estrogens were found mainly as phase II conjugated metabolites,
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8 which was already noticed by other authors. Saumande et al. developed in 1984 a
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10 radioimmunoassay for free 17β -estradiol, conjugated 17β -estradiol and total (free +
11
12 conjugated) 17β -estradiol in defatted milk of cows. They found that concentrations of
13
14 conjugated 17β -estradiol were higher than those of the free 17β -estradiol. McGariggle
15
16 et al. also demonstrated with RIA that conjugated estrogens comprised more than 90%
17
18 of the total human breast milk. Only few reports deal with androgens in milk. Contents
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20 reported are 20-150 ng L⁻¹ testosterone (Hoffmann et al. 1977, Gaiani et al. 1984) and
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22 100 to 3500 ng L⁻¹ androstenedione (Gaiani et al. 1984). Values obtained in our study
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24 appeared in accordance with these data. At the contrary, Hoffmann et al. found that the
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26 ratio between free testosterone and conjugated testosterone in milk was about 1:1, while
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28 in the present study testosterone was found to be mainly unconjugated (70-90%).
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38 *In eggs*

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41 Maternal transfer of nutrients, including steroid hormones, to embryos during gestation
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43 in viviparous amniotes is well known, but the underlying process is poorly understood
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45 (Janzen et al. 1998). A very significant transfer of 17β -estradiol and testosterone into
46
47 the egg yolk was observed in oviparous animal (Lipar et al. 1999). French et al. studied
48
49 steroid contents in egg collected from two colonies of common terns by
50
51 radioimmunoassay. 4-androstenedione concentrations differed between the geographic
52
53 sites and with egg-laying order (from 200 to 500 $\mu\text{g kg}^{-1}$) whereas testosterone
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55 concentrations did not vary between the two different colonies (only with egg-laying
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57 order: from 5 to 12 $\mu\text{g kg}^{-1}$). Estradiol concentrations were below the limit of detection
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4 (0,1 $\mu\text{g kg}^{-1}$) for 70 % eggs and considering only eggs with measurable estradiol, mean
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7 concentration was near 0,4 $\mu\text{g kg}^{-1}$. Petrie et al. compared hormone concentrations in
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9 the avian yolks of male and female eggs and found that these were significantly
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11 different; estradiol varying from 0,4 $\mu\text{g kg}^{-1}$ in male to 0,8 $\mu\text{g kg}^{-1}$ in female while 17 β -
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13 testosterone was quantified from 1,2 $\mu\text{g kg}^{-1}$ in female to 2 $\mu\text{g kg}^{-1}$ in male. Hartmann et
14
15 al. using GC-MS quantified in eggs from domestic fowl (*Gallus gallus*) estradiol,
16
17 estrone and testosterone to 0,2 $\mu\text{g kg}^{-1}$, 0,9 $\mu\text{g kg}^{-1}$ and 0,5 $\mu\text{g kg}^{-1}$ respectively. All
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19 these results from different authors indicate that there is a great variability in steroid
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21 concentrations from one egg to another depending on sex, sites, and egg laying order.
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23 This can explain the high standard deviation observed in our preliminary results.
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32 **Conclusions**

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34 An analytical method was developed for steroid measurement at ultra-trace level in food
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36 products. On one hand, the application of the developed method to sera samples
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38 confirmed that estrogens endogenous levels in pre-pubertal children are unsurprisingly
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40 extremely low (Courant et al. 2007). On the other hand, this method was applied to milk
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42 samples and egg samples collected in a French supermarket. In milk, estrone was
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44 quantified at levels in-between 100 and 300 ng L^{-1} , while 17 β -estradiol levels were
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46 estimated near 20 ng L^{-1} . 17 α -testosterone was quantified from 50 ng L^{-1} in skim milk
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48 to 85 ng L^{-1} in whole milk. In egg samples, estrone and 17 β -estradiol were quantified at
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50 1.5 and 0.9 $\mu\text{g kg}^{-1}$, respectively, while 17 α -estradiol was found to be in lower
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52 concentrations (i.e. around 0.55 $\mu\text{g kg}^{-1}$). Regarding androgens, 17 α and 17 β -
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54 testosterone were estimated at 1.9 and 1.3 $\mu\text{g kg}^{-1}$, respectively.
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7 These results represent a first attempt to estimate the food intake in steroid hormones
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9 for pre-pubertal children. Analyses are on going in our lab to collect additional data
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11 regarding plasmatic hormonal rates and steroid residues in food. In next future, these
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13 data are expected to be used for establishing a probabilistic modeling of exposure in
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15 which children ages (linked to endogenous production), consumption levels and
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17 residue concentrations will be taken into account. Our final objective will be the
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19 comparison between the dietary intake in steroid hormones (especially estrogens) and
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21 the daily endogenous production of prepubertal children, as a basis of risk assessment
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23 regarding endocrine disruption linked to these molecules for this critical population.
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Table I: GC-MS/MS acquisition parameter used for the measurement of estrogens (Negative chemical ionisation; PFB-TMS derivatisation) and Androgens (Electron ionisation; TMS derivatisation)

Analytes	Transition	Collision Energy (eV)	RT (min)
17 α -estradiol	343>343	20	21.25
17 β -estradiol	343>343	20	21.94
Estrone	269>269	20	21.78
17 β -estradiol-d ₃ (Internal Standard)	346>346	20	21.91
Estriol (External Standard)	431>431	20	24.30
Dehydroepiandrosterone	432.3>327.3	10	17.03
	432.3>417.4	5	
17 α -testosterone	432.3>247.2	15	17.40
	432.3>209.2	8	
17 β -testosterone	432.3>247.2	15	17.94
	432.3>209.2	8	
4-androstenedione	430.3>415.3	5	17.75
	430.3>209.2	10	
17 α -methyltestosterone-d ₃ (Internal Standard)	449.3>301.2	5	19.04
Norgestrel (External Standard)	456.3>316.2	5	20.07

Table IIa: Estrogens concentrations measured in milk (n = 12)

Analytes	Mean concentration (ng L ⁻¹) ±	Min value	Max value	% Conjugates
	standard deviation	(ng L ⁻¹)	(ng L ⁻¹)	
estrone	152.8 ± 60.0	75.8	277.5	96
α-estradiol	39.4 ± 10.7	28.0	63.6	94
β-estradiol	23.0 ± 12.6	9.8	44.3	84

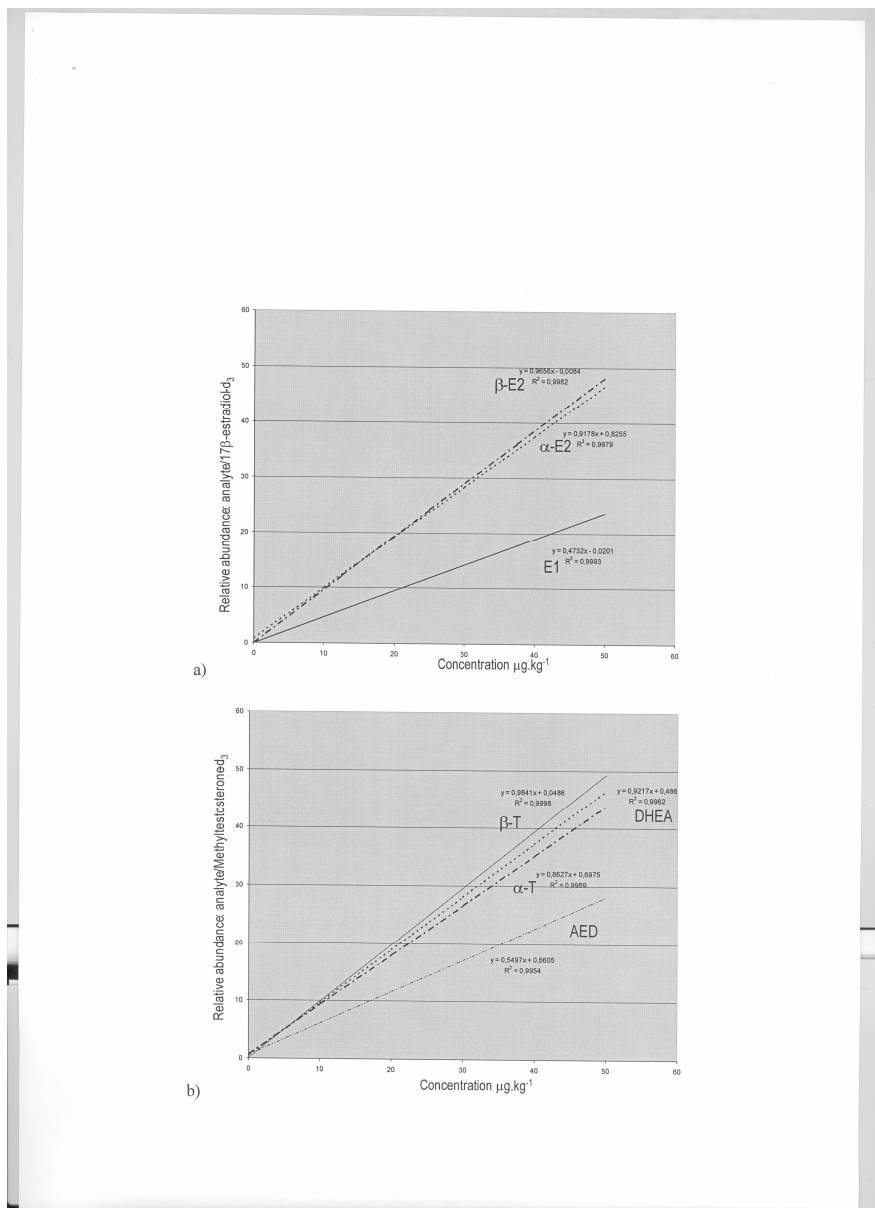
Table IIb: Free androgens concentrations measured in milk (17β-testosterone concentrations are not described because only total 17β-testosterone was quantified in milk samples).

Type of milk	n =	Dehydroepiandrosterone (ng L ⁻¹)			α-testosterone (ng L ⁻¹)			4-androstenedione (ng L ⁻¹)		
		mean±sd	Min Value	Max Value	mean±sd	Min Value	Max Value	mean±sd	Min Value	Max Value
Whole	4	65.4 ± 22.4	38.28	91.71	78.1 ± 21.8	46.51	94.86	934.3 ± 270.9	593.92	1218.9
½ Skimmed	5	52.7 ± 18.0	36.48	67.19	51.3 ± 17.0	40.88	77.67	533.8 ± 101.4	453.89	676.15
Skimmed	3	34.0 ± 12.4	20.85	52.54	31.8 ± 3.6	27.46	36.01	296.3 ± 86.5	191.56	400.62

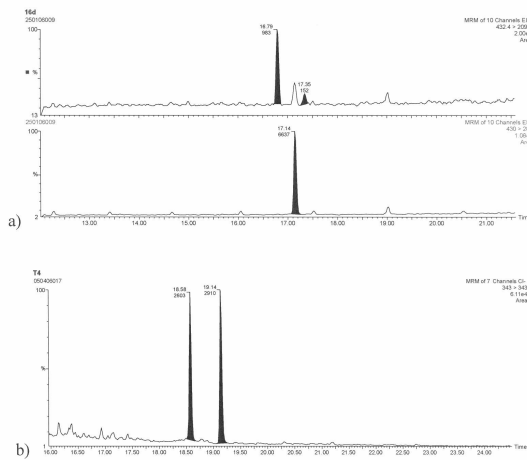
Table III: Estrogens and androgens concentrations measured in hens eggs (n = 8)

Analytes	Mean Concentration ($\mu\text{g kg}^{-1}$) \pm	Min value	Max value
	standard deviation		
estrone	1.44 ± 0.27	1.09	1.91
α -estradiol	0.55 ± 0.15	0.39	0.85
β -estradiol	0.91 ± 0.29	0.47	1.45
Dehydroepiandrosterone	1.48 ± 0.63	0.94	2.68
α -testosterone	1.94 ± 0.31	1.54	2.62
β -testosterone	1.33 ± 0.19	1.06	1.56
4-androstenedione	85.0 ± 22.3	55.8	125.2

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a) calibration curves observed for 17α-estradiol, 17β-estradiol and estrone in the 0 to 50 μg kg⁻¹ concentration range in egg sample b) calibration curves observed for 17α-testosterone, 17β-testosterone, androstenedione and DHEA in the 0 to 50 μg kg⁻¹ concentration range in egg sample 674x928mm (96 x 96 DPI)



a) diagnostic ion chromatogram observed for a milk sample in which 17 α -testosterone and 17 β -testosterone (upper trace) were quantified at 87 and 13 ng kg⁻¹, respectively. Androstenedione (lower trace) was quantified at 536 ng kg⁻¹ b) diagnostic ion chromatogram observed for an egg sample in which 17 α -estradiol and 17 β -estradiol were quantified at 0.85 and 0.87 μ g kg⁻¹, respectively.
674x928mm (96 x 96 DPI)