A Fluorescence Polarization Assay To Detect Steroid Hormone Traces in Milk

Antonio Varriale, ‡ Anna Pennacchio, ‡ Gabriella Pinto, ‡ Giorgia Oliviero, § Stefano D’Errico, § Adelia Majoli, ‡ Andrea Scala, ‡ Alessandro Capo, ‡ Angela Pennacchio, ‡ Stefano Di Giovanni, ‡ Maria Staiano, ‡ and Sabato D’Auria* ‡

†Institute of Food Science, CNR, Via Roma 52, 83100 Avellino, Italy
§Dipartimento di Farmacia, Università degli Studi di Napoli “Federico II”, Via D. Montesano 49, 80131 Napoli, Italy

ABSTRACT: Steroids are a class of hormones improperly used in livestock as growth-promoting agents. Due to their high risk for human health, the European Union (EU) has strictly forbidden the administration of all natural and synthetic steroid hormones to food-producing animals, and the development of new rapid detection methods are greatly encouraged. This work reports a novel fluorescence polarization assay, ready to use, capable of detecting 17β-estradiol directly in milk samples with a low limit of detection of <10 pmol. It is based on the coupling of monospecific antibodies against 17β-estradiol and fluorophores, capable of modulating the fluorescence polarization emission on the basis of the specific binding of antibodies to fluorescence-labeled 17β-estradiol derivative. The successful detection of 17β-estradiol has disclosed the development of an efficient method, easily extensible to any food matrix and having the potential to become a milestone in food quality and safety.

KEYWORDS: fluorescence, biosensors, antibody, estradiol, milk

INTRODUCTION

The well-established trend of extending the milk-producing period of modern genetically improved dairy cows, such as the Holstein, has led to the consumption (roughly 75%) of milk originating from animals in gestation, an event controlled by a large pool of hormones. In particular, steroid hormones, a class of lipid compounds, are able to regulate a huge plethora of physiological functions, primarily correlated to reproduction and growth. The presence of steroids in milk can derive from their active (transport) and passive (diffusion) passage over the blood–milk barrier or from their direct biosynthesis within the mammary gland, and their release in milk fluctuates on the basis of the reproductive status of the dairy animal. In addition, natural and synthetic hormones can even more probably be released in milk as a result of an artificial administration to the food-producing animals. Abusive practices aimed at reducing costs of production and increasing the profitability of the meat and dairy industries are considered illegal because of the use of steroid hormones, strictly forbidden by the European Union (EU). The main aim of these drugs is to speed and improve growth rate in meat animals and milk production by increasing pregnancy rates, as well as to decrease feed costs by increasing the feed conversion efficiency (FCE), a measure of efficiency by which they convert the taken feed into muscle mass. Regardless of the exogenous or endogenous source, extended exposure to hormones can lead to metabolic disequilibria, causing an accumulation of abnormalities in the early phases of development, such as sexual precocity in young females and feminization of the male reproductive system, or irreversible genome and epigenome changes, underlying severe diseases. Indeed, the effects on human health as well as a direct association between breast and uterine cancer and exposure of natural and synthetic hormones are well-known in the literature. Moreover, in the light of a high risk to human health of such substances, even at low levels, steroid hormones are listed within group A in annex I of Council Directive 96/23/EC (group A, substances having an anabolic effect and unauthorized substances). However, the real onset of health damage, following milk intake, remains to be scientifically proven, and no national or European regulation establishes the maximum levels of hormones in milk or an official method of analysis. Increasingly, approaches are supporting the detection and quantification of hormones at low concentration in numerous matrices. The traditional use of an enzyme-linked immunosorbent assay (ELISA) for quantitative targeting of hormonal substances requires several incubation and washing steps, which are not readily compatible to the screening of a large number of samples and to field application. More recent analytical techniques are currently useful for profiling steroid hormones by high-throughput mass spectrometry such as GC-MS or LC-MS, but they require expensive equipment and preliminary procedures of sample purification. Actually, in recent years, several kinds of sample prepnurification such as liquid–liquid extraction (LLE), solid–liquid extraction (SPE), molecularly imprinted solid phase extraction (MISPE), and multistep solid phase extraction (MSPEE) have been combined with mass spectrometry analysis to isolate, purify, and concentrate estradiol from different food sources such as milk.

The determination of 17β-estradiol levels, the predominant and most biologically active estrogen, is a challenging task, due...
to its immiscibility in water, its low concentration, and the complexity of milk matrices. The determination of milk 17β-estradiol has been carried out by immunological techniques, which offer several advantages compared to conventional analysis (i.e., low cost per sample, high selectivity, and high sensitivity) but are time-consuming. A really valid alternative to detect hormonal contaminants in foods, even 17β-estradiol levels in milk, is biosensors. It has recently been acknowledged that biosensor technologies represent a greatly versatile approach for their capability to solve a large number of analytical problems related to agriculture and food safety as well as environmental security, pharmacology, and medicine.

Recently, our laboratory has increased the knowledge about this topic and developed fluorescence polarization assays for the detection of penicillin G directly in milk and of patulin in fruit juices. In the present work, we describe a similar fluorescence polarization approach to measure traces of 17β-estradiol in milk. This assay is based on the use of an ad hoc synthesized 17β-estradiol hemisuccinate–glutamine-binding protein (GlnPBP) conjugate labeled with fluorescence probe (Biotium CF647) and of polyclonal monospecific anti-17β-estradiol antibodies. Then, a competitive immunosassay has been performed with the aim to detect 17β-estradiol in milk sample without pretreatment steps. This method is rapid and innovative, and the analysis can be conducted directly in the real matrix with no interference due to different milk compounds, using an infrared emitting probe.

In conclusion, this technique represents a novel approach that allows the detection of any contaminant in a food matrix only by changing fluorescent conjugate molecules and specific antibodies; thus, it is a promising analytical tool in food quality and safety control.

**MATERIALS AND METHODS**

**Reagents.** All commercially available reagents were chosen for the highest quality. 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide (EDC), bovine serum albumin (BSA; fraction V), 17β-estradiol, ovalbumin (OVA), and 3,5-tetramethylbenzidine (TMB) were purchased from Sigma-Aldrich (St. Louis, MO, USA). A PURE1A Protein A Antibody Purification Kit was purchased from Sigma. Goat polyclonal to rabbit IgG–HRP conjugate (secondary antibody) was from Abcam (Cambridge, UK). Affinity resin EAH Sepharose 4B and ECL detection reagents used in Western blot experiments were purchased from Amersham Biosciences (GE Healthcare Switzerland). Protran nitrocellulose transfer membranes were purchased from Schleicher & Schuell (Dassel, Germany). Microplates (96-well), Nunc LockWell MaxiSorp form, and a microplate reader, Multiskan EX from Thermo Scientific, were used for ELISA experiments. UV measurements were carried out on a Varian Cary 50 Bio spectrophotometer (Agilent Technologies, Santa Clara CA, USA). Fluorescence probe CF647 was purchased from Biotium (Hayward, CA, USA). Fluorescence experiments were performed with an FP-8600 fluorescence spectrometer (Jasco, Japan).

**Synthesis of 17β-Estradiol-hemisuccinate.** The 17β-estradiol-hemisuccinate (Figure 1B) was synthesized from 17β-estradiol (Figure 1A) following the previously reported procedure but with minor modifications. Briefly, the 17β-estradiol (270 mg, 1.0 mmol) was dissolved in 12 mL of a mixture of benzene/pyridine (8:2, v/v) and refluxed with a succinic anhydride solution (600 mg, 6 mmol) for 24 h. TLC analysis showed the disappearance of the 17β-estradiol and the formation of two bands with a Rf lower than that of 17β-estradiol. The reaction mixture was dried under reduced pressure, and the residue was dissolved in acetone/ethanol (3 mL, 1:1, v/v) and purified by silica gel chromatography. For this purpose, the solution was absorbed on a small amount of silica gel (5.0 g) and, after evaporation of the solvent, the silica was applied on the top of a silica gel column, which

was eluted with an increasing amount of methanol in dichloromethane (DCM) (from 0 to 5% of MeOH). The purification furnished the estradiol-3,17β-bis-succinate (70% yield, lower Rf) and a product (at higher Rf), which was identified as a mixture of β-estradiol-3-hemisuccinate (15% yield) and β-estradiol-17-hemisuccinate (15% yield). The estradiol-3,17β-bis-succinate (300 mg), suspended in 10 mL of MeOH, was then treated with 10 mL of a 1.2 M solution of NaHCO3, and the mixture was kept at room temperature under stirring for 15 h. The selective hydrolysis of phenolic ester furnished, as expected, a mixture of estradiol-3β and estradiol-17β-hemisuccinate.

The latter (95% yield from bis-succinate) was further purified on a silica gel column (as previously described) eluted with an increasing amount of MeOH in DCM (from 0 to 20%, v/v). 1H NMR and MS results were in agreement with those reported previously.

**Preparation of the 17β-Estradiol-hemisuccinate–BSA Conjugate for the Immunoassay Procedure.** The 17β-estradiol-hemisuccinate–BSA conjugate (Figure 1C) was prepared by adding a solution of 17β-estradiol-hemisuccinate (2 mg) in 1 mL of 0.1 M MES buffer, pH 6.0, to a BSA solution (2 mg/mL) in 0.5 mL of the same buffer and 0.1 mL of an EDC solution in H2O (10 mg/mL). The reaction mixture was incubated for 16 h at 4 °C and then extensively dialyzed for 3 days, against potassium phosphate buffer (20 mM), pH 7.4. The obtained conjugate was spectrophotometrically analyzed at λ = 278 nm (2 mg/mL), but the efficiency of conjugation could not be determined because the maximum absorptions at 278 nm of estradiol (275 nm) and BSA are overlapped.

**Antibody Production and Purification.** Two rabbits were immunized following a standard protocol by intradermal inoculation of an antigen (0.5 mg per rabbit). After the immunization period, the rabbits were sacrificed, and their blood was recovered and centrifuged to separate blood cells from serum. A 1.5 mL sample of rabbit serum diluted with 1.5 mL of binding buffer Tris (50 mM), pH 7.0, was applied to a protein A column of the PURE1A Protein A Antibody Purification Kit, Sigma, and the IgG fraction was purified in agreement with the manufacturer’s instructions. The IgG fraction was eluted with glycine (0.1 M) at pH 3.0, and immediately buffered in Tris-HCl (1.0 M), at pH 9.0, and its concentration and purity were checked by absorbance measurement at λ = 278 nm and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis, respectively.

**Affinity Column Preparation of 17β-Estradiol-hemisuccinate–EAH Sepharose 4B.** The affinity column was obtained by conjugating 17β-estradiol-hemisuccinate to EAH Sepharose 4B. Briefly, Sepharose resin (0.6 mL) was washed with distilled water adjusted to pH 4.5 (20 mL) and with HCl, followed by 0.5 M NaCl (80 mL), and then added to a solution of 17β-estradiol-hemisuccinate (2 mg in 0.5 mL of H2O), and the resulting suspension was gently shaken. The slurry was cooled to 0 °C, and EDC was added to a final concentration of 0.1 M. The reaction mixture was incubated overnight at 4 °C and then it was taken to room temperature. The suspension was washed with H2O at pH 4.5, 0.1 M acetate buffer containing 0.5 M NaHCO3, and the obtained suspension was kept at room temperature.

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Chemical structure of the 17β-estradiol (A), 17β-estradiol-hemisuccinate derivative (B), and the GlnPBP conjugate used for the production of the rabbit anti-17β-estradiol antibodies (C).
NaCl (20 mL), pH 4.0, and 0.1 M PBS containing 0.3 M NaCl, pH 7.4, and finally packed into a polystyrene column (2 mL, Bio-Rad). For the affinity chromatography purification, a solution of IgG (8.0 mg/mL), obtained from protein A purification, was applied dropwise to the previously prepared affinity column. Before elution of the monospecific IgG, three high-salt buffers at pH 7, all containing 10 mM PBS but increasing concentrations of NaCl (0.1, 0.5, and 1.0 M, respectively), were used to wash away antibodies weakly and unspecifically bound to the column. Instead, monospecific IgG was eluted with 0.1 M glycine, at pH 3.0, and appropriately monitored by absorbance measurements at 278 nm and SDS-PAGE. The fractions containing the antibodies were collected and dialyzed against a buffer containing 0.01 M PBS and 0.1 M NaCl, pH 7.4. The concentration of the monospecific antibodies, spectrophotometrically determined at A = 278 nm, was 1.3 mg/mL.

Preparation of 17β-Estradiol-hemisuccinate—GlnBP Conjugate. The 17β-estradiol-hemisuccinate was conjugated to Escherichia coli GlnBP, a protein different from that used for the immunization process, with the aim of avoiding interferences due to BSA during the polyclonal antibody detection. The synthesis of 17β-estradiol-hemisuccinate—GlnBP conjugate (Figure 1C) was carried out following the same procedure previously described for the 17β-estradiol-hemisuccinate—BSA conjugate.

Antibody Titration Experiments. The antibody titer was determined by an indirect ELISA. The antigen (17β-estradiol-hemisuccinate—GlnBP), dissolved in 0.1 M PBS, pH 7.4, was used to coat 96-well microplates, at increasing concentrations from 125 to 5000 ng/mL, and incubated overnight at 4 °C. Control wells were incubated for the same period with GlnBP dissolved in the same buffer. The wells were flushed three times with TBS 1× containing 0.05% Tween (TBST), pH 7.4, and blocked by incubation for 1 h at room temperature with TBST containing OVA (1%). After two washings with TBST, polyclonal and polyclonal monospecific anti-estradiol antibodies were serially diluted in blocking buffer, added to the wells, incubated at room temperature for 1 h, and then washed twice with TBST. Horseradish peroxidase-conjugated anti-rabbit IgG antibodies, diluted 1:12000 in blocking buffer, were added to the wells and incubated at room temperature for 1 h. After two washings with TBST, the enzyme substrate TMB was added, and the color reaction was quenched by adding 2 M HCl, for 5 min. The absorbance measurements were recorded at 450 nm.

Western Blot Experiments. Proteins (BSA, GlnBP, and 17β-estradiol-hemisuccinate—GlnBP, 20 μg for each lane) were separated by 12% SDS-PAGE and then transferred overnight at 4 °C onto a nitrocellulose membrane. Membranes were blocked for 30 min at room temperature in 50 mL of the blocking buffer (TBS containing 5% of milk), washed with TBST (10 min for each washing), and incubated with purified monospecific IgG (1:100000 in the blocking buffer containing Tween 0.05%) for 1 h at room temperature. After three washings with TBST, the filters were incubated with secondary antibody (goat anti-rabbit HRP conjugate, 1:6000 in the blocking buffer containing Tween 0.05%) for 1 h at room temperature and washed three times as described above. Finally, proteins were visualized by chemoluminescence using the Amersham ECL plus and X-ray films developed on the Compact X4, Xograph Imaging systems.

Fluorescence Labeling of 17β-Estradiol-hemisuccinate—GlnBP and Steady-State Fluorescence Measurements. A solution of 17β-estradiol-hemisuccinate—GlnBP (2.0 mg/mL) was concentrated by nitrogen flow and diluted with 0.1 M sodium bicarbonate buffer, pH 7.0, at a final concentration of 4.0 mg/mL. This solution was mixed with the CF647 dye (Biotium) at a protein/dye molar ratio of 1:10 and incubated for 1 h at 37 °C. The labeled molecules were separated from unreacted probe by gel filtration and extensive dialysis in PBS buffer at pH 7.0. Steady-state fluorescence experiments were performed by using an FP-8600 fluorescence spectrometer, equipped with a thermostatic cell holder. The excitation wavelength was fixed at 630 nm, and emission spectra were recorded between 650 and 800 nm with excitation and emission slit widths of 5 and 2.5 nm, respectively. The fluorescence measurements were performed at 25 °C on five times diluted solutions of milk with PBS at pH 7.4. 17β-Estradiol-hemisuccinate—GlnBP-CF647 was incubated with a range of concentrations of antibodies against 17β-estradiol from 0.0 to 2.2 pmol for 10 min, and polarization fluorescence measurements were acquired by inserting a Glan polarizer between the excitation source and the sample, with a vertical (0°) excitation and emission polarization filter.

RESULTS AND DISCUSSION

Steroids have been widely used as growth-promoting agents in livestock with the principal aim to reduce the FCE and increase the animal weight gain and the milk yield in breeding of meat and milk animals. The administration of naturally and synthetic anabolic growth promoters in food-producing animals is now prohibited by the EU because of their potential risk to consumer’s health. Nevertheless, illegal use or abuse of 17β-estradiol as an additive in animal feed or as subcutaneous injections, in hormonal treatments, implies a contamination of produced milk with several consequences for human health. In the absence of a precise regulation that sets the maximum limit of such substances in milk and official methods of analysis, the development of procedures addressed at this aim is of great importance. A rapid and simple method to detect the concentration of 17β-estradiol in milk is urgently needed to control all phases of milk production, from the cattle shed to the consumers’ table.

Antibody Fluorescence Polarization-Based Sensor for 17β-Estradiol Sensing. In the context of high competence in the biosensor field, we propose a new method of 17β-estradiol detection that combines fluorescence polarization assays and a strategy well-established by our research group (Figure 2), starting from the functionalization of the 17β-estradiol to a more reactive carboxylic derivative with a significantly higher antibody response. Actually, in this context, the 17β-estradiol derivatization process is markedly necessary to obtain a derivate exhibiting a higher stability and a short arm to be conjugated to the protein carrier while maintaining the original skeleton of estradiol. 17β-Estradiol binding to a carrier protein through a spacer such as a hemisuccinic arm is necessary to distance the antigen from the same protein. On the other hand, the presence of a carrier protein conjugated to the hormone was then strongly required for amplifying the immunological response of antibodies against 17β-estradiol that would otherwise be too small because of its very low molecular weight. Then, the synthesis of 17β-estradiol-hemisuccinate was preliminarily carried out with the aim of preparing the antigen 17β-estradiol-hemisuccinate—BSA conjugate (BSA was chosen as carrier protein) to be used in the standard protocols of immunization on two different rabbits (Figure 2). At the end of the immunization period, a protein A antibody chromatograph was used to separate the IgG fraction from the complex pool of serum protein, whereas a further affinity chromatography by EAH Sepharose 4B resin was carried out to isolate specifically
The preliminary SDS-PAGE analysis (Figure 3A) of BSA, GlnBP, and 17β-estradiol-hemisuccinate–GlnBP demonstrated the purity of the preparations, used in the Western blotting experiments (Figure 3B) to verify the specificity of the produced antibodies. The response to antibody binding was observed only for the conjugate 17β-estradiol-hemisuccinate–GlnBP, and a negative response was registered for BSA and GlnBP (Figure 3B). This confirms the specificity of antibodies versus the only estradiol. In addition, an indirect ELISA test was performed to evaluate the titer of polyclonal monospecific purified antibodies. The results have shown that no signal was registered as a consequence of incubation with different diluted samples of IgG for noncoated wells, whereas an increase of the absorbance was registered as a function of decreasing the dilution factor of polyclonal monospecific antibodies against 17β-estradiol (data not shown). The titer was considered as the maximum antibody dilution able to give a reading of 0.1 absorbance unit. The experiment, performed in triplicate, showed that the titer of anti-17β-estradiol antibodies was excellent. In fact, it was possible to perform the ELISA test with mono-specific IgG dilutions up to 1 to 100000.

Fluorescence Steady-State Measurements of 17β-Estradiol-hemisuccinate–GlnBP-CF 647 Conjugate and Competitive Immunoassay. 17β-Estradiol-hemisuccinate–GlnBP was labeled with a fluorescence probe (CF647 dye), possessing near-infrared emission, to record changes in the emission processes on the basis of specific binding to anti-estradiol antibodies. The polarized emission spectra of 17β-estradiol-hemisuccinate–GlnBP-CF647 fluorescent conjugate were acquired in the absence and in the presence of increasing concentrations of antibodies against estradiol ranging from 0.0 to 2.2 pmol (Figure 4). The fluorescence polarization spectrum of milk samples showed a maximum of fluorescence emission, centered at 668 nm, and revealed an increase of 17β-estradiol-hemisuccinate–GlnBP-CF647 fluorescence polarization emission as a consequence of binding with anti-17β-estradiol antibody, present in solution. Subsequently, a fluorescence polarization competitive immunoassay was used to measure unlabeled 17β-estradiol in solution. To evaluate the competition between 17β-estradiol-hemisuccinate–GlnBP-CF647 and unlabeled 17β-estradiol for binding with monospecific antibodies against 17β-estradiol, different samples at a fixed concentration of antibody (2.2 pmol) were incubated in the presence of increasing concentrations of unlabeled 17β-estradiol in a range between 0.0 and 47 pmol, before the fluorescence polarization measurements were performed. The polarized fluorescence spectrum in the presence of increasing concentrations of unlabeled 17β-estradiol showed a reduction of fluorescence emission following increasing concentrations of unlabeled 17β-estradiol (Figure 5). The presence of increasing concentrations of the analyte in the milk samples allowed return to the minimum measurable value of the analyte. The

Figure 2. Flowchart of strategy for antibody fluorescence polarization-based sensor.

Figure 3. SDS-PAGE (A) and nitrocellulose filter (B) after incubation with 17β-estradiol antibodies. Lanes: 1, molecular weight standards; 2, BSA; 3, GlnBP; 4, 17β-estradiol-hemisuccinate–GlnBP.

Figure 4. Polarization emission spectra of 17β-estradiol-hemisuccinate–GlnBP-CF647 in the absence and in the presence of increasing concentration of antibodies against 17β-estradiol.
Recently, immunosensors and nanobiosensors have been developed for the determination of 17β-estradiol in food. In the other groups to identify and quantify the presence of 17β-estradiol, a DNA-Aptamer optical biosensor has been realized for the detection of 17β-estradiol. These biosensors are highly sensitive but unfortunately not suitably specific due to the wide range of affinity of estrogen receptor to other xenoestrogens.25, 26 Recently, immunosensors and nanobiosensors have also been developed for sensitive and rapid detection of 17β-estradiol by utilizing antibodies or functional polymers,27,28 and a DNA-Aptamer optical biosensor has been realized for the detection of 17β-estradiol in water.29 The present paper shows a fluorescence polarization competitive immunoassay for detecting 17β-estradiol up to a concentration of <10 pmol. This assay can be carried out directly in a real matrix, without any sample pretreatment, providing great advantages in time and cost terms. In addition, the simplicity and quickness of 17β-estradiol quantification, performable during every step of milk production from milking to the commercial product, offer further reasons why our procedure could be preferred to others. Actually, it is not the first time that we have developed a fluorescence polarization-based method to measure a contaminant analyte in a food matrix. During past years, several papers from our laboratory have demonstrated the possibility of detecting other contaminants such as penicillin G20 and patulin,21 in milk and in juice, respectively, by using a very similar strategy (Figure 2), even at concentrations much lower than the established EU limit. Our expertise in this field gives us great confidence that this approach can be extended to any contaminant and to any food by carrying out minor modifications, thanks to the high flexibility of the developed method. Therefore, it is our opinion that the fluorescence immunoassay is a valid alternative to the analytical techniques, currently in use, and it can become a promising tool in the control of food quality and safety.

### AUTHORS INFORMATION

**Corresponding Author**

*(S.D.A.)* Phone: +39-0825 299101. Fax: +39-0825 78158. Email: sabato.dauria@cnr.it.

**Funding**

This project was realized within the framework of the CNR Commessa “Progettazione e Sviluppo di Biochip per la Sicurezza Alimentare e Salute Umana (SD; MS; AV)” and partially funded by the “SAFE &SMART” project – Nuove tecnologie abilitanti per la food safety e l’integrità delle filiere agro-alimentari in uno scenario globale” (CTN01_00230_240864).

**Notes**

The authors declare no competing financial interest.

### ABBREVIATIONS USED

FCE, feed conversion efficiency; ELISA, enzyme-linked immunosorbent assay; GlnBP, glutamine-binding protein; EDC, 1-[3-(dimethylamino)propyl]-3-ethylcarbodiimide; BSA, bovine serum albumin; OVA, ovalbumin; TMB, 3,5-tetramethylbenzidine; DCM, dichloromethane; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris-buffer saline Tween

### REFERENCES

7. Key, T. J.; Pike, M. C. The dose–effect relationship between ‘unopposed’ oestrogens and endometrial mitotic rate: its central role in...