Determination of Steroids by High Performance Liquid Chromatography-Fluorescence

Subjects: Chemistry, Analytical | Others | Endocrinology & Metabolism

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Steroids are compounds widely available in nature and synthesized for therapeutic and medical purposes. Although several analytical techniques are available for the quantification of steroids in clinical samples, their analysis is challenging due to their low levels and complex matrices. The efficiency and quick separation of the high performance liquid chromatography (HPLC) combined with the sensitivity, selectivity, simplicity, and cost-efficiency of fluorescence, make HPLC coupled to fluorescence detection (HPLC-FLD) an ideal tool for routine measurement and detection of steroids for clinical and medical applications.

high performance liquid chromatography | clinical | steroids | fluorescence | quantification

1. Clinical Applications of HPLC-FLD Techniques

Quantification of steroids plays an important role in the diagnosis and treatment of endocrine disorders. Diseases, such as cancer, metabolic syndromes, and neurodegenerative diseases, are associated with abnormalities in the endocrine system. Steroids also play an important role in biochemical processes, such as aging, reproduction, and metabolic pathways [1]. Furthermore, disturbances in the endocrine system due to EDC from environment and food are becoming a major clinical concern. HPLC-FLD has been employed in many research studies, both in humans and animal models, to quantify the various types of steroids and investigate their role in diseases and clinical conditions.

2. Detection of Glucocorticosteroids

Glucocorticosteroids are widely recognized as markers for adrenal activity. Cortisol can reflect the short-term changes in the activity of the hypothalamic–pituitary–adrenocortical axis (HPA axis), making it a valuable surrogate marker for stress and glucose metabolism [2].

Many methods have been developed to detect unconjugated cortisol levels in various biological samples. However, the use of certain samples, such as urine, may not be recommended as these samples may contain many interfering substances thus complicating steroids extraction procedure [3]. In addition, the concentrations of corticosteroids in urine are low compared with those found in plasma, which makes the latter a better medium for

measurement. Steroids have been measured by HPLC-FLD methods for various clinical purposes, such as investigating disease courses, pathogenesis, and metabolic processes [4].

The detection of corticosteroids in biological samples can be done directly depending on fluorescence signal enhancement upon the treatment of samples with the deproteinizing agent (ethanol) and sulfuric acid (**Figure 1**). A study to quantify corticosteroids in serum samples was performed using C_{18} analytical column after treatment with ethanol and sulfuric acid [5]. Cortisol, testosterone and corticosterone emitted fluorescence, with LOD for cortisol = 0.3 pg/dL (S/N = 3). In another study, the same group measured cortisol in urine samples producing a LOD = 0.26 pg/dL (S/N = 3) [6]. Sudo et al. used post-column derivatization with sulfuric acid to measure cortisol and corticosterone in rat urine (LOD was 0.5 pmol for corticosterone at (S/N = 5)) [3]. They analyzed other corticoids, such as prednisolone, 6α-methylprednisolone, dexamethasone, and betamethasone, but with lower sensitivity. Moreover, ethyl acetate was used for extraction after sulfuric acid hydrolysis to prevent the acid from entering the HPLC system. It was noticed that the fluorescence intensities were dependent on the reactor temperature and sulfuric acid [3][7].

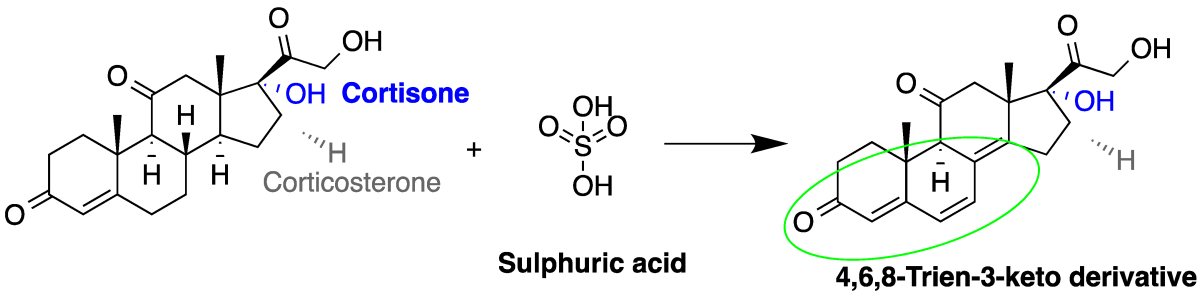

Figure 1. Fluorescence emission of steroids after treatment with sulfuric acid.

Since cortisol levels in hair might be used as a biomarker of chronic stress, a study developed a HPLC-FLD method to measure cortisol distribution in human hair samples. The method has achieved equal precision to mass spectrometry. Samples were prepared by pulverization and incubation in 0.1 M HCl, and extraction with ethyl acetate followed by derivatization with sulfuric acid. A detection limit of 1 pg/mg was achieved [2].

Derivatization with fluorophore-containing reagents was employed in many studies to detect and quantify corticosteroids in serum and urine samples. The derivatization process depends on functionalization of specific groups in corticosteroids structure (**Figure 2**). The primary alcohol functionality can be esterified through its reaction with either 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) (**Figure 2**), 9-anthroyl nitrile (9-AN) (**Figure 3**), or 1-anthroyl nitrile (1-AN) (**Figure 3**). These derivatization processes proved to be selective as neither secondary alcohol, nor tertiary alcohol can react with fluorophore-containing reagents (**Figure 3**).

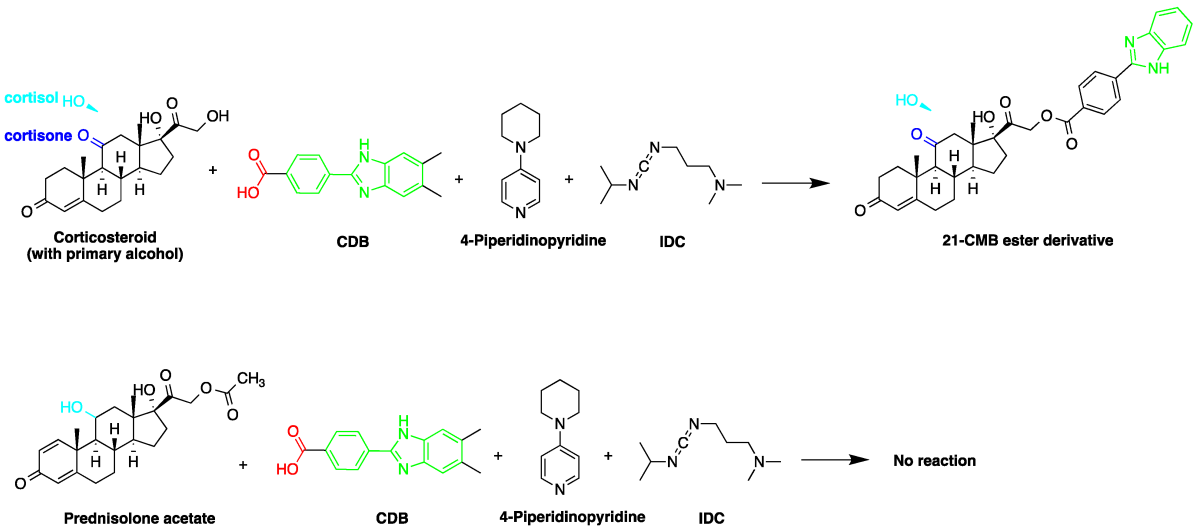

Figure 2. Esterification of primary alcohol functionality of cortisol and cortisone by 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) in the presence of 4-piperidinopyridine and 1-isopropyl-3-(3-dimethylaminopropyl) carbodiimide (IDC) perchlorate. Prednisolone secondary alcohol shows no reaction.

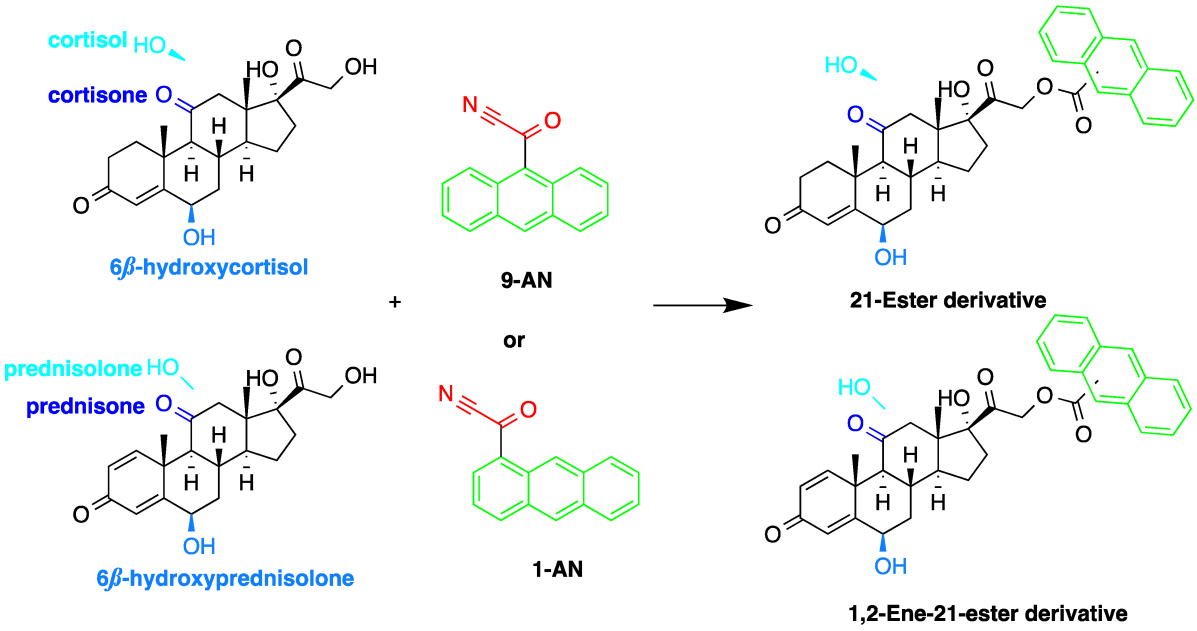

Figure 3. Esterification of primary alcohol functionality of cortisol and cortisone by 9-anthroyl nitrile (9-AN) or 1-anthroyl nitrile (1-AN).

The derivatization through esterification of corticosteroid primary alcohol functionality with CDB was studied by Katayama et al. [8]. The results showed that the detector response to secondary alcohols was less than one fiftieth that of the primary alcohol. However, certain secondary alcohols in steroids, such as prednisolone acetate and testosterone, and tertiary alcohols, showed no reaction. Later, Katayama et al. used human plasma to detect eight corticosteroids by derivatization with CDB to their esters in acetonitrile. The esters were separated on a reversed-phase column (Zorbax ODS) with water:methanol (25:75, *v*/*v*) containing 5 mmol/L tetramethylammonium hydrogen sulfate as a mobile phase. The LOD ranged between 0.06 and 0.3 pg per 100 µL of plasma (S/N = 3) [9].

The use of 9-AN as a derivatizing agent for corticosteroids was reported in several studies. Neufled et al. used 9-AN to derivatize the primary alcohol in C_{21} corticosteroids. The reaction was carried out at 45 °C for 2 h and the fluorescent derivatives were separated on silica stationary phase with a mixture of 2-propanol and hexane as a mobile phase in the gradient mode. The low temperature in derivatization prevented the thermal degradation of corticosteroids and avoided reaction with secondary hydroxyl groups [10][11].

Measuring endogenous glucocorticoids and their metabolites is important as disturbances in the enzyme responsible for their metabolism can cause hypertension. One study described an HPLC-FLD method using 9-AN derivatization for the determination of cortisol, cortisone, and their tetrahydro- and allo-tetrahydro-metabolites in plasma and urine [12]. Following extraction with dichloromethane and SPE, 9-AN was used to derivatize the steroids in the samples to their fluorescent products. LOD (S/N = 3:1) achieved with this method was 3.0 ng/mL for all analytes. Similarly, Shibata et al. employed 9-AN for fluorescent derivatization and developed a method to investigate cortisone levels in plasma and urine samples of renal transplant patients who received prednisolone [13]. Cortisol, cortisone, prednisolone, prednisone, 6β-hydroxycortisol, and 6β-hydroxyprednisolone were derivatized to their fluorescent esters after extraction with ethyl acetate (**Figure 4**). The 6β-hydroxycortisone was used as an internal standard. LODs achieved for cortisol, cortisone, prednisolone and prednisone in plasma or urine were 0.1 ng/mL, while those for 6β-hydroxycortisol and 6β-hydroxyprednisolone in plasma or urine were 0.5 ng/mL.

Similar to 9-AN, fluorescent derivatization can be achieved using 1-AN. A study described the use of 1-AN to derivatize 18-oxygenated corticosteroids: 18-hydroxycortisol, 18-hydroxycortisone and 18-oxocortisol in human urine into their fluorescent 21-anthroyl esters [14] (**Figure 4**). A mixture of ether and dichloromethane was used to extract the steroids, and the anthroyl derivatives were enriched by SPE using a CN cartridge column. The LOD was 0.1 pmol (S/N = 5).

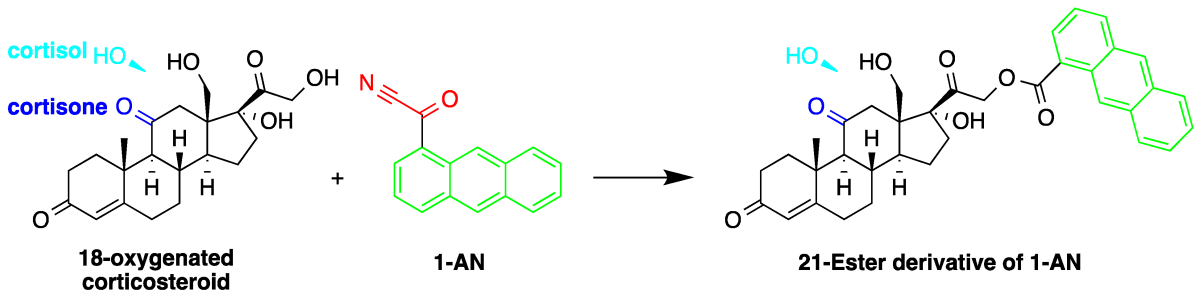

Figure 4. Fluorescent derivatization of 18-oxygenated corticosteroids by or 1-anthroyl nitrile (1-AN).

Fluorescent derivatization of corticosteroids can also be carried out using dansyl hydrazine targeting the carbonyl groups (**Figure 5**). In a study to determine corticosteroids in human plasma and urine samples were derivatized by dansyl hydrazine and quantified by HPLC-FLD, following extraction with methylene chloride. The linearity range of the method was between 0.5 and 60 ng of cortisol, proving it to be suitable for the routine analysis of cortisol in plasma and urine [15].

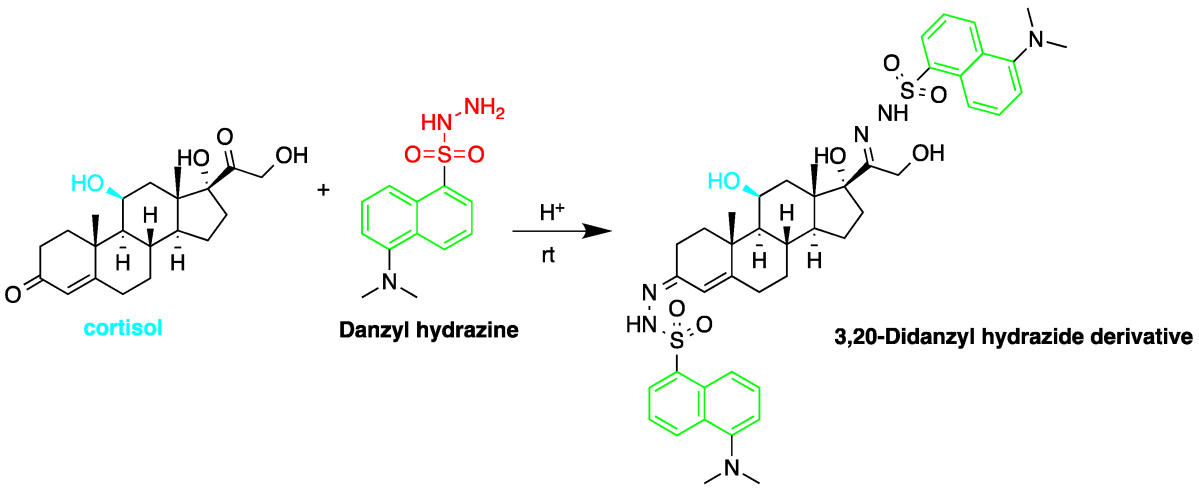

Figure 5. Fluorescent derivatization of carbonyl functionality of cortisol by dansyl hydrazine.

The detection of corticosteroids with ketolic groups in urine is key part in diagnostic procedure of Cushing's syndrome. Ketol-containing corticosteroids, such as 17-hydroxycorticosteroid (breakdown product of cortisol), are usually excreted in urine as tetrahydro form making their detection using UV absorption at 240 nm not possible. To detect these steroids in urine, derivatization with amidine-containing compounds was used yielding fluorescent compounds [16] (**Figure 6**). Using this approach, the best sensitivity was achieved for cortisol.

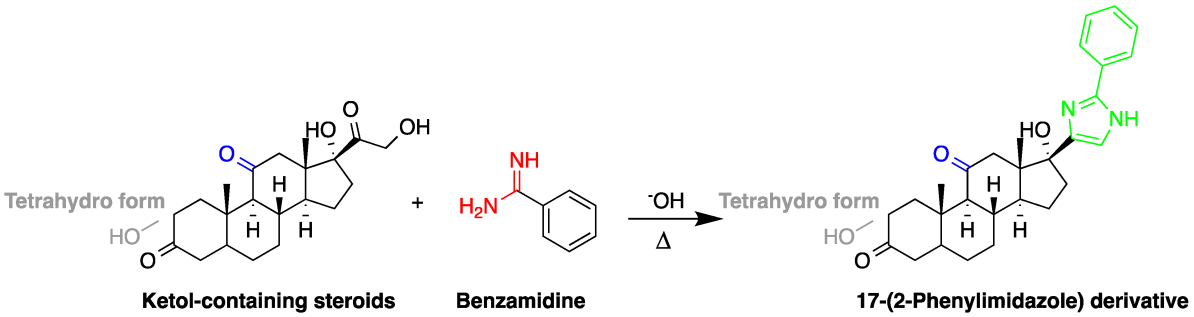

Figure 6. Fluorescent derivatization of ketolic-containing corticosteroids by benzamidine.

The utilization of ketolic groups was also performed by Yamaguchi et al. to detect and quantify corticosteroids in urine after their conversion into fluorescent quinoxalines (**Figure 7**) [17]. They detected nineteen 21-hydroxycorticosteroids in human urine samples achieving LOD = 0.14–29.4 pmol/50 µL injection volume (S/N = 3). In another study by the same group, prednisolone and prednisone were quantified in plasma samples following similar derivatization procedure and analyzed by reversed-phase liquid chromatography with isocratic elution. The LOD of prednisolone and prednisone was 3 ng/mL in plasma (S/N = 3) [18].

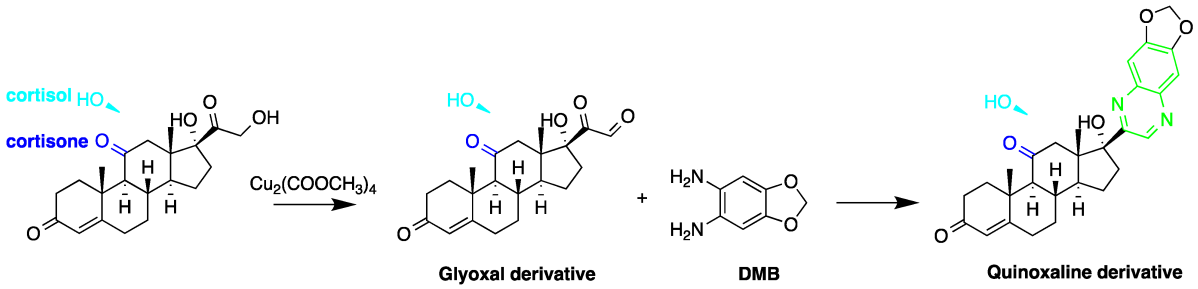

Figure 7. Fluorescent derivatization of ketol-containing corticosteroids to quinoxaline derivative.

Cholesterol quantification is commonly required in the analysis of biosamples. A study described a simple and sensitive method for the determination of cholesterol and phytosterols, such as β-sitosterol in biosamples (e.g., saliva and urine matrices) and food samples (cow and soybean milk) after derivatization with naproxen acyl chloride in toluene (**Figure 8**). The method employed a C_8 column with a mixture of methanol, isopropanol, and water, achieving a LOD of about 25 nM (S/N = 3). The analysis of cholesterol and sitosterol is usually time-consuming, but using the method, a relatively short period of time was required since solvent concentration, evaporation, and replacement steps were not necessary [19].

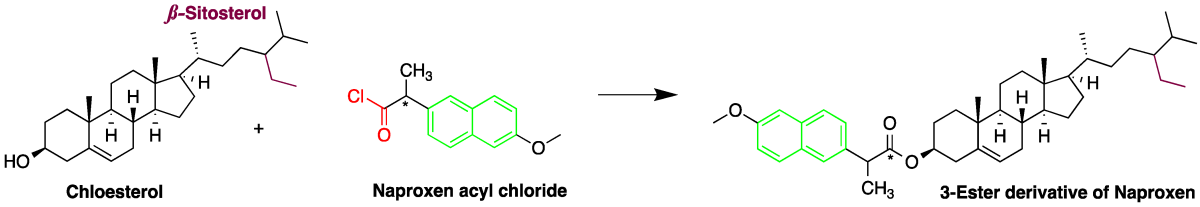

Figure 8. Derivatization of cholesterol by naproxen acyl chloride.

Free 7α-hydroxycholesterol (7-HC) levels in human serum were reported to be a good indicator for bile acid synthesis. An HPLC-FLD method was developed by Saisho et al. to quantify 7-HC levels in dog plasma, with the purpose of studying the effect of cholestyramine on plasma levels of 7-HC. 7-HC was converted to its fluorescence derivative, by two procedures, after being extracted and then purified [20]. The two derivatization reagents used were 1-AN and 7-methoxycoumarin-3-carbonyl azide (MC-CON_3). The MC-CON_3 derivatization resulted in higher fluorescence intensity compared to 1-AN route. 1-AN produced only a C-3 fluorescent derivative due to the bulky anthracene group and steric hindrance around the C-7 position. In comparison, MC-CON_3 yielded a double coumarin derivative at the C-3 and C-7 positions (**Figure 9**) producing a LOD of 4 pg (S/N = 5).

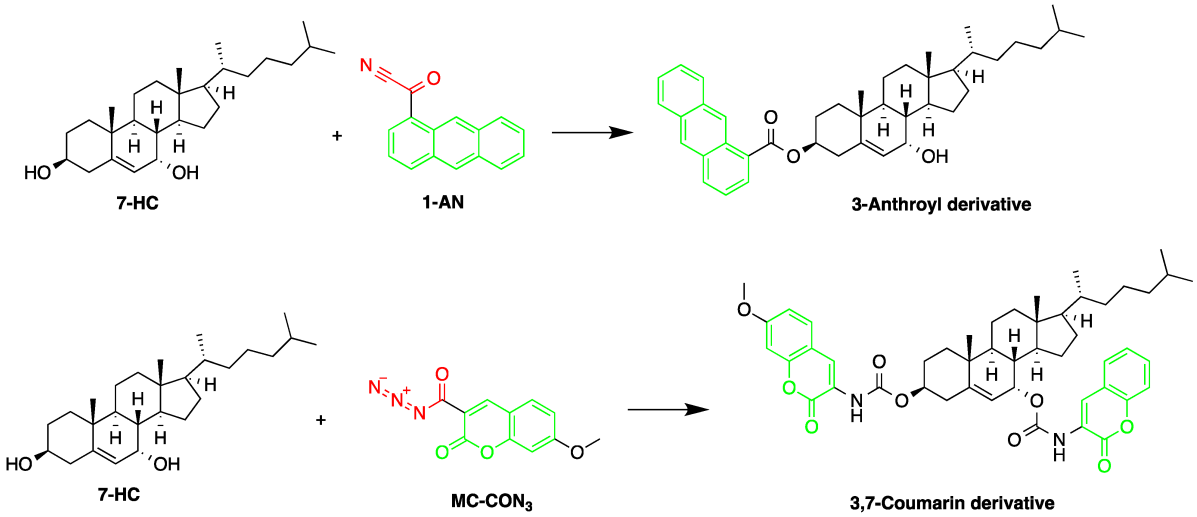

Figure 9. Derivatization of 7α-hydroxycholesterol (7-HC) by 1-anthroyl nitrile (1-AN) or 7-methoxycoumarin-3-carbonyl azide (MC-CON_3).

3. Detection of Steroid Hormones

Detection of steroid hormones and their metabolites are important in diagnosis of metabolic diseases. Several HPLC-FLD methods were described for the detection of steroid hormones. A study developed a method for monitoring progesterone and 17-hydroxyprogesterone in the serum from pregnant women. The quantification employed fluorescent derivatization using 4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-*s*-indacene-3-propionohydrazide (BODIPY^{TM} FL hydrazide) [15] (**Figure 10**). The derivatization was carried out in ethanol at room temperature (about 22 °C) for 15 h. This derivatization method was reported to be 50 times faster than that using dansyl hydrazine. The LODs for progesterone, 17-hydroxyprogesterone, dehydroepiandrosterone, androstenedione, testosterone and 17-methyltestosterone were in the range of 550–3700 fmol per 10 µL injection (S/N = 5).

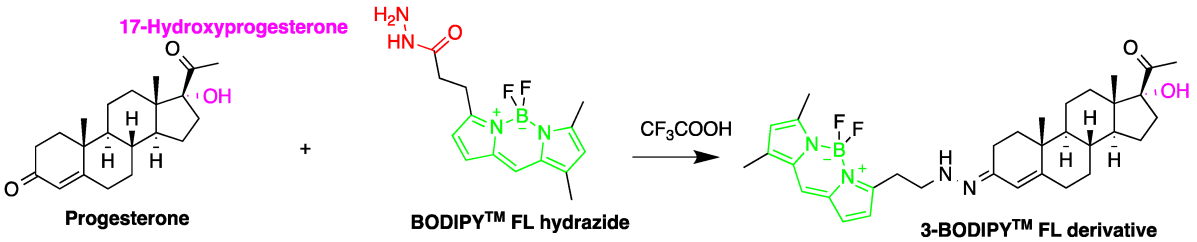

Figure 10. Derivatization of progesterone by 4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-s-indacene-3-propionohydrazide (BOD-IPYTM FL hydrazide).

The derivatization reagent 1-AN was used by Shimada et al. to determine the endogenous steroid pregnenolone in Wistar and Sprague–Dawley rat brain samples [10]. The samples were homogenized in isotonic saline then deproteinized with methanol, before subjected to sequential steps of extraction and derivatization. The samples were derivatized with 1-AN (**Figure 11**) and the excess reagent was removed by purification, carried out on two

successive silica gel columns. The 3β-hydroxy-16-methylpregna-5,16-dien-20-one was used as an internal standard.

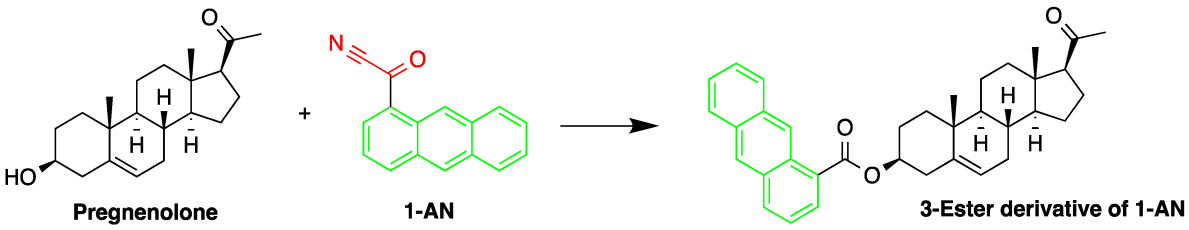

Figure 11. Derivatization of pregnenolone by 1-anthroyl nitrile (1-AN).

Several steroids in animal tissue were detected in another study using ultraviolet, fluorometric, and electrochemical detectors [21]. The study demonstrated the selectivity of FLD in determination of estradiol by exploiting its fluorescence emission, although it was eluted with the same fraction containing other steroids and their respective metabolites. Extraction with methanol allowed the separation of acidic corticosteroids (diethylstilbestrol, estradiol, zeranol/zearalenone, and their metabolites) from their neutral anabolic counterparts (testosterone, trenbolone and progesterone).

Urine is the best source for the estimation of estrogen concentrations, since they are primarily excreted renally as glucuronides and sulfates. Previous studies used urine samples for the determination of estrogen levels in their studies, albeit with different extraction methods [22][23]. Mao et al. used chemical hydrolysis in methanol and concentrated hydrochloric acid at 70 °C for 1 h to release the conjugation before SPE sample preparation, whereas Kumar et al. used a fabric phase sorptive extraction (FBSE) procedure, which offered shorter sample preparation times and 400 times higher sorbent loading. This method achieved a lower detection limit and analysis time, proving to be greener and more economical. The LODs for β-estradiol, 17α-ethinylestradiol, and bisphenol A were 20 pg/mL, 36 pg/mL, and 42 pg/mL, respectively.

In another study, Mao et al. used *p*-nitrobenzoyl chloride at 25 °C as a derivatization reagent, which can easily react with the hydroxyl and phenolic hydroxyl groups of organic chemicals (**Figure 12**) without the need for a catalyst [22]. Moreover, 4-nonylphenol, bisphenol A (BPA), 17α-ethinylestradiol, and three endogenic estrogens, including 17α-estradiol, 17β-estradiol, and estriol, were determined in urine samples collected from 20 healthy volunteers. Samples were hydrolyzed with HCl and subjected to SPE purification. Separation was performed on a C_{18} column with gradient elution using acetonitrile and water as a mobile phase. The LODs of the method were 2.7 µg/L for BPA and 17β-estradiol, 2.9 µg/L for 4-nonylphenol, 4.6 µg/L for 17α-estradiol and 17α-ethinylestradiol, and 8.3 µg/L for estriol.

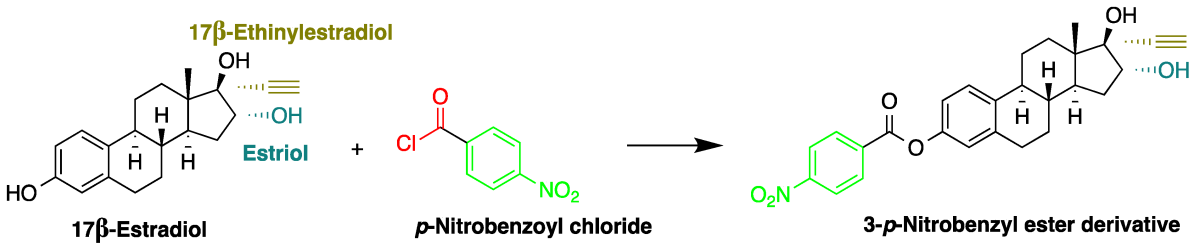

Figure 12. Derivatization of 17β-estradiol by *p*-nitrobenzoyl chloride.

A recent study examined the determination of estrone (E1), 17β-estradiol (E2), and estriol (E3) and their conjugated metabolites levels in cow and river buffalo meat [24]. Samples were extracted with methanol, enzymatically deconjugated and purified by C_{18} SPE before they are analyzed by HPLC-FLD. The effect of temperature on the studied steroid concentrations was also investigated and it was shown that heating processes was not able to significantly affect the level of phenolic estrogens in meat.

Estrogens in human urine samples were determined by HPLC-FLD following vortex-assisted dispersive liquid–liquid microextraction (VA-DLLME). Fine droplets of nonanoic acid floating on the top of sample solution were used to extract the estrogens using vortex-mix to assist the dispersion of the extraction solvent into the aqueous sample. Derivatization was not required in this work as the high extraction efficiency of DLLME improved the sensitivity and shortened the analysis time. LOD values were 0.01 ng/ml for E3, 0.01 ng/mL for βE2, and 0.06 ng/mL for E1, respectively [25].

The abuse of anabolic steroids has gained worldwide concerns, as frequent high doses can irreversibly affect the endocrine system, mineral metabolism, and may result in hepatic carcinomas [26]. Therefore, several committees have prohibited or imposed strict rules on their use for illegal purposes, such as increasing performance activity for athletes and stimulating meat production in cattle and poultry. Amin et al. used micellar chromatography with the detergent solution as the mobile phase to detect testosterone and bolasterone in human urine samples [26]. The method made use of fluorescence by means of energy transfer from the aromatic carbonyls in the anabolic steroids to the terbium ion in micellar media. No sample preparation was required and urine samples were injected directly onto the HPLC column. Excitation of terbium by means of energy transfer from steroids resulted in 183-fold fluorescence enhancement. The detection limits were 10 ng/200 µL injection volume for testosterone and 2 ng/200 µL for bolasterone.

4. Detection of Endocrine Disruptive Chemicals (EDCs)

The analysis and detection of steroid blood levels is vital for the investigation of food safety and effect on health [22][27]. Many studies have reported the presence of synthetic steroids in our daily food, both from plant and animal sources [28][29][30][31]. BPA, an industrial chemical, was found in biological fluids because of its ability to leach into food or liquids or through dental sealants into patient's saliva [24][22][28][32][33]. One of the reasons behind their existence is that farmers increase their profit by using endocrine disruptive chemicals (EDCs) to support the feed

conversion and growth rate in animals. Substances with hormonal actions are prohibited in the European community for use in animals intended for meat production due to their possible toxic effects on public health [27].

When bound to human estrogen receptors, they can stimulate the transcriptional activity of various estrogen receptor subtypes. The increase in levels of estrogen may be linked to the possibility of cancer occurrence among meat consumers [30].

In addition, studies have proved the ability of BPA to cross the placenta and blood brain barrier, in turn affecting the endocrine organs in animals and humans. This includes increasing prostate size, decreasing the number of produced sperms, and causing early puberty in females, in addition to effects on sexual differentiation [32][34]. Therefore, several HPLC-FLD methods were developed and used for quantification of EDC's.

A sensitive HPLC method was developed for quantification of BPA and eight compounds of alkylphenol [35]. The alkylphenols assessed were; 4-*sec*-butylphenol, 2-*tert*-butylphenol, 3-*tert*-butylphenol, 4-*tert*-butylphenol, 4-n-pentylphenol, 4-*tert*-pentylphenol, 4-n-hexylphenol, and 4-n-heptylphenol. Derivatization was done using 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) at 40 °C for 60 min. A C_{18} stationary phase was used for separation. The reported detection limits were in the range of 0.1–10.0 pg/mL. The method was applied to determination of bisphenol A in mother and infant rat serum samples.

The fluorescent reagent 4-(4,5-diphenyl-1*H*-imidazol-2-yl) benzoyl chloride (DIB-Cl) (**Figure 13**) was used by Sun et al. to detect EDC's. To the evaporated sample residue, DIB-Cl suspension in acetonitrile and triethylamine in acetonitrile were added and reacted at room temperature [33].

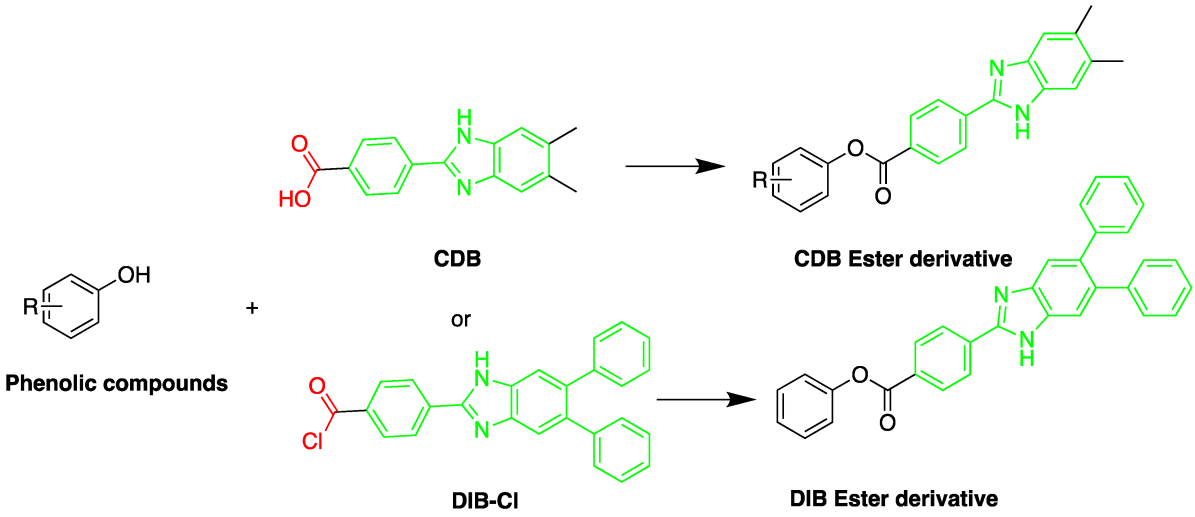

Figure 13. Derivatization of alkyl phenols by 2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole (CDB) or 4-(4,5-diphenyl-1*H*-imidazol-2-yl) benzoyl chloride (DIB-Cl).

The study determined BPA in rat brain samples using HPLC-FLD coupled with a microdialysis [33]. A microdialysis probe was inserted into the hypothalamus of rat brains and artificial cerebrospinal fluid was used for perfusion. After the administration of a single intravenous or oral dose of BPA, concentrations were monitored in brain and

plasma for 8 h. The obtained data proved that BPA could penetrate the blood brain barrier. The LOD of BPA was 0.3 ppb in 60 µL brain microdialysate at (S/N = 3).

Kuroda et al. used a similar method for determination of BPA in human blood serum and ascitic fluid samples. Samples were extracted by LLE using chloroform [32]. The LOD of BPA for both samples was 0.04 ppb at (S/N = 3). Human breast milk was also used to investigate the presence of BPA employing DIB-Cl as a fluorescent derivatizing agent [28]. Two steps of LLE were applied, and two C_{18} columns were used to separate DIB-BPA from the endogenous material in breast milk. The detection limit in 23 samples of healthy lactating women was 0.11 ng/mL at (S/N = 3).

The chromatographic conditions, sample preparation and detection limits of the HPLC-FLD methods that were employed in the steroid studies are summarized and presented in **Table 1**.

Table 1. HPLC-FLD method conditions used for steroid detection.

Detected Steroids	Sample Type	Column Chemistry	Mobile Phase (*v*/*v*)	Derivatization Agent	Excitation (λex) and Emission Wavelengths (λem) (nm)	Extraction Method	LOD	References
PL and PN	Human plasma	C_{18}	MeOH:ACN:1.0 M ammonium acetate (38:25:45)	DMB	350, 390	LLE	3 ng/mL	Yamaguchi et al. 1991 [18]
F and E	Biological samples	Keystone Hypersil	H_2O:MeOH:ACN (50:33.3:16.7)	9-AN	305–395, 430–470	SPE	F: 50 pg E: 70 pg	Haegele et al. 1991 [36]
F	Human serum	C_{18}	1: 10 mM potassium biphthalate 2: ACN Tetrahydrofuran: 19 mM potassium biphthalate (40:6:54) Both adjusted to pH 1.85 with trifluoroacetic acid	Sulfuric acid-ethanol	365, 520	-	0.30 pg/dL	Nozaki et al. 1991 [5]
F	Human urine	C_{18}	Gradient of ACN: 36.4 mmol/L phosphate (45:55; pH 1.85 with trifluoroacetic acid)	Sulfuric acid-ethanol	365, 520	SPE	0.26 pg/dL	Nozaki et al. 1992 [6]
7α-Hydroxycholesterol	Dog plasma	Develosil Ph-5	Acetonitrile: Water (5:2)	1-AN	338, 411	LLE	4 pg	Saisho et al. 1998 [20]
Corticosteroids	Urine	Silica	2-Propanol–hexane	9-AN	370, 470	Enzyme hydrolysis,	NR	Neufeld et al. 1998 [11]

Detected Steroids	Sample Type	Column Chemistry	Mobile Phase (*v*/*v*)	Derivatization Agent	Excitation (λex) and Emission Wavelengths (λem) (nm)	Extraction Method	LOD	References
						extraction with 0.5 M NaOH		
F and E	Human plasma	C_{18}	ACN: 0.3 mM *ortho*-phosphoric acid (470:530)	9-AN	360, 460	SPE	3.0 ng/mL	Glowka et al. 2009 [12]
Corticosterone	Rat urine	CN	ACN: H_2O (24.5:75.5)	post-column reaction with sulfuric acid	460, 510	LLE	0.5 pmol	Sudo et al. 1990 [3]
F	Human hair	C_{18}	MeOH:H_2O 60:40	sulfuric acid	360, 480	LLE	1 pg/mg	Gao et al. 2010 [2]
EE2, E2, and BPA	Human urine and aqueous samples	C_{18}	ACN:MeOH:H_2O (30:15:55)	-	280, 310	FPSE	E2: 20 pg/mL EE2: 36 pg/mL BPA: 42 pg/mL	Kumar et al. 2014 [23]
BPA, NP, E2, EE2, and E3	Human urine	C_{18}	Gradient elution of ACN and H_2O	*p*-nitrobenzoyl chloride	E2, E3: 282, 315 BPA, NP, EE2: 228, 316	SPE	BPA and E2: 2.7 µg/L NP: 2.9 µg/L E2 and EE2: 4.6 µg/L E3: 8.3 µg/L	Mao et al. 2004 [22]
E, testosterone, methyltestosterone, bolasterone, testosterone acetate, progesterone	Urine	C_{18}	0.01 M $Tb(NO_3)_3$, 0.1 M sodium dodecyl sulfate (SDS), and 20% acetonitrile	-	245, 547	SPE	Down to 100 pg/mL	Amin et al. 1993 [26]
BPA and 8 alkylphenols (4-*sec*-Butylphenol, 2-*tert*-Butylphenol, 3-*tert*-butylphenol, 4-*tert*-butylphenol, 4-*n*-Pentylphenol, 4-*tert*-pentylphenol, 4-*n*-hexylphenol, and 4-*n*-heptylphenol)	Rat plasma and blood	C_{18}	MeOH:Water (10:90)	2-(4-carboxyphenyl)-5,6-dimethylbenzimidazole	Derivatized: 336, 440 Native: 275, 315	LLE	BPA: 0.1 pg/mL Alkylphenol: 0.7–10 pg/mL	Katayama et al. 2001 [35]
progesterone and 17-hydroxyprogesterone dehydroepiandrosterone, androstenedione, testosterone and 17-methyltestosterone	Serum from pregnant and non-pregnant women	Wakosil 5C4	Acetonitrile:Water (7:3)	BODIPY FL hydrazide	495, 516	LLE	550–3700 fmol per 10 µL	Katayama et al. 1998 [37]

Detected Steroids	Sample Type	Column Chemistry	Mobile Phase (*v/v*)	Derivatization Agent	Excitation (λex) and Emission Wavelengths (λem) (nm)	Extraction Method	LOD	References
aldosterone, corticosterone, F, E, dexamethasone, fluocinolone acetonide, triamcinolone and triamcinolone acetonide	Human plasma	C_{18}	Water:MeOH (25:75) containing 5 mmol/L tetramethylammonium hydrogen sulphate	CDB	334, 418	LLE	0.06–0.3 pg per 100 µL	Katayama et al. 1992 [9]
BPA	Breast Milk	C_{18}	1: ACN:H_2O:MeOH (72:13:15) 2: ACN:0.1 M acetate buffer (pH 5.5):MeOH (55:12:33)	DIB-Cl	350, 475	SPE then LLE	0.11 ng/mL	Sun et al. 2004 [28]
BPA	Human blood serum and ascitic fluid samples	C_{18}	1: ACN: H_2O:MeOH (72:13:15) 2: ACN:0.1 M Acetate buffer (pH 5.5):MeOH (55:12:33)	DIB-Cl	350, 475	LLE	0.04 ppb	Kuroda et al. 2003 [32]
BPA	Rat brain rat plasma	C_{18}	1: ACN: H_2O:MeOH:Tetrahydrofuran (55:10:35:2.5) 2: ACN:0.1 M Acetate buffer (pH 3.0):MeOH (35:10:55)	DIB-Cl	350, 475	LLE	0.3 ppb in 60 µL rat brain 4.6 ppb in 50 µL rat plasma	Sun et al. 2002 [33]
F, E, PL, PN, 6β-OHF, 6β-OHP and 6β-OHE	Human plasma and urine	Cosmosil 5SL	diethylene dioxide: ethyl acetate:chloroform:*n*-hexane:pyridine (500:100:100:1400:21)	9-AN	360, 460	LLE	F, E, PL and PN: 0.1 ng/mL 6β-OHF and 6β-OHP: 0.5 ng/mL	Shibata et al. 1997 [13]
Corticosterone	Rat serum	C_{18}	60% MeOH: 40% 5 mM triethylamine, pH 3.3	-	375, 485	LLE	0.1 ng	Mason et al. 1992 [7]
18-Oxygenated corticosteroids, 18-hydroxycortisol, 18-hydroxycortisone and 18-oxocortisol	Human urine	µBondasphere phenyl	A: 10 mM ammonium acetate: MeOH (50:50) B: ACN	1-AN	370, 470	LLE and SPE	0.1 pmol	Kurosawa et al. 1995 [14]
Cholesterol and sitosterol	Saliva and urine biosamples, Cow milk, and Soybean milk	C8	MeOH:isopropanol:H_2O (90:5:5)	naproxen acyl chloride	231, 352	LLE	25 nM per 10 µL injected volume	Lin et al. 2007 [19]

Detected Steroids	Sample Type	Column Chemistry	Mobile Phase (*v*/*v*)	Derivatization Agent	Excitation (λex) and Emission Wavelengths (λem) (nm)	Extraction Method	LOD	References
Pregnenolone	Rat brain	C_{18}	MeOH:H_2O (9:1)	1-AN	370, 470	SPE	NR	Shimada et al. 1996 [38]
C21 steroids; corticoids	Steroid standards	C_{18}	MeOH:H_2O:cyclodextrin	1-AN	360, 460	NR	NR	Shimada et al. 1991 [39]
Triamcinolone	Human plasma	C_{18}	ACN and 0.3 mM ortho-phosphoric acid	9-AN	360, 460	SPE	1 ng/mL	Glowka et al. 2006 [40]
Butane acid-(5-androsten-17-one-3beta-ol)-diester (A1998)	Rat plasma	C_{18}	25 mM acetate buffer (pH 3.7):ACN Alfaxalone: (45:55) Pregnanolone: (40:60)	Dansyl hydrazine	332, 516	LLE	10 ng/mL	Visser et al. 2000 [41]
Alfaxalone and pregnanolone	Rat plasma	C_{18}	Gradient mixture of ACN and H_2O	Dansyl Hydrazine	350, 520	-	0.025 µg/mL	Peng et al. 2007 [42]
EED	Oral contraceptive tablets	STAR RP-18e	ACN:H_2O (47:53)	-	EED: 285, 310	-	EED: 0.0538 µg/ml	Sarafinovska et al. 2006 [43]
EED and drospirenone	Oral contraceptive tablets	STAR RP-18e RP	ACN:H_2O (47:53)	-	285, 310	-	EED: 0.00065 µg/mL DROSP: 0.0774 µg/mL	Sarafinovska et al. 2009 [44]
EED	Coated tablets	LiChroCART 100RP	ACN:H_2O (50:50)	-	280, 310	-	EED: 0.02 µg/mL	Silva et al. 2013 [45]
Sodium E1 sulphate, sodium equilin sulphate, E1 and equilin	Raw materials and Pharmaceuticals	5 ODS_2	TEA phosphate buffer (pH 4.0; 0.05 M):ACN 1—(70:30, *v*:*v*) 2—for unconjugated estrogens: (66:34)	Postcolumn on line photochemical derivatization	280, 410 or 312	-	0.01–1.38 pmol	Gatti et al. 1998 [46]
E1, 17β-Estradiol, E3, BPA, NP, OP	Fish, chicken, aquaculture pond water sample	C_{18}	Gradient program of 1: H_2O: 5% ACN 2: H_2O: ACN	BCEC-Cl	279, 380	DLLME	0.02–0.07 µg/L	Wu et al. 2015 [47]
E1, E2, and E3	Cow and river Buffalo	C_{18}	1: ACN:H_2O:Formic Acid (40:60:0.4)	-	280, 310	LLE and SPE	5–10 ng/kg	Shahbazi et al. 2016 [48]

Abbreviation: solid phase extraction (SPE), liquid–liquid extraction (LLE), dispersive-liquid–liquid extraction (DLLME), ultrasonic-assisted dispersive liquid–liquid microextraction (UA-DLLME), vortex-assisted dispersive liquid–liquid microextraction method based on floating organic acid droplet (VA-DLLME-FOA), ionic liquid foam floatation coupled with an ionic liquid-based homogeneous liquid–liquid microextraction (IF-IHLME), magnetic solid phase extraction (MSPE), fabric phase sorptive extraction (FPSE), molecularly imprinted solid-phase extraction (MISPE), hollow fiber liquid-phase microextraction (HF-LPME), 4-octylphenol (OP), 4-tert-octylphenol (4-t-OP), 4-

Detected Steroids	Sample Type	Column Chemistry	Mobile Phase (*v/v*)	Derivatization Agent	Excitation (λex) and Emission Wavelengths (λem) (nm)	Extraction Method	LOD	References
			2: ACN:H_2O:Formic Acid (90:10:0.4)					
α- and β-Trenbolone	Bovine muscle and liver	C_{18}	MeOH:H_2O (60:40)	-	364, 460	LLE then SPE	bovine muscle: 0.2 ng/g liver: 1.0 ng/g	Yoshioka et al. 2000 [49]
NP, 4-nonylphenol mono-(NP1EO), diethoxylates (NP2EO), BPA, TBP, and OP	Fish and shellfish	Inertsil PH	Gradient program of A: H_2O B: MeOH	-	275, 300	LLE	NP NP1EO and NP2EO : 2 ng/g BPA, BP and OP: 1 ng/g	Tsuda et al. 2000 [24]
Nonylphenol and its ethoxylates	Fish tissue	Hypersil APS	Hexane:ethanol (98:2)	-	230, 300	Pressurized fluid extraction	4–15 ng/mL	Datta et al. 2002 [50]
E2 and EE2	Poultry litter	C_{18}	Gradient program of A: H_2O B: ACN	-	280, 312	LLE	E2: 4.0 µg/kg EE2: 2.6 µg/kg	Lu et al. 2014 [51]
E2 and EE2	Waste Water	C_{18}	Gradient program of A: H_2O B: ACN	-	282, 306	SPE	2.5 ng/L	Liz et al. 2017 [52]
E3, E2, EE2, HEX, mestranol	Water, sediment	Poroshell 120 EC	H_2O:ACN 50:50	-	275, 310	SPE	Water: 6–24 ng/L Sediment: 0.1–0.9 ng/g	Perez et al. 2015 [53]
E2, 17α-EE2, and E1	Water	C_{18}	Gradient program of A: ACN B: H_2O acidified at pH 3.6 with glacial acetic acid	-	230, 302	SPE	10 to 1100 ng/L	Patrolecco et al. 2013 [54]
OP, NP, BPA, diethylstilbestrol, E1, EE2, E2, and E3	Wastewater samples	C_{18}	Gradient program of A: 5% ACN B: ACN	EASC	262, 430	SPE	0.3–0.7 ng/L	Zhang et al. 2012 [55]
E2 and EE2	Tap, surface and waste water	C_{18}	Water:acetonitrile mixture (50:50)	-	280, 310	DLLME	E2: 2.0 ng/L EE2: 6.5 ng/L	Lima et al. 2013 [56]
BPA, 17β-estradiol, and 17α-ethynyl estradiol	Drinking water	LiChro-sorbs RP18	10 mM H_3PO_4:55% MeOH (45:55)	-	280, 310	PAC	BPA: 201 ng/L E2: 313 ng/L EE2: 284.5 ng/L	Yoon et al. 2003 [57]
17α- and 17β-Trenbolone	River water	C_{18}	Gradient program of MeOH:H_2O	-	359, 458	SPE	4 ng/L	Durhan et al.

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Detected Steroids	Sample Type	Column Chemistry	Mobile Phase (*v/v*)	Derivatization Agent	Excitation (λex) and Emission Wavelengths (λem) (nm)	Extraction Method	LOD	References
								2005 [58]
NP, OP, NP polyethoxylates	municipal wastewater treatment plants	C_{18}	Gradient program of H_2O:ACN	-	229, 310	LLE	OP: 2 ng/L NP: 11 ng/L NPE: 52 ng/L	Snyder et al. 1999 [59]
E2, E3, BPA, and 17β-ethinylestradiol	environmental waters	C_{18}	ACN:0.02 mol/L phosphate solution (45:55)	-	227, 315	Synthesized in-tube SPME device	0.006–0.10 ng/mL	Wen et al. 2006 [60]
E2, E3, EE2, 3-methyl ether EE2, NP, OP, POE(1-2) nonyl phenol and BPA	River water	C_{18}	Gradient program of Milli-Q H_2O and ACN	-	230, 290	on-line SPE	20–50 ng/L	Ying et al. 2002 [61]
Endocrine disruptors; BPA and EE2	Environmental water samples	C_{18}	MeOH:0.025 mol/L Na_2HPO_4 buffer (70:30)	-	220, 315	A poly(acrylamide-vinylpyridine) monolithic capillary column	BPA: 0.064 ng/mL 17α-ethinylestradiol: 0.12 ng/mL	Fan et al. 2005 [62]
Brassinosteroids	Plant: (*Vicia faba* L.)	C_{18}	ACN: H_2O (90:10)	9-Phenanthreneboronic acid	305, 375	LLE	50 pg	Gamoh et al. 1989 [63]
Brassinolide	Arabidopsis thaliana, Daucus carota and Brassica campestris L. leaves' samples	C_{18}	Gradient program of A: H_2O and ACN B: ACN and 0.1% Formic Acid	-	305, 375	UA-DLLME	8.0 ng/L	Lv et al. 2014 [64]
Brassinolide and castasterone	Pollen of orange (Citrus sinensis Osbeck)	C_{18}	ACN:H_2O (80:20)	Dansylaminophenylboronic acid	345, 515	LLE	NR	Motegi et al. 1994 [65]
Norgestrel, norethindrone, EE2, gestodene, and norethisterone acetate	Meat samples	Hypersil GOLD	Gradient program of (A) H_2O containing 5% ACN (B)ACN	Fmoc-Cl	250, 395	MSPE	1.4×10^3–8.7×10^3	Qianyu Li et al. 2018 [66]
F and E	Human urine samples	C_{18}	ACN:0.3 mM orthophosphoric acid (470: 530)	9-AN	360, 460	LLE	LLOQ: F: 27.6 nmol/L E: 27.7 nmol/L	Kosicka et al. 2018 [67]
FFA	Edible oils and foodstuff	C_8	Gradient system: A: H_2O	BCETS	279, 380	Supercritical CO_2 and	0.22–1.06 ng/mL	Li et al. 2011 [68]

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Detected Steroids	Sample Type	Column Chemistry	Mobile Phase (*v/v*)	Derivatization Agent	Excitation (λex) and Emission Wavelengths (λem) (nm)	Extraction Method	LOD	References
			B: ACN/DMF (1:1) C: ACN (100%)			organic solvent extraction		
OP, NP, TBP, BPA, E1, E2, E3	Milk samples	C_{18}	Gradient program of A:5% ACN in H_2O B: ACN	BQEIC	302, 401	LLE	10.5–13.8 ng/L	Liu et al. 2018 [69]
E3, 2-OHE_2, 17β-E2, 17α-E2, EE_2, HEX	Dairy products	C_{18}	Gradient Flow A: 1 mM formic acid in ACN B: 1 mM formic acid at pH 3.50	-	280, 310/320	HF-LPME	0.23–14.8 µg/kg	Bárbara Socas-Rodríguez et al. 2014 [70]
Zearalenone	Edible oil	C_{18}	Gradient program of A: H_2O, B: ACN		274, 456	SPE	10 µg/kg	Drzymala et al. 2015 [71]
EE2, E1, E2, E3, and progesterone	Drinking water and wastewater samples	C_{18}	Gradient program of A: H_2O/CH_3CN 90/10 v/v B: CH_3CN		200, 315	SPE	Drinking water: 1–3.8 ng/L Sewage water: 3.8–7.5 ng/L	Kozłowska-Tylingo et al. 2015 [72]
E3, E2, E1	Human urine	C_{18}	Gradient program of A: H_2O, B: ACN		280, 310	VA-DLLME-FOA	E3: 0.01 ng/mL β-E2: 0.01 ng/mL E1: 0.06 ng/mL	Wang et al. 2015 [25]
17-α-E2, 17-β-E2 benzoate and quinestrol	Environmental water samples	Zorbax Eclipse SB-C_{18}	Gradient program of A: ACN, B: H_2O		265, 311	IF-IHLME	17-α-Estradiol: 0.04 ng/mL E2 and Quinestrol: 0.05 ng/mL	Zhang et al. 2017 [73]
E2 and EE2	Tap water samples	Pursuit 5 C_{18} column	ACN:H_2O (50:50), with 200 µL of H_3PO_4		230, 306	Nanoparticles of graphene oxide/ γ-Fe_2O_3 as a sorbent for SPE	E2: 2.7 ng/L EE2: 0.8 ng/L	Fernanda Nunes Ferreira et al. 2020 [74]
17-E2 and E3	Water samples	C_{18}	H_2O:MeOH:ACN (50:30:20)		280, 310	ultrasonication assisted DLLME	DLLME-HPLC/FLD: 7.16–69.22 ng/L	Zhang et al. 2020 [75]
E2, 1,3,5(10)-Estratriene-3,17β-diol	Fish and prawn tissue samples	ODS C_{18}	H_2O:MeOH (30:70)		280,310	MISPE	0.023 mg/L	Jiang et al. 2009 [76]
BPA, EE2, 4-t-OP, 4-OP, and 4-NP	River water	C_{18}	Gradient program of A: ACN B: H_2O		277, 307	Disposable pipette extraction (DPX)	BPA, EE2, 4-OP and 4-NP: 0.30 µg/L	Gabriela Corazza et al. 2017 [77]

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Detected Steroids	Sample Type	Column Chemistry	Mobile Phase (*v/v*)	Derivatization Agent	Excitation (λex) and Emission Wavelengths (λem) (nm)	Extraction Method	LOD	References
							4-t-OP: 0.60 µg/L	
E1 and EE2	Digested sludge	C_{18}-PFP	A: H_2O B: ACN E1: (50:50) EE2 (55:45)		280, 310	ultrasonic liquid extraction	E1: 0.305 µg/g EE2: 0.052 µg/g	Vitória L. Louros et al. 2019 [78]
E2	Milk sample	XDB-C_{18}	MeOH:H_2O (70:30)		280, 310	SPE	0.7 ng/mL	Yanan Yuan et al. 2019 [79]
Nine BPs	milk samples	C_{18}	Gradient program of 0.1% formic acid: ACN		230, 305	ultrasonically with acetonitrile and cleaned using the QuEChERS technique.	1.0–3.1 µg/kg	Xiong et al. 2017 [80]
EE	river water samples	5C_{18} MS-II	ACN: 5.0 mM Tris-HNO_3 buffer, pH 7.4 (60:40)		310, 400	C_{18} SPE disk	7.4 ng/L	Ali et al. 2020 [81]
E3, 17β-estradiol glucuronide, 17β-E2, 17α-E2, 17β-E2-3-methyl ether	wastewater	UPLC C_{18}	Gradient program of water with 0.1% of ammonia: ACN		280, 310	Molecularly Imprinted SPE	1.4 to 2.5 ng/mL	Rayco Guedes-Alonso et al. 2015 [82]

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Determination of ethinylestradiol and drospirenone in oral contraceptives with HPLC method with UV and fluorescence detection. Arch. Public Health 2009, 1, 67.

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