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Design and validation of a novel immunological test for enterolactone

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ABSTRACT

Enterolactone (ENL) is produced by the gut microflora from lignans found in edible plants. ENL is estrogenic with no effect on the E-screen test and is a natural Selected Estrogen Receptor Modulator (SERM) with health interests that have to be checked in clinical studies with bioavailability assessment. Two haptens of ENL were synthesized, with a spacer arm at the C5 position having either 2 or 4 carbon atoms (ENLΔ2 and ENLΔ4, respectively). Hapten coupling to bovine serum albumin (BSA) was characterized by MALDI mass spectrometry. Polyclonal antibodies were obtained against the BSA conjugates. Additional conjugates were generated by coupling to swine thyroglobulin (Thyr). Homologous and heterologous competitive ELISAs were developed with Thyr or BSA conjugates as coating. The best assays were validated on biological samples from mice. Both antibodies exhibited the same IC_{50} at 1.5 ng mL^{-1} with a detection limit below 0.5 ng mL^{-1}. Most cross-reactions with structurally related lignans were lower than 0.03%. This new assay type is faster, more specific and more reliable than existing ones.

1. Introduction

Lignans are plant polyphenolic compounds. Some of them, including secoisolariciresinol diglucoside, matairesinol, lariciresinol, pinoresinol, syringaresinol, arctigenin, and sesamin can be metabolized in the mammalian gut into estrogenic enterolignans such as enterodiol (END) and enterolactone (ENL, Figs. 1 and 3) [1,2]. The ability to convert precursors into ENL and END is highly variable among humans [3]: the conversion measured on urinary excretion varies from 3- to 285-fold for the same ingested amount of precursors. Nevertheless, it was shown for a same individual that the excretion rate is proportional to the ingested amount of precursor [1]. Amounts of lignans are found to decrease along a series including flaxseeds, sesame seeds, nuts, cereals and breads, legumes, fruits, vegetables, processed foods, alcoholic, and non-alcoholic beverages [4], but yet unidentified sources of ENL precursors probably exist.

ENL displays modest estrogenic properties in the uterotrophic test [5], but exhibits a particular interaction with the estradiol receptor α [6], known to be involved either in cell differentiation or cell proliferation [7]. At physiological doses, ENL does not prevent estradiol induced MCF-7 proliferation (E-Screen test), but has no effect on this proliferation when tested alone [6]. In addition, Power et al. [8] showed *in vivo* that ENL does not induce the proliferation of xenografts from human breast cancer cells in ovariectomized athymic nude mice at physiological concentrations. Several epidemiological studies reported an inverse relationship between plasma ENL and breast cancer risk [9,10] or breast cancer morbidity [11]. This suggests that ENL could be a compound of interest for menopausal women to counteract estrogen deficiency without any effect on breast cancer cells proliferation. Previous ENL assays were developed using GC [12], LC–MS–MS [13,14] and time resolved immunoassay (TR-FIA) [15]. Except for TR-FIA which leads to a technique with rather similar advantages, the physicochemical techniques are highly time-consuming and require sophisticated equipment and skilled staff, a situation that

Abbreviations: Bovine Serum Albumin, (BSA); Enzyme Linkage Immuno-Sorbent Assay, (ELISA); Enzymatic Immuno-Assay, (EIA); Enterolactone haptens with a spacer arm of 2 and 4 carbon respectively, (ENLΔ2 and ENLΔ4); High Resolution mass spectroscopy, (HR-MS); Optical Density, (OD); Phosphate Buffer Saline, (PBS); Selected Estrogen Receptor Modulator, (SERM); Swine Thyroglobuline, (Thyr); Time Resolved Immunoassay, (TR-FIA); Variation Coefficients, (VC)

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often prevents them to be used in bioavailability studies where high numbers of samples must be considered. An EIA is commercialized and used here as the validating method. In that case the primary antibodies are coated onto the plates and the competition is performed between a free antigen and another one coupled to acetylcholine esterase. The enzyme takes place in the revelation step converting the Ellman reagent into a chromogenic substance in a dose dependant manner.

To be able to assay ENL in human plasma and urine on large scale studies, we propose a simple, sensitive and reliable method based on polyclonal antibodies, used in homologous or heterologous competitive ELISAs. These assays were fully characterized and validated on mice plasma.

2. Material and methods

2.1. Reagents and chemicals

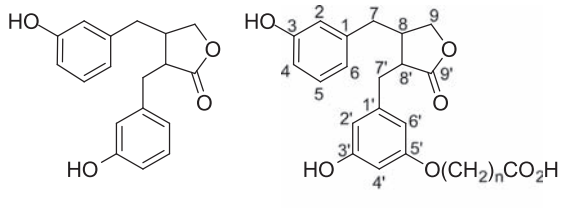
All chemicals were from Sigma Aldrich Chemical Co. (Saint Quentin Fallavier, France) and solvents were from Acros Organics (Noisy, France). All moisture-sensitive reactions were performed under argon atmosphere in oven-dried or flame-dried glassware. Biological reagents were purchased from Sigma-Aldrich except for the secondary antibody *i.e.* goat anti-rabbit IgG-horseradish peroxidase purchased from DAKO (Trappes, France) and β-D-glucuronoside glucuronosohydrolase-arylsulfate sulfohydrolase from *Helix pomatia* which was purchased from Roche Diagnostic (Mannheim, Germany). Lariciresinol, pinoresinol, isolariciresinol, hydroxymatairesinol used for specificity tests were from Arbonova (Turku, Finland). Secoisolariciresinol and matairesinol were from Fluka (Saint Quentin Fallavier, France).

2.2. NMR

^{1}H and ^{13}C NMR spectra were recorded with a Bruker AC-300 FT (^{1}H: 300 MHz, ^{13}C: 75 MHz) or with a Bruker A C 250 (^{1}H: 250 MHz, ^{13}C: 63 MHz). The spectra were calibrated using the frequency of the deuterated solvent (lock frequency). The chemical shifts (δ) and coupling constants (J) are respectively expressed in ppm and Hz, see Fig. 1 for atom numbering in NMR signal description.

2.3. Mass spectrometry

Electrospray (ESI) mass spectra were performed on a QStar Elite mass spectrometer (Applied Biosystems) equipped with an ESI source; spectra were recorded in the positive mode (CESAMO, Bordeaux, France). The electrospray needle was maintained at 5000 V and operated at room temperature. Samples were introduced by injection through a 20 μL sample loop into a 400 μL/min flow of methanol from the LC pump.



Enterolactone ENL 1 **ENLΔ2 : n=1 ENLΔ4 : n=3**

Fig. 1. Mammalian lignan, and the two related haptens synthesized in this study for antibody production.

Field desorption (FD) mass spectra were acquired with a TOF mass spectrometer (AccuTOF GCv, JEOL, CESAMO). Samples (1–2 μL) were deposited on a 13 μm emitter wire and desorbed with an emitter voltage of 10 kV.

Matrix Assisted Laser Desorption-Ionisation Mass Spectrometry (MALDI-MS) spectra of the hapten–BSA conjugates were recorded in the linear mode with a Bruker Reflex III mass spectrometer equipped with a UV laser (337 nm). Samples (10 μM aqueous solutions) were mixed (1/1 v/v) with matrix solutions (sinapinic acid, 10 mM in water/acetonitrile containing 0.1% trifluoroacetic acid) and 1 μL of the mixture was applied on a stainless steel target and left drying at room temperature. Spectra were acquired in the external calibration mode using BSA as a reference.

2.4. Synthesis of haptens and standards

The full description of the synthetic work is given in the Supplementary information. The synthesis steps of the *O*-benzylated γ-butyrolactone 5 and of the hapten ENLΔ4 are described in Fig. 2. Up to nine steps were necessary to obtain each hapten. The synthesis of standard compounds ENL and END is given in Fig. 3. All structures were characterized using ^{1}H and ^{13}C NMR as well as high resolution mass spectrometry (HR-MS).

2.5. Preparation and analysis of hapten–protein conjugates

2.5.1. Obtention of hapten–protein conjugates

The haptens (ENLΔ2 and ENLΔ4) were coupled to bovine serum albumin (BSA) for injection into rabbits and to swine Thyroglobulin (Thyr) for coating of microtiter plates. Briefly, water-soluble carbodiimide 1-ethyl-3-[3-(dimethylamino)propyl] carbodiimide hydrochloride (EDC · HCl, 5.3 mg, 27.5 μmol) and N-hydroxysuccinimide (NHS, 3.2 mg, 27.5 μmol) were added to a solution of ENL hapten (ENLΔ2 or ENLΔ4, 25 μmol) in dry DMF (2 mL) at 4 °C and stirred on an ice bath for 60 min. The resulting activated hapten solution in DMF was added dropwise to the protein solution (BSA or Thyr, 0.5 μmol) in 5 mL of a borate buffer (borate–boric buffer 0.2 M, pH 8.7). The mixture was stirred for 4 h at 4 °C, dialysed twice against phosphate buffer saline (PBS: 0.01 M, pH 7.4, 0.9% NaCl) and then against distilled water. The hapten–protein conjugates were lyophilized and stored at −20 °C.

2.5.2. Characterization of the conjugates by hapten density analysis

Matrix assisted laser desorption-ionisation mass spectrometry (MALDI-MS) was used to check for the efficiency of hapten–BSA conjugation. The hapten/carrier protein ratio of each conjugate $(MW_{conjugate} - MW_{BSA})/MW_{hapten}$ was estimated by measuring the molecular weight increase due to covalent hapten attachment to lysine ε-amino groups of BSA, using hapten molecular weights of 372 and 400 Da for ENLΔ2 and ENLΔ4 respectively.

2.6. Obtention of the polyclonal antibodies

Immunization of the rabbits was performed as described previously [16,17]. For each hapten–BSA conjugate, two rabbits received injections and only one serum was retained based on a cross-test (direct binding of the antibody onto the coated conjugate) checking for antibody titer. Titers of all antisera obtained against ENLΔ2 or ENLΔ4 were compared using the homologous coupled molecule (Thyr–ENLΔ2 and Thyr–ENLΔ4 respectively, the immobilized antigens exhibiting a homologous length of the spacer arm). OD were compared for a 1/10,000 2nd antibody dilution at 490 nm.

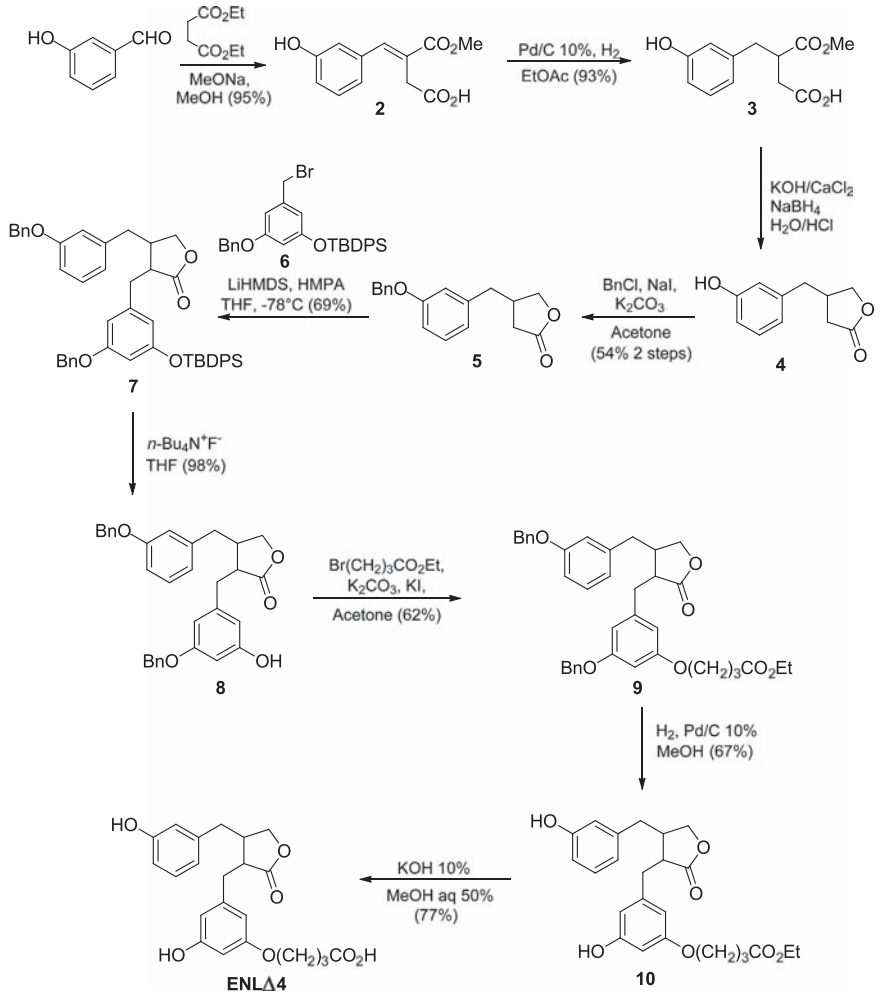


Fig. 2. Synthesis of hapten ENLΔ4.

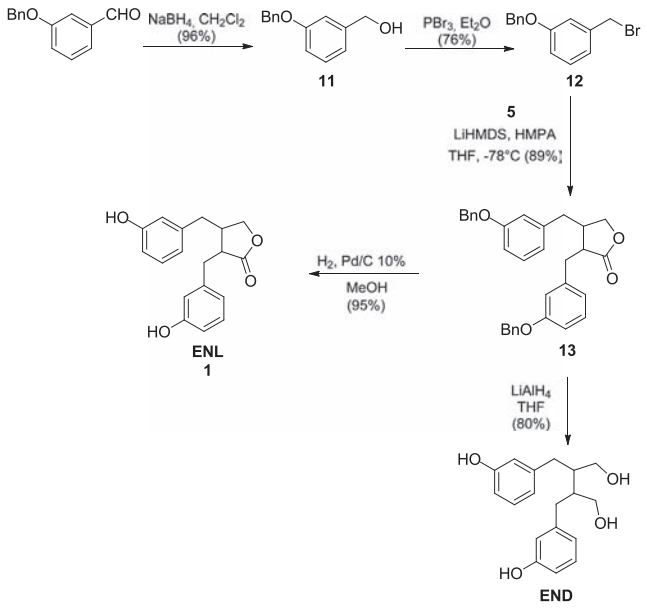


Fig. 3. Synthesis of standard compounds ENL and END.

2.7. Assay procedure

The assay procedure followed that of a homologous competitive ELISA, as described previously [16,17]. It is based on the competition of free ENL for the primary antibody in standards or in samples with coated haptens. Coating of the wells was performed overnight at 4 °C with the homologous hapten (with the corresponding spacer arm) coupled to Thyr or to BSA (*i.e.* Thyr–ENLΔ2 and BSA–ENLΔ2 or BSA–ENLΔ4 and Thyr–ENLΔ4) (200 μL per well). Optical densities (OD) of microtitration plates were read on a Dynex MRX II microtitration plate reader at 490 nm. The plates were NUNC Maxisorp® 96 wells microtitration plates. Blanks were the negative controls obtained in wells with no coating but with the regular adding of the primary antibody. The standard curves were expressed as log[ENL]=*f*((Bi-Blank)/(Bo-Blank)) × 100 where Bi is the OD of the sample or of the standard well, Bo is the positive control *i.e.* the ODmax obtained by allowing the antibody to react on the coated antigen without any added competitor. Blank was obtained without coating.

2.8. Validation of the assays

2.8.1. Origin of samples

All blood samples were collected under isofluran anesthesia by retro-orbital withdrawal and following recommendations from the French Ethical Comity for Animal Experiment by persons fully trained and aware of these ethical rules. Mice blood samples were collected between 9:00 am and 10:00 am on animals injected intraperitoneally 12 h before with 0, 20 or 40 mg kg^{-1} of pure ENL dissolved in corn oil. Plasma from 12 mice on each treatment were pooled by groups of 4. This was done to get enough material for a double analysis using the two assay techniques. The samples

were used for comparison of the present ELISA with a commercial EIA for ENL. The assay was done blindly. The specificity test based on standard-curve and plasma dilution curve parallelism was performed using a plasma sample collected at 10:00 am on a mouse fed a linseed extract Linugin® from Tournay Biotechnology (France) for 1 week. According to the manufacturer Linugin® contains 20% of SGD among other lignans. The mouse was a 9-weeks-old ovariectomized female. In this case and to collect more plasma the mouse was killed by cervical dislocation according to the ethical rules for animal experimentation. Blood was collected on ice in a heparinized tube after decapitation.

2.8.2. Hydrolysis of lignan glucuronides

ENL-glucuro and sulfo-conjugates in plasma were hydrolyzed using β-glucuronidase aryl-sulfatase from *H. pomatia* (Roche Diagnostic). The hydrolysis recovery ratio was monitored as described previously [18].

2.8.3. Lignan extraction from plasma

Free aglycone lignans were extracted using a liquid–liquid extraction with acidified ethyl acetate (1% HCl) as described previously [18]. The recovery ratio was monitored using glycosides compounds run in parallel. The extracts were dissolved in assay buffer and stored at −20 °C until assay. In addition, three external standards of extraction, *i.e.* control plasma containing ENL in known quantities, were run in parallel to the sample extraction to check extraction recoveries.

2.8.4. Cross-reactivity tests

The cross-reactivity of an antibody is defined as its ability to react with a molecule distinct from the one it was initially raised against. The cross-reactivities of the antibodies were tested using a competitive procedure. Following the previously optimized conditions, the antibodies were challenged with ENLΔ2 and ENLΔ4 coupled either to Thyr or to BSA as coating, and to serial dilutions of other compounds diluted in assay buffer as competitors. Eight concentrations of the various competitors were tested (from 10,000 ng mL^{-1} to 0.6 ng mL^{-1}) with a 4-fold decrease between each concentration. For these tests the antibodies and coating were at their working dilution. The secondary antibody dilution was 1/10,000 and the peroxidase revelation lasted 20 min at 37 °C.

2.8.5. Validation tests

The assays of ENL were undertaken on plasma samples from mice (see above). Data obtained using the new ELISA techniques on plasma samples were compared to values obtained using the EIA specific for ENL (Cayman, purchased from Bertin Pharma, France).

3. Results and discussion

3.1. Immunogen design

The hapten structures were designed as ENL analogs bearing an alkyl chain with a carboxylic function at the end, required for covalent coupling to carrier proteins. The linker position is crucial to preserve and favorably expose the discriminating functions of ENL for specific antibody recognition. A previous study on the design and synthesis of four ENL haptens demonstrated a significant difference in immunogenic activity in relation to the linker position on the lignan skeleton [19]. Linkers bearing a carboxylic function required for covalent coupling to carrier proteins were introduced at C3′-, C4′-, and C5′-positions (Fig. 1). Considering the chemical structures of plant lignans and ENL, constituted of two phenylpropanoid units with different functional groups on aromatic rings at C3-, C3′- and C4-, C4′- positions, it was obvious that placing the linker at these positions could affect the specificity of antibodies. However, it was also very important to maximize the exposure of the γ-butyrolactone moiety of ENL to avoid the recognition of END, the reduced form of ENL with the same aromatic units. Indeed, END is produced by the gut microflora and can be found in plasma, although at a lower concentration, together with ENL.

Therefore, it was decided to synthesize ENL haptens with the linker on the C5′-position, located as far as possible from the discriminating functions of plant and mammalian lignans. The length of carboxylic alkyl chain was also varied in order to study its influence on the specificity and sensitivity of the antibodies, as well as to develop heterologous immunoassays [16].

3.2. Synthesis of ENL haptens

As ENL is found in human organisms as a mixture of *trans* (−)-(*RR*)-ENL and (+)-(*SS*)-ENL [20], we were interested in the diastereoselective synthesis of ENL haptens. Two ENL haptens were synthesized through a convergent strategy using benzyl and silyl ether hydroxyl protecting groups (Fig. 1). The ENLΔ2 hapten, 5-carboxymethoxyenterolactone, was prepared using the method described by Mäkelä et al. [19]; the various steps of the protocol were optimized, resulting in yields higher than the described ones [19]. The total synthesis of the hapten ENLΔ4, 5-carboxypropoxyenterolactone, was carried out through a new strategy starting from commercial 3-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde to give two key intermediates: a functionalized benzyl bromide (compound 6 in Fig. 2) and a hydroxybenzylbutyrolactone (compound 5 in Fig. 2), respectively. The last one was obtained *via* Stobbe condensation, the phenol hydroxyl group was protected by benzylation just before the condensation of the butyrolactone intermediately with substituted benzyl bromide.

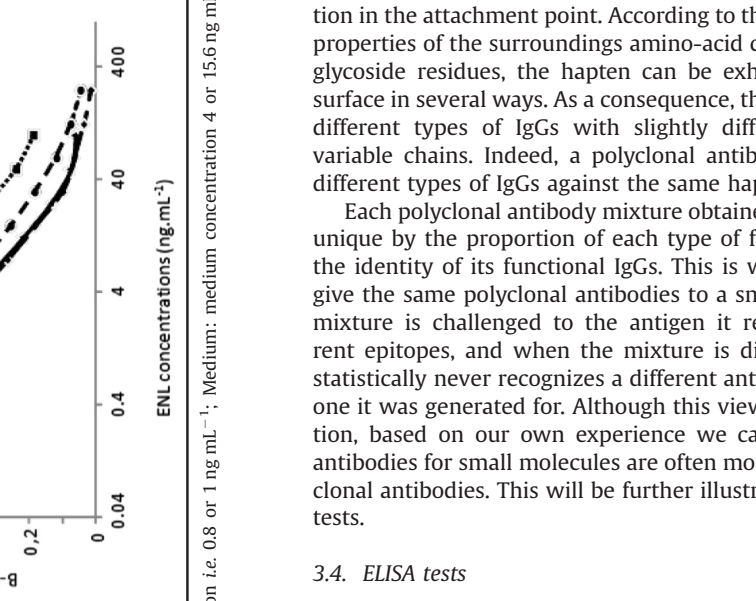
Haptens were then reacted with a carrier protein, *i.e.* BSA, to produce immunogenic constructions in order to obtain anti-ENL antibodies. Hapten/protein molecular ratios were determined by MALDI-MS. The hapten/protein ratios in both cases were 10.4 ± 0.5 and 13.4 ± 0.5 haptens per BSA molecule, for ENLΔ2 and ENLΔ4 respectively, a value considered to be adequate to obtain a good antibody quality [21].

3.3. Immunization tests

The titers of the antibodies were rather similar (Table 1). Anti-ENLΔ2 and Anti-ENLΔ4 were used at 1/20,000 and 1/15,000 dilution respectively, in homologous tests challenged to Thyr–hapten coating. Both antibodies were tested in ELISA challenged against BSA–haptens as coating in homologous or heterologous conditions (data not shown). The only combination which gave relevant results is the one presented in Table 1, *i.e.* Anti-BSA–ENLΔ4 on BSA–ENLΔ2 used as coating. Interestingly, when the BSA–hapten complexes were used as coating, with the antibodies harvested with BSA, the dilution was higher (1/300,000) preventing non-specific binding characterized by the blank values. In those conditions, with relevant dilutions, a valuable assay was developed. This means that the immunization protocol was suitable for the generation of specific antibodies to lignans, to be used in ELISA tests. As mentioned we chose to generate polyclonal antibodies on purpose. Indeed, previous works by our team mentioned the great specificity achieved with polyclonal antibodies to small polyphenol molecules [16,18]. It is known that the variable chain of an IgG recognizes a small decapeptide as epitope. Estrogenic polyphenols exhibit their properties because

Table 1
Characteristics of the assays.

Antibody		Anti-ENLΔ2		Anti-ENLΔ4		Standard curves obtained using the two antibodies raised against BSA–ENLΔ2 and BSA–ENLΔ4 and using either Thyr–ENLΔ2, Thyr–ENLΔ4 or BSA–ENLΔ2 as coating
Coating		Thyr–ENLΔ2	Thyr–ENLΔ4	Thyr–ENLΔ4	BSA–ENLΔ2	
Coating concentration (μg mL^{-1})		2	0.06	1	0.01	
Antibody dilution		1/20,000	1/80,000	1/15,000	1/300,000	
2nd Antibody dilution		1/10,000				
Standard curve limits (ng mL^{-1})		250–0.122				
IC_{50} (ng mL^{-1})		1.5	1.5	1.95	3.9	
Detection limit (ng mL^{-1})		0.488	0.488	0.244	0.488	
Variation coefficients intra-assay variation ($n=8$)	Low	9.929	17.362	8.47	13.28	
	Medium	4.880	6.217	4.13	14.07	
	High	5.902	7.593	8.72	14.96	
Variation coefficient inter-assay variation ($n=7$)	Low	4.493	7.618	13.90	14.99	
	Medium	8.598	8.316	10.25	15.60	
	High	4.671	7.704	14.82	16.92	



IC_{50}: Concentration of the analyte required for 50% inhibition of the antibody binding to the coating antigen; Low: low concentration *i.e.* 0.8 or 1 ng mL^{-1}; Medium: medium concentration 4 or 15.6 ng mL^{-1}; High: high concentration: 390 or 800 ng mL^{-1} diluted 1/100.

they present two hydroxyl groups at a distance of 10 Å like those present on the estradiol molecule [22]. Therefore, it is likely that, after ENL hapten coupling to a carrier protein, each ENL moiety is presented for antigen production in a particular chemical environment depending on amino acid sequence and possible glycosylation in the attachment point. According to the steric and electronic properties of the surroundings amino-acid chains with or without glycoside residues, the hapten can be exhibited on the protein surface in several ways. As a consequence, the hapten can generate different types of IgGs with slightly different complementary variable chains. Indeed, a polyclonal antibody mixture contains different types of IgGs against the same hapten.

Each polyclonal antibody mixture obtained on a given animal is unique by the proportion of each type of functional IgGs and by the identity of its functional IgGs. This is why two rabbits never give the same polyclonal antibodies to a small hapten. When the mixture is challenged to the antigen it recognizes it *via* different epitopes, and when the mixture is diluted appropriately it statistically never recognizes a different antigen, as it does for the one it was generated for. Although this view still needs confirmation, based on our own experience we can say that polyclonal antibodies for small molecules are often more specific than monoclonal antibodies. This will be further illustrated by the specificity tests.

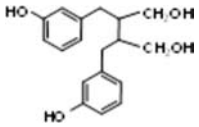
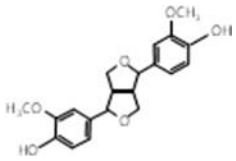
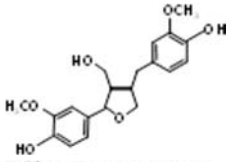
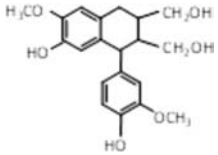
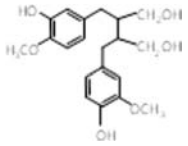
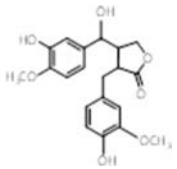
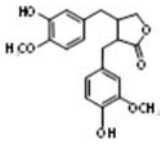
3.4. ELISA tests

The optimal conditions of the four ELISAs are shown in Table 1. Before ELISAs, the recovery ratios of the extraction procedure registered in parallel to all tested samples, were between 98 and 100%. For each conjugate of BSA–Δ2-ENL and BSA–Δ4-ENL, two antibodies were generated, each on two different rabbits. They were selected for their relative sensitivity in homologous conditions and the best were used thereafter. The standard sigmoid curves obtained for the best antibodies (anti-Δ2-ENL and anti-Δ4-ENL challenged with either BSA–haptens or Thyr–haptens as coating) are presented in Table 1. Both antibodies challenged with Thyr–hapten as coating gave the best sensitivities as measured by the IC_{50} values (1.5 ng mL^{-1} for both Anti-ENLΔ2 on Thyr–ENLΔ2 and 1.95 ng mL^{-1} for Anti-ENLΔ4 on Thyr–ENLΔ4). The detection limits obtained for 80% binding are 0.488 and 0.244 ng mL^{-1} for Anti-ENLΔ2 and Anti-ENLΔ4 respectively. In comparison, the assays developed by challenging the antibodies with their homologous BSA–hapten conjugates as coating were less sensitive. The one described in Table 1 was the best, with a sensitivity (IC_{50}) of 3.9 ng mL^{-1} when challenged in heterologous condition on BSA–ENLΔ2 as coating. This can be explained by considering that for high sensitivity the recognition of the free antigen has to be higher than that of the coated competitor. When BSA–haptens were used, because the polyclonal antibodies were obtained specifically against these complexes, they were able to react with the epitopes recognized during the immunization process. Therefore, the displacement of the IgGs from the coated antigen requires higher concentrations of free antigens. As the combinations of Anti-ENLΔ2 and Anti-ENLΔ4 with their homologous hapten coupled to BSA were less sensitive than the others, their specificity was not tested.

3.5. Specificity tests

These tests were performed with lignans, known to be END and ENL precursors [14] (results presented in Table 2). As these precursors can be encountered in human plasma [23], it is important for an accurate quantification to test the ELISA tools for their specificity toward them. Here, the tests were performed against END, pinoresinol, lariciresinol, isolariciresinol, secoisolariciresinol,

Table 2
Specificity tests.

Antibodies	Competitors	Anti-ENLΔ2		Anti-ENLΔ4	
Coating	Structures[a]	Thyr–ENLΔ2	Thyr–ENLΔ4	Thyr–ENLΔ4	BSA–ENLΔ2
Enterodiol		0.77%	0.5%	0.5%	0.5%
Pinoresinol		0.004%	0.003%	0.01%	0.01%
Lariciresinol		0.003%	0.003%	0.01%	0.01%
Isolariciresinol		0.003%	0.004%	0.01%	0.01%
Secoisolariciresinol		0.002%	0.002%	0.03%	0.01%
Hydroxymatairesinol		0.003%	0.004%	0.95%	1%
Matairesinol		0.03%	0.048%	0.01%	0.01%

[a] Structures from CAS database.

matairesinol, hydroxy-matairesinol. All tested antibodies exhibited a recognition rate for END below 0.5%, except for the combination Anti-ENLΔ2 on Thyr–ENLΔ2 for which the greatest cross reaction rate with ENL was observed, but was still far below 1% Hydroxymatairesinol also shows low cross reactions. This can be explained by the fact that the chemical structure of hydroxy matairesinol is very similar to ENL. These results allowed using our new ELISA tools with good accuracy on human or animal plasmas.

The present ELISAs exhibit good sensitivities, comparable to the HPLC technique associated with a coulometric detection developed previously, with a reported sensitivity of 1.55 nmol L^{-1} *i.e.* 0.46 ng mL^{-1} [23]. Using LC–MS–MS analysis, other authors found that concentrations of ENL in human plasma were not below 65 ± 16 nmol L^{-1} [24]. This is equivalent to 19.4 ng mL^{-1}. This value is above our IC_{50} values and will allow previous dilution steps. This indicates that our assays are relevant for human plasma analysis. Our team previously developed immunological tests based on polyclonal antibodies [16,17,25–27] and we were able to show that, using such tools, the achieved specificity was high and their sensitivity was at least as good as that obtained with HPLC–MS–MS methods if not better. These tools were then used to assay the corresponding polyphenols in animal and human plasmas in clinical studies [28,29].

3.6. Intra and inter-assay variation

Coefficients listed in Table 1 were determined for both antibodies using either high (390 or 800 ng/mL^{-1}), medium (4 or 15.6 ng mL^{-1}) or low (0.8 or 1 ng mL^{-1}) concentrated solutions. The highest concentrations required previous dilutions which uncertainty was included in the corresponding variation coefficients (VC). Inter-plate variations were calculated as VC of measurements carried out on 7 different microtitration plates. Intra-assay variations were calculated as the VC obtained on 8 values of the same sample. These variations were always higher using the BSA-conjugate than when using the Thyr conjugates. With the Thyr conjugates used in a homologous fashion, the intra-assay variations did not exceed 14% which is reasonable (Table 1). The highest VCs were recorded for the lowest value for which the % of

binding was close to 70%. When inter-assay variations were calculated, they were also higher with the BSA-conjugates than with the Thyr-conjugates. In their work, Prasain and co-workers [14] found inter-assay CVs for Enterolactone from 0.35 to 9.23%. These values are of the same order of magnitude as those obtained here; therefore, the present ELISAs could be considered to be as reliable as other physicochemical techniques [13,14]. Nevertheless, they are cheaper, easier to handle, and sometimes more accurate and sensitive since they do not require long sample preparation steps and thus avoid losses.

3.7. Validation of the assays

Beside the assessment of intra and inter-assay variation coefficient and the comparison of results obtained with the present ELISA and a previous EIA, a test was performed to check the specificity of one of the best assays obtained, with a plasma containing ENL and its parent metabolites. Serial dilutions of this plasma were prepared and run in parallel to a standard curve of ENL to check for parallelism. Indeed, if the antibody cross-reacted with compounds present in the plasma which are not ENL, the curves would significantly divert. Fig. 4 shows the result of this comparison. Indeed, the dilution test performed with Anti-ENLΔ4 on Thyr–ENLΔ4 as coating using serial dilutions of a mouse plasma beside an ENL standard curve (see Fig. 4) exhibits a nice parallelism with no significant differences between the two curves. This indicates that in the plasma collected from a mouse fed a complex lignan mixture (Linugin®), the antibody only recognized ENL and did not cross react significantly with the other lignans potentially present.

Another validation was undertaken on plasma of mice injected 12 h before with 0, 20 or 40 mg kg^{-1} of pure synthetic ENL. The results are presented in Table 3. It can be seen from this table that the treated mice exhibit large plasma concentrations of ENL which are highly different from the placebo treated mice. The small amount of ENL found in non-treated mice can be explained by the composition of their diet. Indeed the lab diet, namely Teklad Total 18% from Harlan (Gannat, France) contains ground wheat, ground corn, wheat middlings, dehulled soybean meal and corn gluten meal. These cereals and oleaginous are known to contain small amounts of lignans [29].

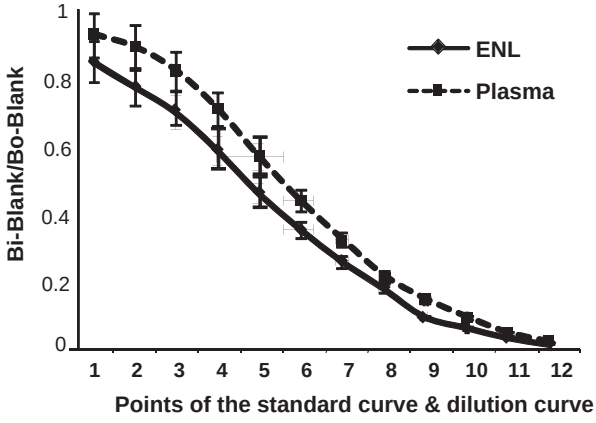


Fig. 4. Standard curve of Enterolactone and mice plasma dilution curve with Anti-ENLΔ4 and Thyr–ENLΔ4 as coating. Mice were fed linugin® enriched diet for one week and therefore it is expected that ENL and other precursors are present in mice plasma. This test is required to check for specificity of the antibody in *in vivo* conditions. Each value is presented with its SEM variation calculated on three values.

3.8. Comparison between EIA and ELISA

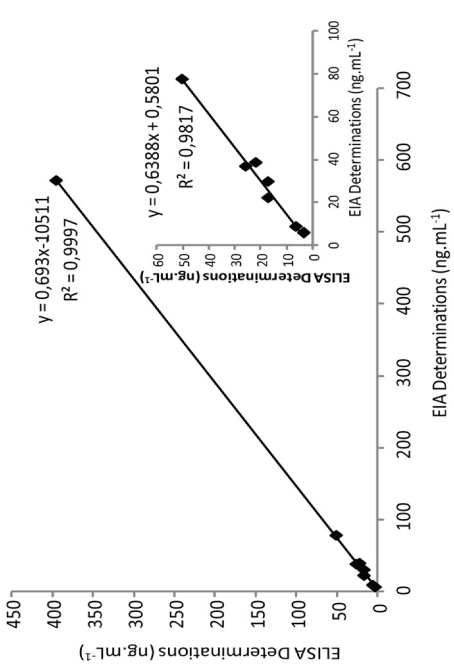
The EIA specificity for ENL was tested against END, chlorogenic acid, cortisol, daidzein, estradiol, estriol, estrone, matairesinol, phenyl caffeinate, resveratrol and rosmarinic acid. Except for END, all cross-reactions were below 0.01%. However, except for END and matairesinol, the chemical structures of the test compounds greatly differ from ENL. Therefore, the cross-reactions levels reported by the manufacturer were expectable. No data were given in the EIA kit about the specificity of the antibody toward the compounds we decided to check here, *i.e.* pinoresinol, lariciresinol, isolariciresinol, secoisolariciresinol, hydroxymatairesinol. The structures of these lignans are more closely related to ENL (see Table 2) and these compounds can possibly be present, together with ENL, in the plasma of consumers eating flaxseeds, fruits or vegetables containing ENL precursors. In addition, these compounds were presented as natural precursors of ENL, possibly found in human plasma [23,30]. A good correlation was obtained when comparing the new ELISA assay with EIA on the same plasma samples (Table 3): the correlation coefficient r^2 was 0.999 or 0.981 according to the values taken into account. However, EIA individual concentration values were always higher than those of ELISAs. The mean superiority of concentrations obtained by EIA was 50.42% when all samples were considered and it was 51.26% when the highest value was not taken into account. This can be explained since the results presented here with the EIA kit were obtained with the kit standard stock solutions. We also used the kit with our own standard solutions and although the values were a bit lower (data not shown) they still were higher than the one obtained with the present test. In that case the superiority of the EIA concentrations was 34.93% when all samples were considered and was 35.72% when the highest sample was omitted. In that case the r^2 was 0.9998 or 0.9795 if the highest concentration was or was not taken into account. This suggests that at least a part of the difference observed is due to differences in the standards used in both assays. Secondly, the discrepancy between EIA and ELISA values could be the result of a lower specificity of the kit antibody. Then, this antibody may cross-react systematically with other compounds from the plasma samples. Looking at the VCs for each value measured in quadruplicates, those obtained by ELISA tend to be lower (mean VCs for ELISA and EIA were 6.33 and 8.60, respectively). Both assays however, exhibited higher VCs for low concentration measurements. Finally, EIA uses acetylcholine esterase-bound to ENL as tracer. According to our own experience, such complexes have a reduced half-life when diluted in water. In our case, we observed that Thyr–hapten conjugates could be lyophilized and stored for at least 10 years at −20 °C, and that a stock solution prepared in distilled water could be stored in small aliquots at −20 °C for several years. The antibodies are used under high dilution allowing them to be used for several years since they are stable for at least 20 years. The ELISAs based on the competition of free ENL with Thyr–hapten complexes are more stable along time, as shown by inter-assay variations that are lower than those reported for EIA by the manufacturer.

In conclusion, the assays developed here for ENL were shown to be sensitive and reliable enough for assays in mice plasmas injected with pure ENL, or fed a diet containing ENL precursor. Their sensitivity and specificity are equivalent to that obtained using physicochemical techniques and seem to be sufficient for assay in human plasmas. The cross reaction tests showed that they are specific of ENL and are robust even if other precursors or parent compounds are present in the media to analyze. Therefore, they can also be used for currently higher concentrated samples such as urine. Polyclonal antibodies were shown to allow a good specificity of the assays, as demonstrated by a good parallelism between standard curve and plasma dilution curve obtained after feeding mice with a mixture of lignans. This specificity is important, since ENL exhibits peculiar estrogenic properties that are

Table 3
Comparison of the new and existing assay on ENL content of mice plasma samples.

Samples	EIA determination (ng mL^{-1})[a]	Variation coefficient (%)[b]	ELISA determination (ng mL^{-1})[a]	Variation coefficient (%)[b]	Correlation between the plasma ENL concentration values obtained either using EIA or using ELISA.
1	21.7	6.4	17.23	2.87	
2	77.7	2.9	50.57	2.74	
3	38.3	4.9	21.95	5.18	
4	29.4	5.1	17.34	6.18	
5	36.6	17.0	18.62	8.3	
6	572	1.36	395.7	1.22	
7	5.58	22.9	3.35	14.81	
8	8.34	15.5	7.31	12.21	
Mean VC		8.60		6.33	

[a] Measurements performed in duplicates.
[b] Variation coefficient obtained from 3 or 4 data.



valuable for health issues. The comparison between the ELISA and the commercial EIA is in favor of the new ELISA. The intra and inter-assay variations are lower with the new tool. It is also more stable, quicker and simpler.

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Appendix. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2013.10.034.

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