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Review

Application of coulometric electrode array detection to the analysis of isoflavonoids and lignans

José L. Peñalvo [a,*], Tarja Nurmi [b]

[a] *Institute for Preventive Medicine, Nutrition and Cancer, Folkhälsan Research Center, and Division of Clinical Chemistry, University of Helsinki, Biomedicum, P.O. Box 63, 00014 Helsinki, Finland*
[b] *Research Institute of Public Health, University of Kuopio, P.O. Box 1627, 70211 Kuopio, Finland*

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Abstract

A comprehensive review of the available methods for the analysis of isoflavonoids and lignans in biological matrices based on coulometric electrode array detection (CEAD) is presented. Different aspects such as sample extraction and purification procedures as well as instrumental settings and method validation are discussed. Comparisons with other available protocols using different detection techniques such as mass spectrometry or immunoassay are also reported to underline the versatility and reliability of the detector. Practical notes and tips for scientists working with CEAD are also provided.

Keywords: Coularray; Coulometric; Isoflavonoids; Lignans; Phytoestrogens

Contents

1. Introduction

The rapidly growing field of phytochemical research is constantly seeking for sensitive protocols to study the presence of bioactive compounds in foods as well as their absorption and metabolism in human subjects. Two groups of compounds, isoflavonoids and lignans, often classified under the traditional term of phytoestrogens, have lately been the object of intensive research. The isoflavones belongs to the family of flavonoids, a large group of plant-derived compounds that have been related to the induction of a physiological response in humans after consumption. Specifically, isoflavones and some of their metabolites

* Corresponding author. Tel.: +358 9 19125454; fax: +358 9 19125452.
E-mail address: Jose.penalvo@helsinki.fi (J.L. Peñalvo).

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are considered to act as selective estrogen-receptor modulators, interacting with estrogenic receptors in humans and therefore capable to exert protective effects against hormone related diseases, even though a number of other biological activities and therapeutic uses have been proposed (see [1] for review). Lignans are fibre-related polyphenols widely distributed in the diet, although predominantly present in whole grain products. After intake, dietary lignans are transformed by the gut microflora to the enterolignans enterodiol (End) and enterolactone (Enl). When these compounds were first discovered [2] it was soon found out that the urinary levels of Enl correlated with fibre intake [3,4], and because of their resemblance with endogenous 17β-estradiol they were also described as phytoestrogens. Although structural similarity exists, enterolignans do not seem to bind to the estrogen receptors whereas the other main groups of phytoestrogens, isoflavones and coumestans, have showed different degrees of affinity to the estrogen-receptor β [5]. To date many studies have been based on their possible estrogenic action, but it seems now clear that non estrogenic mechanisms should be also investigated to explain the reported relation between the consumption of lignan-rich food and lowered risk of chronic diseases such as cardiovascular disease [6,7] and breast cancer [8]. The research on isoflavones and lignans needs robust and sensitive analytical protocols to further confirm the epidemiological data. A number of methods supported by different instrumental techniques have been developed [9], and some of the major improvements in this field have been made by using coulometric electrode array detection.

Dietary isoflavones and lignans and their respective metabolites are aromatic compounds bearing polyphenolic structures that are electrochemically active and inclined to oxidation (Figs. 1 and 2). Each isoflavone or lignan possess a characteristic redox potential that, together with their chromatographic behaviour should permit a highly selective and sensitive quantification. The intrinsic nature of these compounds made of them especially suitable for electrochemical detection and particularly for a multichannel coulometric system and so, a number of methods have been reported. Our aim is to carefully review the so far reported methods for their performance in an attempt to unify criteria on sample pre-treatment for different matrices as well as instrument settings and protocol validation.

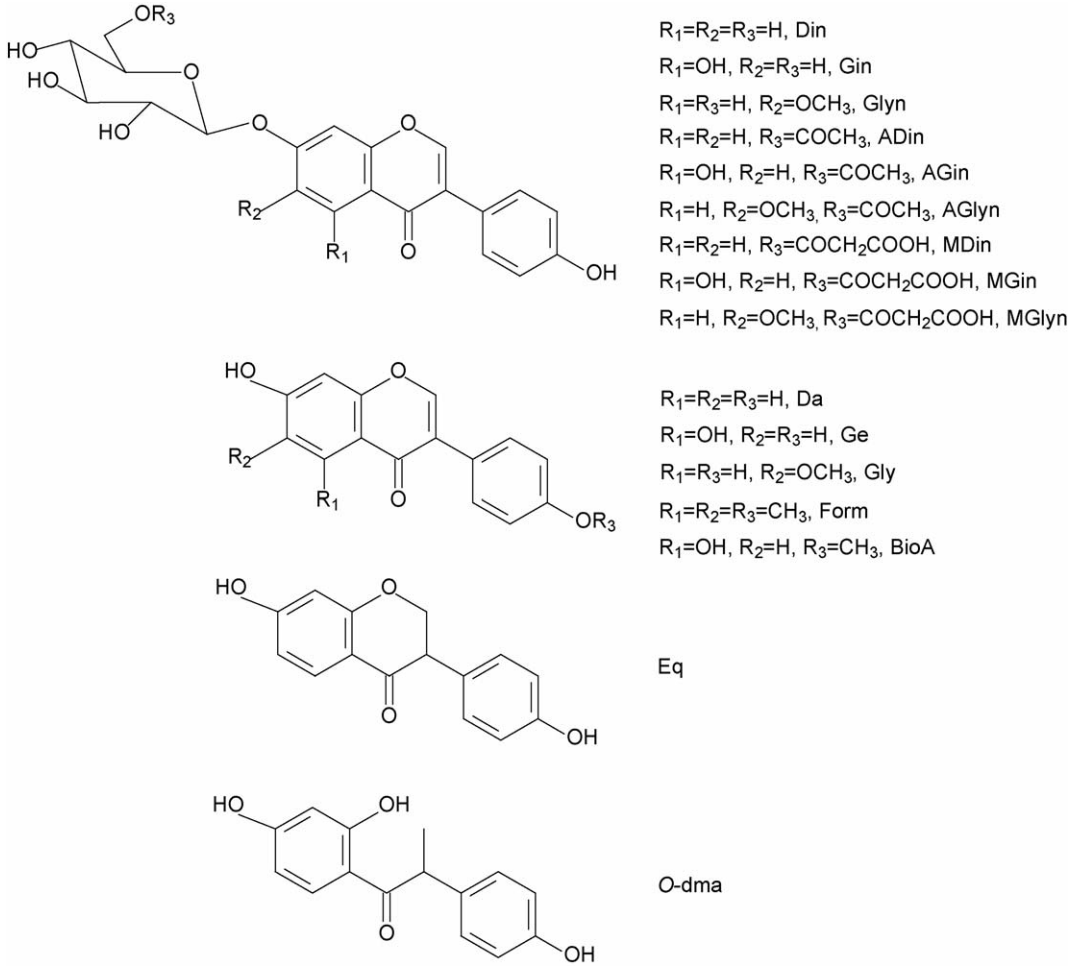

Fig. 1. Chemical structures of isoflavonoids. Din = daidzein-7-*O*-glucoside = daidzin, Gin = genistein-7-*O*-glucoside = genistin, Glyn = glycitein-7-*O*-glucoside = glycitin, Adin = acetyldaidzin, Agin = acetylgenistin, Aglyn = acetylglycitin, Mdin = malonyldaidzin, Mgin = malonylgenistin, Mglyn = malonylglycitin, Da = daidzein, Ge = genistein, Gly = glycitein, Form = formononetin, BioA = biochanin A, Eq = equol, and *O*-Dma = *O*-desmethylangolensin.

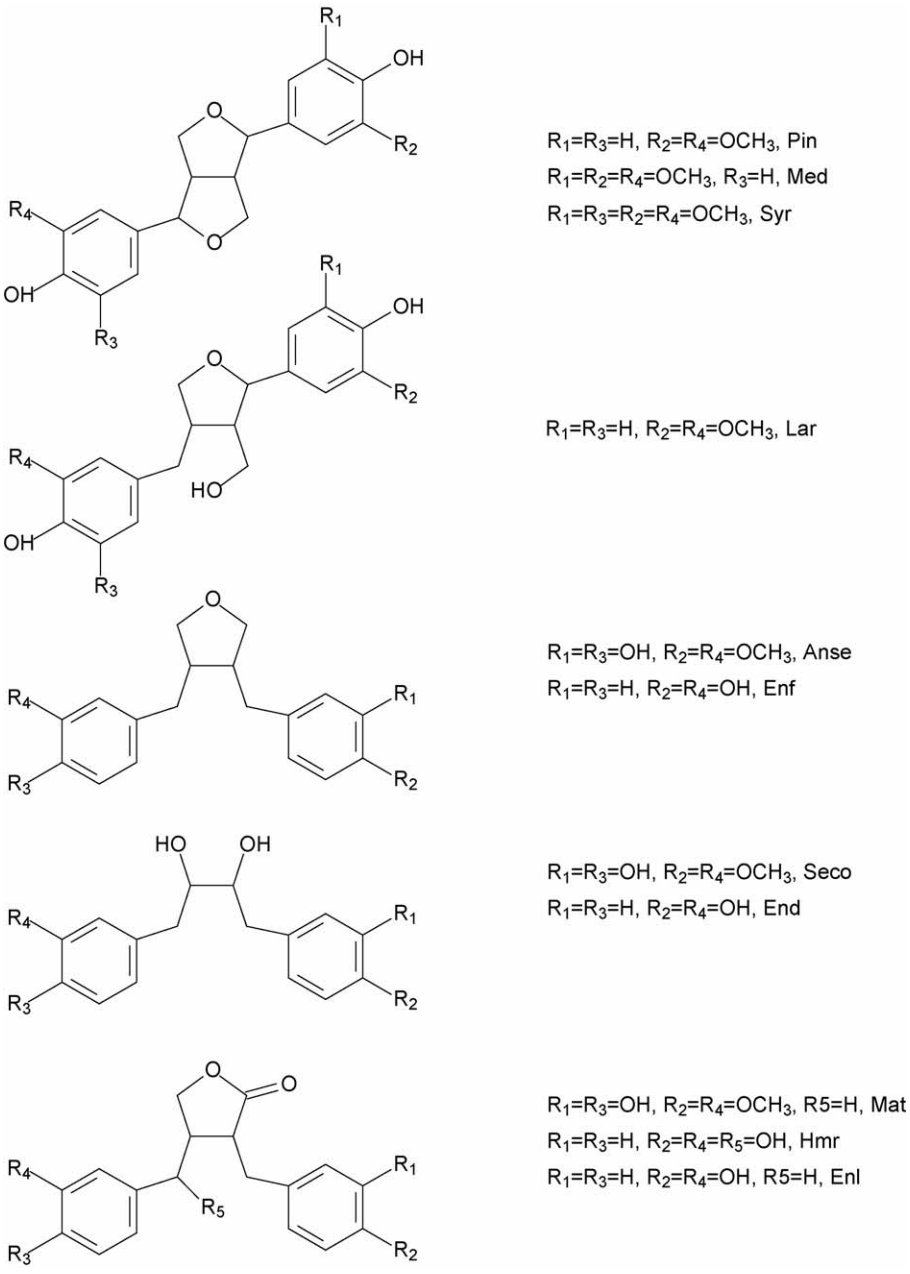

Fig. 2. Chemical structures of lignans. Pin = pinoresinol, Med = medioresinol, Syr = syringaresinol, Lar = lariciresinol, Anse = anhydrosecoisolariciresinol, Enf = enterofuran, Seco = secoisolariciresinol, End = enterodiol, Mat = matairesinol, Hmr = hydroxymatairesinol, and Enl = enterolactone.

2. Coulometric electrode array detection

2.1. Working principle

There are two types of electrochemical detectors that can be coupled to a liquid chromatograph: amperometric and coulometric detectors. Both are based in the same principle of oxidation/reduction of the analyte induced by an electrode held at a fixed potential. In an amperometric detector, mobile phase containing analytes flows over a planar electrode and only partial oxidation/reduction occurs, whereas in a coulometric detector the carrier passes through a porous electrode, resulting in an increased reactive surface and therefore almost complete oxidation/reduction that increases the sensitivity of the detector [10]. On the basis of the coulometric detection, the development of the coulometric electrode array detector (CEAD) constituted a major step towards improved selectivity and versatility. Analogue to the perhaps more commonly used diode array detector (DAD), the CEAD simply consists of a series of coulometric electrode pairs placed in series performing a multichannel (or array) detection. The system is based on analytical cells containing a platinum reference electrode that sets the electrochemical zero and four working electrodes that measure the redox reaction of interest. Each analytical cell therefore provides four channels (Fig. 3) and the systems available at present can bear up to four cells, that is up to 16 channels. The working range of the elec-

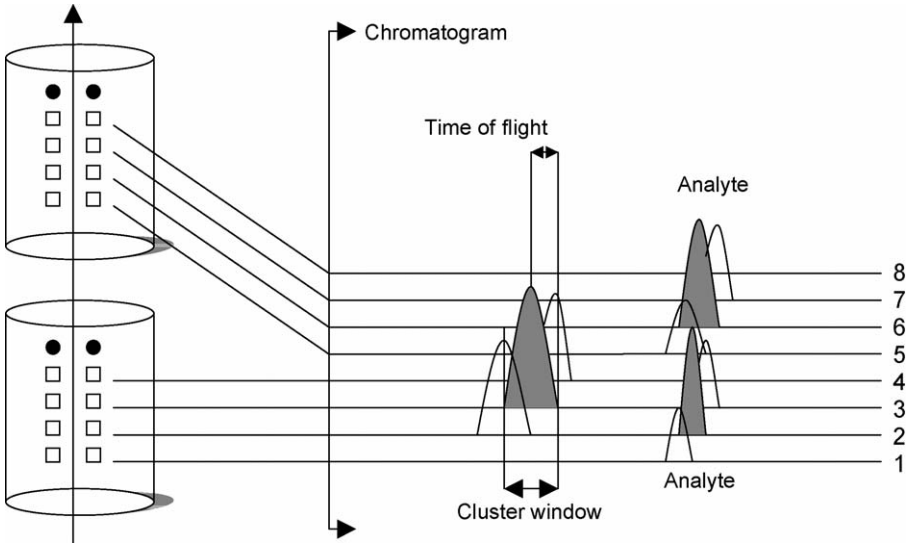

Fig. 3. Schematic structure of the coulometric electrode array detector and the principle of the signal formation [11].

trodes can be varied from −1.0 to +2.0 V, depending on the electrochemical properties of the analyte.

In a single coulometric detector, a potential will be selected based on maximum response (oxidation/reduction) of the analyte by means of the study of hydrodynamic voltamograms (HDV) formed by plotting accumulative peak heights, represented in amperes or peak areas in ampere seconds, i.e. coulombs, after a certain number of injections at fixed increasing potentials (Fig. 4). HDV represents the redox properties of a given analyte and from it the minimum potential that results in maximum response, at the plateau of the sigmoidal shaped curve, can be selected as working potential in a single electrode system. In coulometric electrode array detection however, the array of potentials should be arranged so that contains not only a mainly responsive potential but also potentials adjacent to this so-called main channel selected so that a sufficient response is achieved to originate a constant relationship (ratio) with the aforementioned main channel. When the responses of the array to the analyte are graphically represented a characteristic current voltage curve (CVC) is originated and this, together with the ratio of the three measuring channels constitute the most important feature of the CEAD and the basis of the higher selectivity of the array when compared with a single coulometric detector.

The principle is schematically explained in Fig. 3 where the basic structure of an eight-channel array and the formation of the multichannel chromatogram are presented [11]. After chromatographic separation, the analyte 1 reaches the first electrode (channel 1) where it begins to oxidize passing consecutively through increasing potentials until complete oxidation occurs at channel 3. The potential on channel 2 is selected so that a major part of the analyte 1 is oxidized. The signal on channel 2 will be used for quantification and the signals on channels 1 and 3 are used to confirm the identification of the analyte 1. The identification of the analyte therefore depends on the retention time and the ratio accuracy. The ratio accuracy is a percentage match of the oxidation pattern of the standard and analyte, similar to a match of the UV spectra obtained with a DAD. The signals on the adjacent channels are divided with the signal on the quantification channel to obtain the peak ratios of the analyte. In Fig. 3, the peak ratios of the analyte 1 are 1/2 and 3/2. These values are compared to the peak ratios of the standard and the match of the ratio accuracy should be ≥70% [10,12]. The percentage of ratio accuracy achieved largely depends on the studied matrix and the number and nature of purification steps included in the sample pre-treatment, as will be commented in the following sections. Two analytes could thus be separated if they have different redox properties even though they present similar chromatographic properties. Higher potentials are needed to oxidize the analyte 2 compared to analyte 1. The obtained chromatogram is two-dimensional where separate analytes can

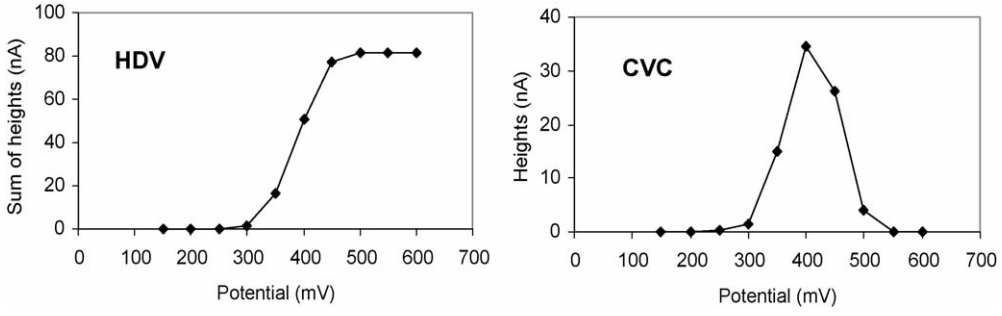

Fig. 4. Hydrodynamic voltamogram (HDV) and current voltage curve (CVC) for pinoresinol under the chromatographic conditions reported by Peñalvo et al. [25].

occur at the same retention time on the different channels. The electrode pairs are placed in line and therefore the retention times of the peaks differ slightly on consecutive channels. That difference in the retention times represents the time of flight and the basic value is 0.5 s, which is applicable to flow rates of 0.5–1.5 mL/min. The signals, preferably from three channels are grouped by the width of the cluster window. This is the time span, in which the retention times of the signals from the consecutive channels must fit. The signals with approximately the same retention times are grouped only when they clearly represent one analyte. Even if the signals of the analytes 1 and 2 in Fig. 3 fitted into the same cluster window, they would be divided into separate clusters, because two oxidations peaks occur and there is a channel without signal in between.

2.2. History and applications

The first applications of the CEAD system or Coularray® (ESA Inc., Chelmsford, MA) were reported in the mid-1980s [13] and since then many different applications have been described for this very versatile system such as the analysis of amino acids, catecholamines, vitamins (see [14–16] for latest examples) and in general redox-active species, a required property for electrochemical detection. Isoflavones were for the first time measured electrochemically, when the sensitivity of UV and amperometric detection was compared in the analyses of soy protein product [17]. The second isoflavonoid method using electrochemical detection was developed for plasma samples [18], and the first using a coulometric detector was reported by Franke and co-workers for isoflavones present in human milk after soy consumption [20]. The first method using CEAD for the analysis of enterolignans in urine and plasma was reported by Gamache and Acworth [22] and afterwards other methods using CEAD have been developed for the analysis of lignans in flaxseed [23], urine [24] and plasma [25] and for lignans and isoflavonoids in dried blood spots [26].

3. Application in the analysis of isoflavonoids and lignans in biological samples

3.1. Food matrices

Isoflavones constitute a numerous group of compounds almost entirely restricted to the family of the leguminosae [27]. Two genera though have attracted most of the attention because of their high isoflavone content. Red clover (*Trifolium pratense* L.) and related species, are especially rich in the isoflavones formononetin (Form) and biochanin A (BioA) [28] that can also appear as their respective glucosides, ononin and sissotrin [29], whereas soybean (*Glycine max* L.) and soy products which are the most relevant dietary sources of isoflavones contain daidzein (Da), glycitein (Gly) and genistein (Ge) that may occur in foods as aglycones, 7-*O*-glucosides, acetyl glucosides and malonyl glucosides [30]. Therefore, altogether 12 isoflavone variations can be found, although the predominant forms in soybeans and non-fermented soy products are glucosides, and fermented soy foods contain mainly aglycones [30]. The natural diversity of isoflavone forms raises a challenge for their quantification. Either a number of standards are used or the sample pre-treatment process involves total or partial hydrolysis of the conjugates to reduce the number of compounds to be analysed.

Electrochemical detection was for the first time applied to isoflavones by the group of Setchell et al. [17]. They determined Da and Ge from soy protein products and noted that sensitivity of electrochemical detector was superior in contrast to UV detector. Over ten years passed before HPLC-CEAD was applied again to determine isoflavones in food. Müllner and co-workers quantified Da and Ge in soy-derived products [31] by reflux extraction (84 °C) with 1.1 M HCl in ethanol. Later on it was reported that those conditions did not hydrolyse 7-*O*-gluosides to aglycones [32] in every situation, and therefore Müllner and co-workers should have evaluated if their conditions apply also for more concentrated sources of isoflavones. The same authors published the same method a year later, with the only variation being the use of 1 M HCl instead of 1.1 M in EtOH to extract analytes [33], and the application of the method to real samples. Probably the most widely applied sample pre-treatment method for soy isoflavones is a method for the 12 forms of isoflavones originally developed for HPLC-DAD by Wang and Murphy [30]. Nurmi et al. applied CEAD to analyse soy based supplements, but they only identified malonyl and acetyl forms [34]. Soon after Peñalvo et al. introduced an adapted method that combined ethanol extraction and mild acid hydrolysis (1 M HCl in 80% EtOH for 1 h at 80 °C) to convert malonyl and acetyl forms quantitatively to 7-*O*-glucosides and aglycones [32]. This is still the only CEAD-based method where attention has been paid to different conjugates of isoflavones, and the effects of sample pre-treatment on hydrolysis of the conjugates. Recently, Klejdus et al. presented a method for Da and Ge in soy foods [35] where isoflavones were extracted with methanol and 0.3% formic acid. As formic acid is not very efficient hydrolyzing reagent, authors should have commented whether the sample pre-treatment was sufficient to achieve complete hydrolysis and reliable results, since the conditions used were clearly insufficient in comparison with Peñalvo et al. report [32]. In the same line, Preinerstorfer and Sontag [36] applied very similar sample pre-treatment as did Müllner and Sontag earlier [31,33] but still the authors did not comment at all whether 7-*O*-glucosides were hydrolyzed or not. Solid phase extraction was applied in both methods after the acid hydrolysis. A sample was dissolved in approximately 23% ethanol when loaded in C_{18} cartridges, and it was then washed with 20% methanol prior to elution with methanol. Therefore, 7-*O*-glucosides might have eluted from the column during loading and washing, and subsequently discarded. Attempts have been made to include Form and BioA in some of the above mentioned protocols [17,31] and even methodological and instrumental parameters have been reported in some cases [35] but, although this would be very convenient in order to analyse in a single assay the most abundant plant-derived isoflavones, it is not feasible in practise due to the poor oxidizability of Form and BioA that made their quantification by CEAD unreliable. As for method validation procedures, different internal standards such as bisphenol A [31] and flavone [35] have been used to correct for possible losses. Only in one

method [36] an internal standard (estriol) was used for quantification purposes by addition to the sample extracts just before the chromatographic run. However, it has to be pointed out that CEAD is linear over several magnitudes of concentrations, and quantification can be easily carried out with external calibration.

Lignans are polyphenolic compounds widely distributed through the plant kingdom, although the number of representatives that can be found in human diets seems to be more restricted [37,38]. Dietary lignans constitute a relatively new research topic and our knowledge is evolving rapidly assisted by new analytical protocols. Only two HPLC-CEAD methods for lignans in foods have been published [23,39]. The first method by Kraushofer and Sontag was developed to quantify matairesinol (Mat) in flaxseed [23]. Although flaxseed was already known at the time to be the major dietary source of lignans because of its high content of secoisolariciresinol (Seco) [40–42] and even other lignans such as pinoresinol (Pin) were also previously quantified [43], the authors did not consider to include all flaxseed lignans in their report. A year after, Nurmi and co-workers reported the levels of five different plant lignans in selected wines [39]. The method applied was a combination of different methods. The hydrolytic steps were obtained from a GC–MS method for lignans in food [44] and liquid chromatography was based on a previous HPLC-CEAD method for plasma phytoestrogens [45]. Enzymatic hydrolysis of the conjugates with *Helix pomatia* was found efficient for all individual lignans, but the applied conditions during acid hydrolysis with 2 M HCl for 2.5 h at 100 °C [39,46] were known to induce the degradation and conversion form Seco to Anse and Lar to Isol, the extent of which depended on the food matrix involved [44,47]. Sample pre-treatment based on acid hydrolysis was discarded and nowadays new methods for lignans in food involving alkaline extraction are in use [38,48] but none of has been applied yet to HPLC-CEAD. Although the instrument should be suitable for these extracts, the complexity and diversity of the food matrices require a tailored sample pre-treatment for each type of samples to achieve clean chromatograms as well as a carefully chosen internal standard that could be used in most of the matrices. CEAD methods for food isoflavones and lignans are briefly summarized in Tables 1 and 2.

Table 1
Isoflavone methods for HPLC and coulometric electrode array detector

Matrix	Analytes	Pre-treatment[a]	Purification[a]	R^b (%), ISTD[c]	Reference
Soy products	Da, Ge	Refluxing in 80% EtOH 2 h, β-glucosidase overnight (37 °C)	SPE	–	[17]
Soy products	Da, Ge, BioA	2 h refluxing in 1.1 M HCl in EtOH	SPE	95–98% for standards and 72–94% from foods, bisphenol A	[31]
Soy products	Da, Ge	2 h refluxing in 1 M HCl in EtOH	SPE	98% for standards and 73–86% from foods, bisphenol A	[33]
Soy products	Da, Ge, Gly (MDin, MGin, MGlyn, ADin, AGin, AGlyn, Din, Gin, Glyn identified)	HP 2 h (60 °C), DEE, 2 M HCl 2.5 h (100 °C), DEE, for conjugates extraction with 80% EtOH		–	[34]
Soy products	Da, Ge, Gly MDin, MGin, MGlyn, ADin, AGin, AGlyn	1 M HCL in 80% EtOH, 1h (80 °C)	–	76–110%, –	[32,46]
Soy products	Da, Ge	Extraction with hexane and 60% MeOH and 0.3% formic acid (100 °C, 130 bar)	–	94–104%, flavone	[35]
Soy products	Da, Ge, Gly	2 h refluxing (84 °C) in 1 M HCl in EtOH	SPE	∼95% for standards and 41–94% from foods, estriol	[36]
Plasma tissue urine	Da, Ge, Eq,	EtOH extraction, β-G 3 h (37 °C), EtOH and centrifuge	–	85–95%, –	[22]
Plasma	Da, Ge, Eq, *O*-dma	β-G + S 16 h (37 °C), DEE	–	93–118%, Estradiol-17β-D-glucuronide	[45]
Dried blood spots	Da, Ge, Eq	HP, 4 h (37 °C) diethyl ether extraction	–	91–94%, estriol-β-glucuronide	[26]
Urine	Da, Ge	Filtration and dilution	–	66–71% and 95–102% after dilution, flavone	[35]
Urine	Da, Ge, Eq	β-glucuronidase 4 h (37 °C) DEE		–, diethylstilbesterol	[58]

[a] β-G = β-glucuronidase; S = sulphatase; DEE = dietehyl ether extraction; HP = *Helix pomatia* enzyme mixture; SPE = solid phase extraction.
[b] R (%) = percentage recovery values.
[c] ISTD = internal standard.

Table 2
Lignan methods for HPLC and coulometric electrode array detector

Matrix	Analytes	Pre-treatment[a]	Purification[a]	R[b] (%), ISTD[c]	Reference
Wine	IsoL, Lar, Pin, Syr, Seco, Mat, Anse, trachelogenin, arctigenin	Alcohol evaporated, SPE, HP 2 h (60 °C), DEE, 2 M HCl 2.5 h (100 °C), DEE	IEC	–	[39]
Flaxseed	Mat	2.4 M HCl in 40% EtOH, 75 min refluxing	–	93%, bisphenol A	[23]
Plasma tissue urine	End, Enl	Extraction with EtOH, centrifuge, evaporate, β-G 3 h (37 °C), EtOH, centrifuge	–	91–96%, –	[22]
Plasma	End, Enl	β-G + S, 16 h (37 °C), DEE	–	69–85%, Estradiol-17β-D-glucuronide	[45]
Plasma	End, Enl, IsoL, Lar, Pin, Syr, Seco, Mat, Anse, Hmr, Med	β-G + S, 16 h (37 °C), DEE	IEC	76–94%, –	[25]
Dried blood spots	Enl	HP, 4 h (37 °C), DEE	–	93%, estriol-β-glucuronide	[26]
Urine	End, Enl, IsoL, Lar, Pin, Syr, Seco, Mat	HP 16 h (37 °C), DEE	IEC	82–102%, –	[24]
Faeces	End, Enl	Water + 0.12 M HCl, DEE		–	[59]

[a] β-G = β-glucuronidase, S = sulphatase, DEE = dietehyl ether extraction, HP = *Helix pomatia* enzyme mixture, IEC = ion exchange chromatography.
[b] R (%) = percentage recovery values.
[c] ISTD = internal standard.

3.2. Plasma

After ingestion, isoflavone glycosides undergo enzymatic metabolism in the small intestine to release the aglycone [49], which is the bioavailable form [19]. Isoflavone aglycones can be thus absorbed or be further metabolised in the large intestine prior to absorption. The human metabolism of isoflavones from soybeans and red clover has been recently studied and numerous compounds have been identified [50]. Few of them though are considered relevant enough at this point to develop quantitative methods, this is the case of equol (Eq) a metabolite of Form and Da that is selectively produced by only some individuals and that has been proposed to be a predisposal factor to the protective effects of isoflavones [51].

Lundh et al. used electrochemical detection for the first time to identify isoflavones in bovine plasma and urine, but they carried out the quantitative analyses with HPLC-UV [18]. The first HPLC-CEAD method for plasma isoflavones was published by Gamache and Acworth [22]. The sample pre-treatment for plasma was completed with a two-fold dilution of the sample prior to HPLC analyses of isoflavones, which made the method applicable only to samples collected after dietary supplementation. Almost at the same time, Franke et al. reported also the use of CEAD for the analysis of isoflavones, but the technique was applied only together with other detectors [21]. Based on the method of Gamache and Acworth [22], Nurmi and Adlercreutz published a method for plasma phytoestrogens, in which the conditions for the quantification of up to 13 compounds including lignans, isoflavones and two isoflavone metabolites were optimized for quantification of non supplemented samples by means of sample concentration during the sample pre-treatment [45]. Recently and also adapted from the above mentioned original methods Yasuda et al. reported a protocol for the simultaneous determination of isoflavonoids and bisphenol A, an industrial chemical classified as a xenoestrogen, in rat serum [52]. The method includes the optimization of the conditions for quantification of Din, Glyn and Gin in serum which does not seem to agree with the already known lack of absorption of isoflavone glycosides [51]. Furthermore, potentials of the array were not appropriately optimized resulting in poor ratio accuracy and thus reliability. CEAD methods for plasma isoflavonoids are summarized in Table 1.

Analysis of enterolignans was also included in the method of Gamache and Acworth [22] and Nurmi and Adlercreutz [45] since the methods were developed for profiling phytoestrogens in human samples. The first HPLC-CEAD plasma method for the analysis of lignans exclusively was recently developed by Peñalvo et al. [25] including all known dietary lignans and enterolignans. Hydrolysis of the samples was similar to that applied previously [45] modified with an additional purification step prior to injection. Ion exchange chromatography with QAE-Sephadex in acetate form has been applied before for analysis of enterolactone in plasma [53] urine [24,54] faeces [55] and foods [39,46], and it has proven its efficacy in removing more polar compounds such as flavonoids and phenolic acids, enabling the extreme sensitivity of the method [25]. CEAD methods for plasma lignans are summarized in Table 2.

3.3. Urine

Very few HPLC-CEAD methods for urinary isoflavonoids and lignans have been published, probably because of the complexity of the matrix when studied with an electrochemical detector. Two CEAD-based methods for urinary isoflavones have been published and both in 2004. Klejdus et al. developed a method to measure Da and Ge in urine after only filtration and dilution [35]. They mainly analysed spiked samples, for which filtration and dilution were suitable sample pre-treatment steps, and they reported also high initial concentrations of Din and Gin in human urine. As mentioned, several studies have been shown that glycosides are not absorbed intact, and therefore they cannot be either excreted as such [51,56,57]. The peaks Klejdus and coworkers observed in their samples might have been glucuronide conjugates of Da and Ge, if the subject has consumed soy, which

authors did not mention. The observed decrease in signals during sample storage was very likely due to the extremely high concentrations of aglycones, which were added directly into urine. Da and Ge do not have a high solubility in water, and therefore signals decreased, while the recovery of spiked Da and Ge increased when dilution of the samples increased, i.e. the amount or organic solvent increased. The second method for urinary Da, Ge and Eq was recently developed by Ouchi et al. [58]. They hydrolyzed the samples and extracted them with diethyl ether. The sample pre-treatment seemed to be appropriate, but the sensitivity of the method was surprisingly low. LOD values were 1 ng on column, and the same on column amount was reported to be LOQ values corresponding to 10 ng/mL of urine, while usual LOD values for isoflavones have been reported to be on low pg level.

Only one HPLC-CEAD method for urinary lignans has been published. Nurmi et al. reported the analysis of seven different lignans and demonstrated the application of the method to non-supplemented samples [24]. Although the visual outlook of the presented chromatogram was busier than those reported in the complementary plasma method by Peñalvo et al. [25], the ratio accuracy of the analytes was regularly above 70%. As in previous methods for urine [54], the use of ion exchange chromatography (QAE-Sephadex) decreased the background signals and increased the reliability of the quantification. The utilization of non-purified *H. pomatia* that is known to contain lignans for the hydrolysis of urinary conjugates resulted in the need of a reagent blank in each assay to subtract the background. During the method development refined enzymes were already available, so the authors could have simplified their method by using those. CEAD methods for the analysis of isoflavonoids and lignans in urine are summarized in Tables 1 and 2.

3.4. Others

One HPLC-CEAD method for the analysis of lignans in faeces has been published, but the method was not fully validated [59]. Lignans occur in faeces as aglycones, so the analytes were directly extracted from the sample. Analytes were separated with a gradient originally developed for plasma phytoestrogens [45], with modified detection potentials for Enl and End [60]. This HPLC-CEAD method for faeces has been applied in several studies over the years [60–62]. Tissue sample pre-treatment procedure for the analysis of Eq in uterine tissue samples was presented by Gamache and Acworth [22]. Rats received a subcutaneous injection of 1 mg of Eq and subsequently, uterine tissue removed, homogenized and further processed according the sample pre-treatment developed for plasma [22]. Eq concentrations increased 10-fold in contrast to controls in which 0.33 nmol/g of tissue was found. Since then this method for uterine tissues has not been applied. Very recently, Melby et al. reported a method to measure isoflavonoids and enterolactone from dried blood spots (DBS) that aimed to profile the levels of such compounds in large population-based studies [26]. Precision of the method is compromised because of the use of β-glucuronidase obtained from *H. pomatia* enzyme mixture, which is known to contain isoflavones and lignans in such concentrations [63] that a reagent blank in the assay would have been definitely needed. Recoveries should have been also investigated at lower levels than reported, since those concentrations cannot be found in individuals without intensive soy or flaxseed consumption. These methods are also summarized in Tables 1 and 2.

4. Sensitivity: comparison with other instrumental techniques

Limits of detection (LOD) for isoflavones in HPLC-CEAD methods are usually sufficiently low to analyse all types of soy food samples. Values ranged from a few pg up to tens of pg on column, but none of the values were even close to 100 pg. Limits of quantification (LOQ) for isoflavones were reported in most of the methods, but they were calculated from LOD values and not separately determined. In some methods LOD values were given for samples, but in practise LOD value is a measure of the device sensitivity and only LOQ values can be used to report the lowest quantifiable concentrations in samples. LOQ values for soy food isoflavones were mainly a few μg/g, but in the method of Klejdus et al. authors reported LOQ values only in pg on column [35]. When these values, injection volume, sample amount and final volume of the sample were taken into account, LOQ values ranged from 2.6 to 8.4 ng/g. Theoretically those values are valid, but when 0.5 g sample is dissolved in 1 mL of solvent prior to HPLC analyses, sample matrix very often provides at least some background, which makes it difficult to quantify so low concentrations in samples. In general the sensitivity of CEAD enables the analyses of soy samples containing very low concentrations of isoflavones, such as e.g. alcohol washed soy protein products. LOD values for isoflavonoids in plasma and urine methods are similar to those reported for food methods, which is expected because the detector is the same. Only one exception has been reported, and that is a urine method for Da, Ge and Eq by Ouchi et al. [58]. They reported LOD values as high as 1 ng on column, which indicates approximately 100–200 times lower sensitivity in contrast to the other published methods for isoflavonoids in human samples. In the method of Melby et al. for dried blood spots [26], on column LODs ranged from 4.5 to 19 pg, but once they proceeded to calculate the corresponding serum equivalents, negative LOD and LOQ values for Eq were reported, which is not logical even considering the authors' explanations regarding negative slopes in the regression lines. Klejdus and co-workers reported LOQ values from 6.3 to 7.9 pg on column for Da and Ge, which corresponded to 2.6 and 3.2 ng/mL when injection volume is 5 μL and dilution of one volume were used. Authors presented results for standard additions only, but if the real samples were analysed after the hydrolysis, they might have found difficulties to quantify such low concentrations of isoflavones in urine, because the number of electroactive analytes present in urine is always very high and, without MS-detector able to perform in selected ion monitoring mode, further purification is needed on those concentration levels. The sensitivity of CEAD for lignans is usually slightly higher in contrast to isoflavones, which means that LOD values are lower for lignans. The peaks of lignans are narrower, which increases the height of peaks increasing the sensitivity.

Kraushofer and co-workers reported LOD for Mat, which was 40 pg on column, and LOQ, which was 5 μg/g. These values were similar to those observed for isoflavones, but further purification of the samples with the ion exchange chromatography increases the sensitivity of CEAD for food lignans. LOD values for lignans in plasma and urine are below 20 pg on column, which correspond to LOQ values of a few nmol/L for plasma samples [25], and tens of nmol/L for urine samples [24].

The highest sensitivity to measure lignans and isoflavonoids is achieved with LC–MS and GC–MS systems, which are able to measure concentration of pg/mL [64–66], while HPLC-CEAD methods are able to measure ng/mL concentrations. Although MS detectors are extremely sensitive, LOQ values are often much higher in contrast to LODs, and in most of the cases they have not been precisely presented. In one LC–MS/MS method LODs values for serum and urine were comparable to those reported for HPLC-CEAD, but those might have been more likely LOQ values [67]. The sensitivity of fluoroimmunoassay method for enterolactone [68] is similar to the HPLC-CEAD method by Peñalvo et al. [25]. The fluoroimmunoassay methods for plasma and urinary Eq [69], and urinary Da and Ge [70] are comparable to GC–MS methods [53,54], which have been applied during the immunoassay method development as reference methods. According to LOD and LOQ values HPLC-CEAD methods are comparable to the other sensitive methods for isoflavones and lignans. Selectivity of CEAD methods is slightly less than for MS methods, but analytical columns with high efficiency increases the selectivity of CEAD methods above that usually observed for conventional HPLC-DAD or UV methods.

5. Notes and tips

The detection in a coulometric electrode array system is based on the transfer of electrons between the analyte and the electrode surface. It is therefore necessary that the mobile phase carrying the analyte allows this transfer and for that it must contain an electrolyte and a solvent of such dielectric constant that permits the ionization of the electrolyte. The mobile phase (electrolyte + solvent) should be electrochemically inert and, in an ideal situation, the working electrodes in CEAD will then measure only the variations in the current caused by the pass of the analyte. In most cases however, a background current is normally detected deriving from the oxidation/reduction of redox active species in the mobile phase. The background current is exponentially related to the applied potential and although the working potential of detector ranges from −1.0 to +2.0 V, an excessive background signal would appear at those limits. It is our experience that a channel is difficult to stabilize when potential is set above 0.7 V, in particular when certain electrolytes such as phosphate-based buffers are used in mobile phase. The concentration of the buffer must not be under 0.01 M in any stage of the isocratic or gradient separation. Too low ion strength in the mobile phase causes signal drifting, in contrast to too high ion strength that causes the mentioned background signal. Sodium acetate buffer pH 4–5 has been popular in mobile phases for lignans and isoflavones, and it was originally introduced by Gamache and Acworth [22]. It is simple to prepare, and it provides stable baseline signals. Also solubility of the buffer is excellent in MeOH and ACN, which are regularly used with this buffer. If the separation of analytes is critical in regard of the pH of the mobile phase, then sodium acetate buffer may cause some problems. Although the pH of the buffer is originally set at 4.8–5.0, the final pH of the mobile phase will differ after MeOH and/or ACN have been mixed with the buffer. MeOH and ACN are weak proton acceptors and that increases pH of the eluent. We have observed that during a gradient from 20% B up to 100% (eluent A: 50 mM sodium acetate pH 5/MeOH 80/20 v/v and eluent B: 50 mM sodium acetate pH 5/MeOH/ACN 40/20/20 v/v/v) pH of the mobile phase varies from 5.3 to 6.2. This does not affect on the retention of lignans and isoflavones, but other phenolic compounds usually need lower mobile phase pH to be eluted. In one method reviewed here sodium acetate buffer was used in concentration of 0.15 M [35], which might be too high to reduce background noise on a low pA level. In two methods buffer solution was prepared from 50 mM sodium acetate solution and glacial acetic acid [31,36], which at least doubled the final concentration of buffer, and might cause noise. In one method mobile phase was prepared from glacial acetic acid and sodium hydroxide [23], which did not form a buffer solution, so final pH value of the mobile phase very likely differed from the expected 3.

Background signal increases with temperature, applied potential, and contamination of the mobile phase. A very intensive background can be seen when the working electrodes are dirty. A normal procedure will be to pump highly organic mobile phase (methanol or acetonitrile) through the array and, if this is not solving the problem, electrodes can be cleaned by the use of high potentials (1.0 V, 30 s) to oxidize/reduce possibly accumulated dirt. In some earlier developed methods, electrode cleaning was routinely applied at the end of run, but recently only one method has been published in which that procedure was used [35]. Authors did not comment whether there was some benefit about the clean cell procedure or not. After electrode cleaning, 10–15 min time is needed to get stable signals in a well balanced system.

An appropriate purification of the sample should be always taken into consideration in sample pre-treatment development, not only for the proper function of the electrochemical cells but also for performance of the whole chromatographic system. Food samples, containing high concentrations of isoflavones and lignans, can be always diluted to avoid column (and detector) overload. Human samples, specially urine and faeces, may contain many co-eluting compounds that will increase background and eventually accumulate in the detector cells, but the inclusion of a purification step such as ion exchange chromatography in lignan methods, improves the baseline so that ratio accuracy is regularly above 70% even in concentrations close to LOQ values [24,25]. The use of a guard column is, of course, recommended.

In CEAD, the selectivity and precision of the analyses are improved by the concept of ratio accuracy (or ratio window), i.e. the constant relationship between the main channel and adjacent channels for a given analyte. To define this ratio, the retention time window should be carefully adjusted so that it only com-

prises the analyte on of interest, and this can only be accurately done if the chromatograms are clean which stresses the importance of sample purification and minimizing the background current. In case of busy chromatograms, as for instance human urine samples usually provide, it is advisable that, to avoid false rejections of preliminarily identified peaks, the ratio window is customized from default (30%) to an appropriate value depending on the situation. In case of uncertainty, current voltage curves could be used to accept the rejection of any peak but should never be used as an identification tool.

Different from the background signal, the term noise refers to a random or periodic pattern in the chromatogram. It usually includes pump pulsation, static electricity, power-line noise, and electronic amplification. Pressure fluctuation occurs as a result of a pulsation in the pistons of the pumps and it can be logically avoided by optimization of the system. Coulometric electrode array detector can be used either with ESA Inc. (Chelmsford, MA) HPLC system or combined with HPLC systems from other manufacturers. Although all HPLC systems should be compatible, in practise many of them cause too much disturbance that is difficult to attenuate even with a pulse damper, and in most of the cases sensitivity is decreased. Leaks and air bubbles are also responsible for pressure fluctuations. A pressure drop may be caused by leak in the fittings or malfunction of the check valves (ESA Inc., Chelmsford, MA) that can be damage or just dirt. Pressure drops in only one of the pistons of a dual system are a sign of a leaking plunger seal that can be easily replaced. If the leak persists it indicates the presence of scratches in the pistons. Air bubbles can be easily detected by the appearance in the chromatogram of a high intensity signal on a regular basis, different from electrical noise which usually causes a very sharp single peak. Air can be trapped in several parts of the system and purging it with degassed mobile phase at high flow rate may sometimes help. Check valves are likely locations for air to accumulate, and a periodical purge for them should be considered. Inadequate degassing of the mobile phase or on-line mixing of aqueous and organic phases are the most common sources of air bubbles. Only in one of the reviewed methods, the mobile phase consisted of plain buffer and plain ACN [35]. Finally, an obvious fact causing system overpressure is a too high flow rate. The detector is described to work within a wide range of flow rates, but lower flow rates provide more stable baseline signal. Low flow rate is easy to apply when narrow (less than 4 mm) analytical column is used, although the most important is to use pumps which produce continuous flow with minimum piston movements. It is also worth to consider the use of methanol specially when added to modify the mobile phase in a gradient run. Methanol increases the viscosity of the eluent and therefore the pressure in the system. This fact can be overcome by slightly warming the system. The most stable baseline signals are obtained when CEAD is thermostated. Different temperatures from 25 up to 37 °C have been applied in methods for isoflavonoids and lignans. In that temperature range all choices are equal if the set temperature is stable throughout the run. Increasing the temperature decreases retention times if column is placed into a thermal chamber together with CEAD. However, higher temperature (above 40 °C) increases the ion mobility in buffer solution, and causes noise in baseline signals. So decreased run time would be achieved by decreasing the sensitivity because of increased noise.

6. Conclusions

The application of HPLC-CEAD to the analysis of isoflavones and lignans in biological samples has proven to be successful in most of the experiments reported so far. Since the first HPLC methods for isoflavones have been published at the beginning of the 1980s, a lot of general facts in regard of isoflavonoids extraction and hydrolysis conditions are known. These previously published methods should be taken into consideration when new methods are developed to avoid repeating mistakes. In HPLC-CEAD, complications might appear when the applied sample pre-treatment is not sufficient to provide purified extracts to be injected into the system, but even in that situation the electrochemical array makes possible the discrimination between co-eluting analytes when the potentials forming the array are carefully optimized.

Therefore the preferred methods based on CEAD to measure isoflavonoids and lignans in foods and human samples would be those that include validated sample pre-treatments. For example, total isoflavonoids in foods are most reliably measured with the method of Peñalvo and et al. [32]. For lignans in food the method by Nurmi et al. [39] is not recommended, although other sample pre-treatments [38,48] could be adopted and applied to the chromatographic procedure described. Plasma isoflavonoids and lignans should be analysed by methods including complete hydrolysis of conjugated forms with β-glucuronidase and sulphates [25,45]. In the case of urinary isoflavonoids the best options are the methods of Gamache and Acworth [22] and Ouchi et al. [58], although only β-glucuronidase is used for hydrolysis. Other sample pre-treatment methods could be applied [66,67] and subsequently carry out the analyses with CEAD. For urinary lignans though the method by Nurmi et al. [24], which includes validated sample pre-treatment and wide variety of plant lignans in addition to enterolignans is recommended.

The main feature of CEAD is an improved selectivity when compared with single electrochemical detectors or the counterpart DAD. It is also a very sensitive detector capable of quantifying isoflavones and lignans biological samples at very low concentrations, although the sensitivity in CEAD is especially dependent on an appropriate sample pre-treatment.

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