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Chromatographic and electrochemical determination of quercetin and kaempferol in phytopharmaceuticals

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Abstract

An NP-HPLC method both with diode-array (DAD) and electrochemical detection (ED) was developed and validated for the determination of quercetin and kaempferol, the principal active constituents in phytopharmaceuticals of *Ginkgo Biloba*. Calculated retention of the two flavonoids was contrasted with experimental values in five different reversed phase columns for methanol–water, acetonitrile–water, THF–water and dioxane–hexane binary mixtures as mobile phases. The capacity factor *k*, selectivity α and asymmetry factor *F* were evaluated and compared in DAD-RP-HPLC, DAD-NP-HPLC, ED-RP-HPLC and ED-NP-HPLC. The methods were used for the quantitative analysis of acid hydrolyzed extracts of tablet phytopharmaceuticals. Calibration curves were linear within the range 10 and 40 $\mu g\,ml^{-1}$ for the DAD and 10–270 $\mu g\,ml^{-1}$ for the ED, whereby limits of detection ranged from 0.5 $\mu g\,ml^{-1}$ (quercetin) to 0.1 $\mu g\,ml^{-1}$ (kaempferol). The electrochemical method based on differential pulse voltammetry (DPV) with a C-PVC electrode resolved the quercetin and kaempferol peaks and exhibited a two orders higher sensitivity in comparison with a carbon fibber electrode. DPV calibration curves were linear within the range 96–300 $\mu g\,ml^{-1}$ for quercetin and 68–960 $\mu g\,ml^{-1}$ for kaempferol. The respective oxidation peaks appeared at 462 and 518 ± 2 mV and were used in the direct determination of quercetin in extracts of commercial phytopharmaceuticals.

Keywords: DAD-NP-HPLC; ED-NP-HPLC; DPV; Composite electrodes; Quercetin; Kaempferol; *Ginkgo Biloba* tablets

1. Introduction

Interest in botanical extracts that have traditionally been used as remedies has tremendously increased in recent years. As a result, manufacture and consume of phytopharmaceuticals or nutraceuticals has exploded in recent years. Extracts of *Ginkgo Biloba* are widely studied due to its important antioxidant and anti-arthritic properties in human metabolism and have been approved for the treatment of dementia [1]. Quercetin and kaempferol may be found as major flavonoids in all extracts of the *Ginkgo Biloba* plant obtained from leaves, rosettes, stems and root bark [2–4] and are considered the major chemical markers in phytopharmaceuticals prepared from this tree.

Analytical and chromatographic research around *Ginkgo Biloba* flavonoids [5] has developed in the last 5 years in three directions: (a) determination in botanical extracts [5–10] and food [11–14]; (b) detection of flavonoids and their metabolites in biological objects like urine [15], plasma [16] and tissue [17] at relative low concentrations; and (c) quality control of *Ginkgo* containing products [3,4,18–21]. Usually, enriched *Ginkgo Biloba* extracts utilized for the preparation of phytopharmaceuticals are standardized to contain 24% flavonoids and 6% terpenoid lactones. High performance liquid chromatography (HPLC) [2,3,6,11,12–14,16,17,19,22], liquid chromatography–electrospray mass spectrometry

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(LC–ES–MS) [4], gas chromatography–mass spectrometry (GC–MS) [20,15], liquid chromatography–mass spectrometry (LC–MS) [6,7,9], thin layer chromatography (TLC) [23], capillary electrophoresis [8,18,21] and combined spectroscopic [23] methods have been developed for the analysis of extracts and biological liquids. In contrast, the quality control standards for phytopharmaceutical preparations in drugstores and small-scale laboratories is somewhat lacking and in many cases it is performed by the laboratory suppliers by UV–vis spectrometry just in case it is accomplished. Products often differ from brand to brand or even from lot to lot. The spectrometric determination of quercetin and kaempferol as an aluminium chloride complex is not very specific, it gives only an estimation of the total flavonoids and due to the interferences is not reproducible. Actually TLC has been adopted as a practical alternative for routine quality control for commercial Ginkgo products [24]. However, the Association of Official Analytical Chemists (AOAC), under contract to the Food and Drug Administration (FDA) and the National Institutes of Health (NIH) of the USA, has embarked on an effort to develop a group of fully validated methods, the AOAC's Official Methods for selected dietary supplements including Ginkgo.

In the chromatographic work the flavonoid glycosides are hydrolyzed to quantify the produced aglycones: quercetin, kaempferol and isorhamnetin. Investigators have studied hydrolysis conditions [11,21], its kinetics [2], argentation [25] and post-column derivatization with fluorescence detection [17]. RP-HPLC peaks of flavonoids and their hydrolyzed products are normally broad and exhibited peak tailing [22] even with the use of an acidified mobile phase. Attempts of optimizing the RP-HPLC separation are reported in the literature by testing the performance of different RP-HPLC columns and mobile phases [3,22]. However, the use of polar stationary phases is only reported in [26] for flavones and the application of a –CN column for the flavonols quercetin, hesperetin and naringenin was noted in [27]. Although much has been learned about the electrochemical detection in liquid chromatography and capillary electrophoresis of biological active compounds, the direct electrochemical analysis of anti-oxidants in *Ginkgo Biloba* phytopharmaceuticals has not been explored. Research in this area has been limited to the analysis of quercetin in lakes' water [28], and wine [29] samples. Interestingly, some studies showed the electrochemistry [29–34] of flavonoids, including quercetin and kaempferol, as well as the relation between the oxidation potentials and the anti-oxidant activity [35]. Cyclic voltammetry (CV) has been commonly used in these studies [21,29–32] to evaluate and identify oxidation peaks, while only in [28] this information was obtained by square-wave voltammetry (SWV) and in [36,37] by differential pulse voltammetry (DPV).

This paper seeks to relate and to compare the chromatographic and the electrochemical determination of quercetin and kaempferol in phytopharmaceuticals. The study is divided into two parts: the chromatographic section compares a novel NP-HPLC with the common RP-HPLC separation and quantitation of quercetin and kaempferol using both diode array detection (DAD) and electrochemical amperometric detection (ED); the electrochemistry part presents the method for these analytes by DPV on the novel C-PVC electrode. Examples of the use of DAD-RP-HPLC, DAD-NP-HPLC, ED-RP-HPLC and DPV are shown by the quantitative analysis of quercetin and kaempferol in Mexican phytopharmaceutical samples containing *Ginkgo Biloba*. The study will attempt to show that DPV can be used as a fast method to investigate phytopharmaceuticals without necessity of any sample pretreatment hydrolysis.

2. Materials and methods

2.1. Chromatographic analysis

Samples were analyzed using a Beckman Gold automated liquid chromatograph (Beckman Coulter, Inc., Fullerton, CA, USA) comprising a Beckman 168 diode array detector, and a Shimadzu electrochemical detector (Shimadzu Scientific Instruments, Columbia, MD, USA), a BAS 480 Liquid chromatograph (BAS, West Lafayette, IN, USA) with an E5 Epsilon electrochemical detector (BAS, West Lafayette, IN, USA), both with a glassy carbon electrode. RP-separations were carried out at 30 °C using the columns described in Table 1 at flow rate 1 ml min^{-1} in isocratic mode, with mixtures of either methanol (MeOH), acetonitrile (MeCN), tetrahydrofuran (THF) in water or dioxane in hexane. All HPLC solvents were purchased from Burdick & Jackson (Michigan, USA). Eluates were monitored by DAD at 355 nm. The ED was set at 500 and 730 mV, respectively, for aqueous (RP-HPLC) mobile phase and non-aqueous (NP-HPLC) dioxane–hexane mobile phase. In ED-RP-HPLC the mobile phase was methanol–acetonitrile–$NaClO_4$ (0.1 M) (30:30:40, v/v/v), while in ED-NP-HPLC it was dioxane–hexane–methanol (40:40:20, v/v/v). Methanol contained LiCl (10%, w/v) as electrolyte. The injection volume was 30 μl for the DAD and 10 μl for the ED detector. Each solution was injected three times.

The asymmetry factor F was calculated according to [38] at the half width as the ratio $F = f/t$, where f is the semiwidth before the peak apex and t the semiwidth after the peak apex. The capacity factor k and the selectivity α were calculated as usually in chromatographic analysis [38].

2.2. Electrochemical analysis

DPV was carried out with a potentiostat (Eco Chemie, Utrecht, The Netherlands) Autolab PGSTAT 10. The GPS 3.1 software package supplied with the instrument was used for control and data storage. The electrochemical measurements were performed on a three-electrode system at room temperature. The working electrodes were a carbon fibber electrode, a carbon fibber coated with Nafion (Aldrich, Mu-

Table 1
Columns used in the experimental determination of the capacity factors, selectivity and asymmetry factor of quercetin and kaempferol peaks

Stationary phase[a]	Packing with particle size 5 μm[b]	Trade	Column length × i.d. (mm)	Carbon loading[b] (%)
Phenyl	Spherisorb	PhaseSep	150 × 4.6	3
Octyl	Ultrasphere	Beckman	150 × 4.6	6
ODS1 (a)	Spherisorb	PhaseSep	150 × 4.6	7
ODS (b)	np	BAS	100 × 3.2	np
C18	np	ISCO	250 × 4.6	12
Diol (c)	LiChrosorb	Phenomenex	125 × 4.0	np

[a] Used in the method validation and comparison: (a) RP-HPLC (DAD and ED detection), (c) NP-HPLC (DAD and ED detection); and used for the analysis of phytopharmaceuticals in: (a) DAD-RP-HPLC, (b) ED-RP-HPLC, and (c) DAD-NP-HPLC systems.
[b] np: not provided by the supplier.

nich, Germany) and a C-PVC electrode described in [39] and applied in [40] for biological active compounds. The working electrode was measured against an Ag/AgCl electrode (3 M KCl, analytical grade, Merck, Darmstadt, Germany). The counter electrode was a platinum spiral foil. The parameters for the DPV used here were a scan rate of 5 mV s^{-1} and pulse modulation of 50 mV in amplitude, 50 ms duration at intervals of 200 ms. DPV was measured for quercetin and kaempferol in two media: (a) polar solution consisting of methanol–acetonitrile–$NaClO_4$ (0.1 M) (30:30:40, v/v/v) and (b) non-polar medium composed of dioxane–hexane–methanol (40:40:20, v/v/v). Methanol contained LiCl (10%, w/v) as electrolyte.

2.3. Materials and software

Quercetin [6151-25-3] was purchased from Aldrich (≥95% Munich, Germany) and kaempferol ≥90% (HPLC) (Cat. # K 0133) was obtained from Sigma (Milwaukee, WI, USA). Water was deionized with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Analytical grade salts LiCl (Merck, Darmstadt, Germany), and $NaClO_4$ (Reactivos Monterrey, Mexico) and analytical grade hydrochloric acid (Reactivos Monterrey, Mexico) were used. The software Chromdream (Knauer, Berlin, Germany) calculated retention of the studied analytes on the basis of the differences in the molecular solvation energies in the mobile phase and in the surface layer. The software MicroCal™ Origin™ Version 5.0 from Microcal Software, Inc. (Northampton, MA, USA) was used for file transformation of the chromatograms and for the linearization procedures.

2.4. Sample preparation

Stock solutions of quercetin and kaempferol were prepared in methanol (HPLC grade) for the RP-HPLC analysis and their concentrations were 0.04 mg ml^{-1} for the chromatographic part and 1 μM for the DPV study performed to assist the ED-HPLC detection. For the NP-HPLC chromatography and DPV in non-aqueous medium the analytes were dissolved in dioxane at the same concentrations. Solutions of the standards were freshly prepared for each analysis and put in dark vials. Six brands of *Ginkgo Biloba* tablets were randomly selected from local markets. For the chromatographic analysis three no film-coated tablets of each pharmaceutical preparation were homogenized together by grinding to represent an average and were dried at 50 °C during 3 h. The sample was then weighed into a 50 ml tared flask and extracted twice with 25 ml methanol or dioxane (both 80% aqueous solution) by sonication at room temperature during 15 min. Solutions were left overnight in the refrigerator allowing the solid to precipitate out upon 24 h storage. The precipitate was filtered and the methanol or dioxane extracts were evaporated to dryness at 45 °C. After addition of 2 ml of methanol and 0.5 ml of hydrochloric acid 2N the extract was stirred for 1 h at room temperature. After cooling it was transferred into a 5 ml flask and made up to the volume with methanol or dioxane. Extracts were kept in dark vials in the refrigerator.

For the electrochemical analysis parameters, a stock solution of quercetin or kaempferol was prepared in 10 ml methanol, shaked and μl aliquots were added to the electrolytic cell to produce a given concentration. For the electrochemical analysis of pharmaceuticals three tablets of each no film-coated pharmaceutical preparation were ground to represent an average and were dried at 50 °C. A mass of 2 mg was immersed in 10 ml methanol, shaken, left overnight and after 24 h, 25 ml of water was added. The solution was transferred to the electrolytic cell to determine quercetin and kaempferol without hydrolysis pretreatment. Calculations were performed accordingly.

2.5. Stability study

No film-coated tablets were homogenized by grinding in a mortar, dried at 50 °C, and 3 mg were subjected to the following accelerated test conditions during 3 h: immersion in 10 ml with continuous stirring in: (a) CH_3COOH (0.1N), (b) CH_3COOH (2N), (c) UV radiation at 255 nm, (d) water at 40 °C, (e) Na_2CO_3 (0.1 mM, pH 8), and (f) H_2O_2 (30%, v/v). UV radiation was prolonged only during 1 h. One blank aqueous solution was also prepared. After treatment the samples reposed during 12 h. For qualitative analysis, the precipitate was filtered and supernatants were analyzed by HPLC. The amount of residual precipitate referred to the tablet weight varied from 92 to 0.3% in the same order as the treatments (a)–(f) are listed.

2.6. Validation

Stock solutions of quercetin and kaempferol were prepared from reagent grade standards in dioxane for the NP-HPLC and in methanol–acetonitrile–acetic acid (0.1N) (25:25:50, v/v/v) for RP-HPLC and were stored at 4 °C. Daily required volumes of each analyte stock solutions were diluted in the same solvent to prepare the standard working solutions. The content of quercetin and kaempferol was calculated from calibration curves constructed individually from six concentration values and their peak areas detected at 355 nm in the concentration range 10–40 μg/L, suitable for the injections of phytopharmaceuticals. Injections were performed in triplicate.

The precision of the used methods were determined, including both intra-day and inter-day variation of the peak areas of standard solutions prepared from the stock solution. The inter-day reproducibility was determined by analyzing the samples daily along 30 days for the NP-HPLC and 10 days for the RP-HPLC. Quantities were calculated from the corresponding calibration curves. Each sample was analyzed five times to determine the intra-day variability. The relative standard deviation (R.S.D.) was taken as a measure of precision. The accuracy of the methods was evaluated by performing recovery experiments.

The limit of detection (LOD) for quercetin and kaempferol was determined as the concentration of the standard solution giving a signal to baseline noise ratio S/N > 3 from the dependence of the peak height with concentration, since noise amplitude is a linear magnitude. The limit of quantitation (LOQ) was calculated as the analyte concentration that give rise to peak heights with S/N > 10. Here injections were made for different volumes with the help of the programmable autosampler, starting at 30 μL of the 10 μg/L solution and decreasing it producing dilution until the peak was indistinguishable from noise. After recalculation of the concentration, noise amplitude was measured using the software and LOQ was evaluated according to the S/N > 10 criterion. In this way LOQ could be found at values inferior than the lowest concentration in the calibration curve. For LOD and LOQ determinations 25 measurements were done by using reference solutions of quercetin and kaempferol.

The data are presented as mean value ± S.D. Statistical analysis was performed by means of Student's test for non-dependent samples.

3. Results and discussion

3.1. Chromatographic analysis

3.1.1. Calculated and experimental retention in different HPLC systems

The theoretically calculated logarithmic dependence of the capacity factor ln *k* of quercetin and kaempferol in RP-HPLC on the Hypersil column produced different curves when methanol, acetonitrile and tetrahydrofuran (Fig. 1A) were introduced as mobile phase. The curves served to illustrate that the greatest separation theoretically occurred when the content of methanol or acetonitrile fluctuated between 50 and 75% on the Hypersil column. In contrast, the tetrahydrofuran content did not theoretically affect the separation of quercetin and kaempferol as it influenced the resolution of quercetin and luteolin and has been demonstrated recently by Wang and Huang [41] (also differing in one OH group, but in different position in the catechol ring). The application of HPLC in the analysis of flavonoids [2–4,9,11,13,14,22] deals commonly with C18 stationary phases. Only Erlund et al. [16] reported the use of ODS3. From the extensive use of reversed phase columns it can be demonstrated repeatedly that the effectiveness of C18 columns varies [22] from trade to trade. On different stationary phases (Fig. 1B–F) the theoretical curves reflected these findings, where the curves additionally illustrated that retention was not described by a simple linear relationship with the organic solvent concentration in the broad concentration range of the methanol–water mobile phase.

Fig. 2 shows the experimental retention values of quercetin and kaempferol on the following RP columns: two C18, one C8 and one phenyl phase with methanol (A), acetonitrile (B) and tetrahydrofuran (C) water mixtures as mobile phases, in the concentrations over which measurements in RP-HPLC are usually performed. Only in some cases experimental and calculated capacity factor was similar and in general the values of ln *k* were much more higher than the calculated ones. The experimental results with THF showed a similar pattern as the theoretical prediction, and then practically no separation could be achieved on the C18 columns (Fig. 2C). Among

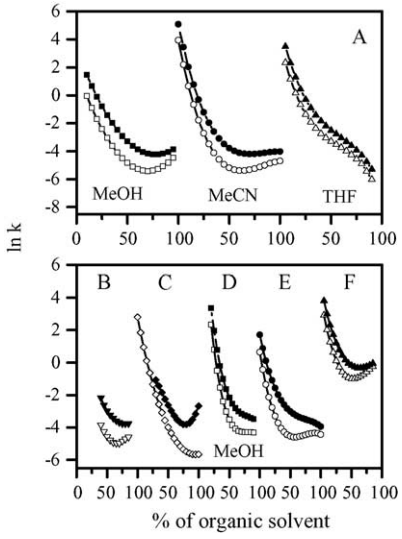

Fig. 1. Calculated ln *k* vs. composition curves for quercetin (open symbols) and kaempferol (solid symbols) on: (A) Hypersil eluting from methanol, acetonitrile and tetrahydrofuran; and on different stationary phases: (B) Spherisorb ODS1, (C) Nucleosil 120-5C18, (D) Nucleosil 100-10C18, (E) Separon SGX C18 and (F) Polyol eluting from methanol.

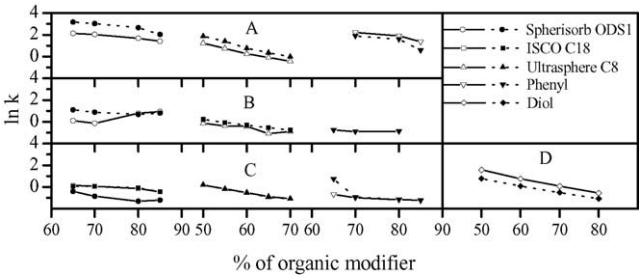

Fig. 2. Experimental capacity factor values of quercetin (continuous line, open symbols) and kaempferol (dotted lines, solid symbols) chromatographed on different columns in dependence on mobile phase composition using (A) methanol, (B) acetonitrile and (C) tetrahydrofuran in water, and (D) dioxane–hexane mixtures.

the three organic solvents, methanol exhibited the better resolution on the tested RP-columns (Fig. 2A). The Spherisorb ODS1 phase showed an enhanced separation of quercetin and kaempferol in comparison with the C8 and phenyl columns and was used further in the next part of this work.

Although flavonoid compounds have been well resolved by silica and cyano columns by Pietrogrande et al. [26], NP-HPLC analysis of quercetin and kaempferol is not popular, mainly because most of the published RP-procedures allowed the analysis. Although also Erlund [27] explored the use of an NP cyano stationary phase for the determination of quercetin, a method validation was not matured in her work. The novel NP-HPLC system we introduced in this work (Fig. 2D) consisting of a diol column eluted by dioxane–hexane mixtures exhibited a lower capacity factor in comparison with the Spherisorb column and judging by the distance between the curves, produced a satisfactory separation ($1.6 < \alpha < 2.2$) in the concentration range from 50 to 80% hexane in dioxane.

Besides high capacity factor values, the common RP-HPLC stationary phases considered in this work produced noticeable peak tailing in the studied mobile phase systems as it can be appreciated in Table 2. Octyl, phenyl and octadecyl moieties on the silica surface produced experimental asymmetry values within 1.1 (C8/MeCN) and 4.4 (ODS1/MeCN), while Crozier [22] calculated an asymmetry value of 10.5 for quercetin on an octadecyl phase. By using acidified mobile phases and C18 stationary phases, selectivity of the pair quercetin/kaempferol varied [3] from 1.07 to 1.17. On the moderately polar diol column we obtained $1.6 < \alpha < 2.2$ and the resolution achieved at composition dioxane–hexane (1:1, v/v) was 2.32. This value was smaller than the value reported by Chin et al. [3] for hydrolyzed samples, but a satisfactory resolution degree. Asymmetry values obtained in NP-HPLC in the 50–80% range were lower than those achieved for some RP-HPLC systems and for all columns using 50% methanol (see Table 2).

3.1.2. DPV assisted electrochemical detection in HPLC

Although considerable progress has been made in the application of the electrochemical detection (ED) to different analytical techniques, most works on the determination of flavonoids report the coupling of ED to capillary electrophoresis [8,18,21,42] and only some authors have utilized methods based on RP-HPLC with coulometric [11,16,27,43–45] and amperometric [46] ED. Nevertheless, since little attention has been paid to NP-HPLC, this explains why there are no examples of ED-NP-HPLC for flavonoids. The hydrodynamic voltammograms obtained in both RP and NP mobile phase systems are depicted in Fig. 3A. Different

Table 2
Asymmetry factor F and selectivity α of four RP-HPLC columns and a diol column tested with quercetin (Q) and kaempferol (K) using different aqueous mobile phases in the percent range indicated

Stationary phase	Mobile phase	F		α
		Q	K	
Spherisorb ODS1	85–65% MeOH	2.4–2.9	2.4–2.7	1.9–2.9
	85–65%MeCN	2.8–3.6	3.1–4.4	1.1–2.7
	85–65%THF	1.3–3.2	1.2–3.4	1.1
ISCO C18	85–65% MeOH	1.1–1.8	1.5–1.7	1.3–1.6
	85–65% MeCN	1.3–1.6	1.7–1.8	1.1
	85–65% THF	1.1–1.7	1.2–2.2	1.1
Ultrasphere C8	70–50% MeOH	1.8–3.2	1.1–3.9	1.5–1.9
	70–50% MeCN	1.1–2.2	1.1–2.8	1.1–1.4
	70–50% THF	1.1–1.4	1.1–1.6	1.1
Phenyl	90–65% MeOH	3.2–4.0	2.1–2.3	1.3–1.4
	90–65% MeCN	1.1–2.4	1.1–1.3	1.3–1.4
	90–65% THF	1.1–2.5	1.3–2.8	1.1
Diol	80–50% hexane–dioxane	2.1–2.6	1.4–1.6	1.6–2.2

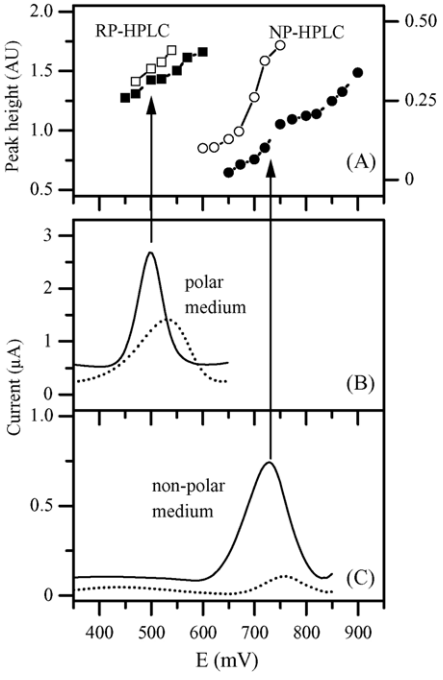

Fig. 3. Hydrodynamic voltammograms (A) of the standard solutions quercetin (□, ○) 0.32 μM and kaempferol (■, ●) 34 μM on the column RP-HPLC Sperisorb ODS1 and NP-HPLC diol and differential pulse voltammograms (B) of quercetin (continuous line) and of kaempferol (dotted line) in methanol–acetonitrile–$NaClO_4$ (0.1 M) (30:30:40, v/v/v) and in hexane–dioxane–methanol with LiCl (0.1%, w/v) (50:25:25, v/v/v) (C) as solvent media. Flow rate 1 ml/min.

curves were obtained for quercetin and kaempferol and no defined plateau was observed. Similar curves can be found in the literature for quercetin [11,31,35], kaempferol [35] in acetonitrile–acidic buffer [11,31] and in methanol–buffer solution pH 7.5 [35] in RP systems, and in for kaempferol [8], quercetin [18,21,42] in capillary electrophoresis; while hydrodynamic voltammograms in an NP-HPLC system are reported here for the first time. Here the potential shifted to higher values with respect to the RP-mobile phase system, but a maximum response could not be clearly observed. Cyclic voltammograms of quercetin and kaempferol in neutral buffer solutions [31,32,37] showed that these compounds are electrochemically active and according to authors [30–32,37] underwent oxidation by a $2e^-$ process while rearranging the flavonoid ring [32,34]. We turned then to DPV with a classical glassy carbon electrode for being a sensitive electrochemical method producing a peak-shaped *I–E* curve, which allowed the precise determination of the potential at which oxidation–reduction occurs. Fig. 3B and C shows the electrochemical response obtained by DPV of quercetin and kaempferol in two different media in the potential range 200–900 mV. Quercetin and kaempferol presented oxidation peaks at 497 and 530 mV, respectively, in aqueous medium (Fig. 3B) and at 729 and 759 mV in the dioxane–hexane medium (Fig. 3C), whereby the response of glassy carbon for quercetin was much higher than that for kaempferol and more sensitive in the polar medium than in the non-polar medium. Electrochemical detection both in HPLC and in capillary electrophoresis has been used at randomly set potential (see Table 3) though the shape of the hydrodynamic voltammograms, which was in stark contrast with the determination we did of the maximum response potential by DPV. This can explain the dispersion in the sensitivity reported by other authors, and why they differed considerably.

Although cyclic voltammetry established a reversible oxidation for quercetin and kaempferol consisting of 2 [44], 3 [31] and 4 [37] oxidation peaks in the potential range 150–900 mV, whereby the peak at 900 mV was more intensive [44]; we set in the RP-HPLC the lower potential value determined by us by DPV since oxidation of the polar solvent could accompany the oxidation of the analytes. Further chromatographic work was performed at 500 mV with the Spherisorb ODS1 column and at 730 mV with the NP-diol column tested above and these results were applied for the analysis of phytopharmaceutical preparations.

3.1.3. Chromatographic systems comparison and validation

Linearity of the NP-HPLC and RP-HPLC was compared. The obtained relationships are summarized in Table 4. The reported statistical data represent the average correlation coefficient, slope and intercept for four curves obtained in four different days.

Table 3
Potential (E_a) used in the electrochemical detection of quercetin (Q) and kaempferol (K) in reversed phase liquid chromatography (RP-HPLC) and capillary electrophoresis (CE) in different media and the achieved sensitivity or the reported limit of detection (LOD) in the coulometric (cdm) or amperometric (adm) detection modes

Method	Detection mode	Electrode	Medium	pH	E_a[a] (mV)		LOD ($\mu g\,ml^{-1}$)	Sensitivity[b]	Ref.
					Q	K			
RP-HPLC	cdm	Porous graphite	Acetonitrile–phosphate buffer	2.4	700	700	0.3 (Q) 0.5 (K)	0.18 (Q) (c) 0.18 (K) (c)	[11]
		Not specified	Methanol–phosphate buffer	2.4	100		0.001 (Q)	4600 (Q) (c)	[16]
		Not specified	Methanol–phosphate buffer	2.6	150		0.001 (Q)		[43]
	adm	Not specified	Methanol–ammonium acetate	5.15	600		0.002 (Q)		[46]
CE	adm	Composite carbon–methyltrimethoxysilane		7.5	800	800	1.7 (K)		[8]
		Carbon disc electrode		9.0	1000	1000	0.5 (Q)	0.14 (Q) (d)	[18]
		Carbon disc electrode		9.0	900		0.1 (Q)	0.18 (Q) (d)	[21]
		Carbon disc	Ethanol–borate buffer	9.0	900		0.1 (Q)	0.22 (Q) (d)	[42]
SWV		Paraffin–impregnated graphite	Sodium acetate–acetic acid		280				[28]
CV		Glassy carbon	Tartaric acid–NaOH	3.6	377				[29]
		Glassy carbon disk	Phosphate buffer	7.5	60 (a)	170 (a)			[30]
		Glassy carbon	Methanol–potassium nitrate	1–10	150, 500				[31]
		Plastic formed carbon and Au electrode	Methanol–phosphate buffer	7.5	60	∼170			[32]
		Glassy carbon	Ethanol–phosphate buffer	5–9	300	330			[33]
		Carbon fibber threads	Methanol–phosphate buffer	7.5	20 (b)	80 (b)			[35]
		Glassy carbon	Phosphate buffer	7.0	290 (b)	390 (b)			[47]
CV + DPV		Mini glassy carbon	Different buffer solutions	2–12	150				[37]
DPV		Carbon paste	Phosphate buffer	7.0	177				[36]

Oxidation peaks observed in square-wave voltammetry (SWV), cyclic voltammetry (CV) and differential pulse voltammetry (DPV).
[a] Corresponds to the: (a) midpoint potential $\{(E_a + E_c)/2\}$, (b) half-wave potential $E_{1/2}$ measured by cyclic voltammetry.
[b] Calibration sensitivity from the slope of the calibration curves in: (c) peak area (AU) units or (d) peak height (nA) units per concentration units ($\mu g\,ml^{-1}$).

Table 4
Quantitative parameters of the analysis of quercetin (Q) and kampferol (K) by NP-HPLC and RP-HPLC

Parameters in RP-HPLC	DAD at 355 nm		ED at 500 mV	
	Q	K	Q	K
Calibration range (μg/L)	10–40	10–40	57–270	57–270
Slope of the calibration curve ± S.D.[a]	$2.5 \times 10^2 \pm 0.4$	$9.7 \times 10^2 \pm 4.8$	$3.9 \times 10^3 \pm 52.3$	$7.3 \times 10^2 \pm 31.5$
Intercept ± S.D.[a]	-0.3 ± 0.3	-4.1 ± 3.7	$-4.7 \times 10^2 \pm 64.0$	$-6.9 \times 10^1 \pm 38.6$
Regression coefficient (R^2)	0.9940	0.93	0.9974	0.9732
Parameters in NP-HPLC	**DAD at 355 nm**		**ED at 730 mV**	
	Q	K	Q	K
Calibration range (μg/L)	10–40	10–40	11–118	11–118
Slope of the calibration curve ± S.D.[a]	$9.9 \times 10^2 \pm 0.9$	$13.3 \times 10^2 \pm 2.0$	$1.4 \times 10^2 \pm 10.7$	–
Intercept ± S.D.[a]	-0.5 ± 0.7	-1.0 ± 1.6	-6.5 ± 13.2	–
Regression coefficient (R^2)	0.9988	0.9932	0.9867	0.992

[a] For probability 95% and $n = 6$.

The precision of the methods was evaluated as described in the experimental part. Repeatability was demonstrated by measuring the peak of four solutions of the calibration curve. The intra-day and inter-day results were reproducible and the statistical parameters are summarized in Table 5.

The average LOD and LOQ values calculated from calibration curves are gathered also in Table 5 both for the two applied detectors and for the two chromatographic systems. They were sufficient for the purpose of this work analyzing the quercetin and kaempferol content in phytopharmaceuticals. In terms of sensitivity, the values reported for DAAD-RP-HPLC [11,12,41] and ED-RP-HPLC [11,16,46] were similar to the magnitudes obtained in this work. In our case sensitivity of the electrochemical detection was greater than that of the DAD detection as it was expected and has been demonstrated before by Jones et al. [46]. A great sensitivity difference could be observed between the proposed here NP-HPLC system and the current RP-HPLC method when the DAD detection was applied. Despite the substantial selectivity of the electrochemical detection, this first report of quercetin determination by amperometric detection using NP-HPLC did not reach the LOD values required for low levels in biological samples [43,46,48], but satisfied the levels expected in phytopharmaceuticals. The values in the ED-NP-HPLC were similar to those we obtained for quercetin in ED-RP-HPLC.

Method validation regarding accuracy was not performed by means of recovery experiments in standard formulations with reliable flavonoids content. Recovery was tested adding known quantities of quercetin and kaempferol to the phytopharmaceuticals studied here and following the four methods described above: DAD-RP-HPLC, DAD, NP-HPLC, ED-RP-HPLC and ED-NP-HPLC. Results will be mentioned in the corresponding section.

3.2. Electrochemical analysis on different electrodes

Based on the DPV results for quercetin and kaempferol applied to the ED in HPLC, we decided to test the application of DPV for the direct electrochemical determination of these analytes. Several anti-oxidants have been determined semiquantitatively by cyclic voltammetry in wines [29] and only the electrochemical work of Filipiak [33] reported the analysis of polyphenol model mixtures.

Results from electrochemical studies (Table 6) have indicated that peak intensity in the voltammograms and the potential at which oxidation of the first hydroxyl group of the flavonol molecule occurs, depended on the medium and on the electrode. These studies also argued the importance of DPV producing narrow peaks at reproducible potential values to generate reliable analysis of mixtures. The examples in Fig. 4 (D and E) demonstrate that the C-PVC electrode produced well-defined, symmetrical and much narrower peaks of quercetin and kaempferol in comparison with a current carbon fibber electrode (Fig. 4A and B). This led to a separation of the DPV-peaks in the binary mixtures (Fig. 4F) on the

Table 5
Comparison of the precision, limits of quantitation (LOQ) and detection (LOD) obtained for quercetin (Q) and kaempferol (K) by liquid chromatography using two detection devices in RP-HPLC (ISCO column, 355 nm) and NP-HPLC (diol column) systems

Detection	HPLC system	n	Inter-day mean peak area $\times 10^2 \pm$ S.D.[a]		LOQ (μg ml^{-1})		LOD (μg ml^{-1})	
			Q	K	Q	K	Q	K
DAD	RP	10	4.25 ± 0.18	6.71 ± 0.08	4.1	35	1.2	10
ED 500 mV	RP	10	3.38 ± 0.15	3.95 ± 0.07	1.7	0.4	0.5	0.1
DAD	NP	30	6.43 ± 0.23	4.08 ± 0.10	12	4	3.5	1.1
ED 730 mV	NP	30	2.23 ± 0.05	1.99 ± 0.02	3		0.3	

[a] For a 10 μg/L concentration.

Table 6
Parameters of the DPV method for quercetin and kaempferol standard solutions using three different electrodes in methanol–$NaClO_4$ (0.1 M) (60:40, v/v)

Working electrode	$E_a \pm$ S.D.[a] (mV)		ΔE_{Q-K} (mV)[b]	W_b (mV)[c]		Concentration range (ng ml^{-1})		Sensitivity[d]		LOD (ng ml^{-1})	
	Q	K		Q	K	Q	K	Q	K	Q	K
Carbon fibber	460 ± 2	543 ± 4	83	245	210	10–50	20–90	31	5	6	20
Carbon fibber/Nafion	438 ± 7	508 ± 3	70	218	215	20–140	11–200	4	7	3	5
C-PVC	462 ± 0	518 ± 2	56	138	107	96–300	68–960	8100	2700	6	6

[a] Observed in the concentration range 40–300 ng ml^{-1} for quercetin and 70–1000 ng ml^{-1} for kaempferol (vs. Ag/AgCl electrode). Standard deviation calculated for $n = 10$.
[b] Potential difference between the peaks of quercetin and kaempferol.
[c] Peak width at the base, evaluated by a Gaussian fit.
[d] Calibration sensitivity obtained from the slope of the calibration curves in response units (nA) per concentration units (μg ml^{-1}).

C-PVC electrode, which could not be achieved on the carbon fibber electrode at a certain concentration relation (Fig. 4C). This becomes clear when one examines the potential difference ΔE_{Q-K} and the peak width W_b values in Table 6. Potentials at which oxidation occurred are presented in Table 6 together with the precision on carbon fibber electrode, a Nafion coated carbon fibber and C-PVC electrode. It could be observed that oxidation of both compounds took place at higher potentials in the studied medium than those reported in the literature (see Table 3). Oxidation of kaempferol occurred at higher potential in comparison to quercetin in agreement with the results obtained in previous studies [30,32–34], due to their relative oxidation feasibility. It was particularly noticeable the higher sensitivity achieved with C-PVC in comparison with the carbon fibber. There was no improvement in the detection limit due to the higher noise level presented by the C-PVC electrode.

3.3. Chromatographic analysis of pharmaceutical preparations

The chromatographic methods described above were applied to analyze four commercial Ginkgo products: G2, G3, G5 and G10. Fig. 5 shows the DAD-NP-HPLC chromatograms of these pharmaceutical preparations, while Table 7 presents the resulting content. The inversion of the elution order of kaempferol first, then quercetin in respect with the elution from RP-HPLC phases (quercetin first, then kaempferol) can be observed here. As shown in the chromatograms, other components than quercetin and kaempferol could be detected and resolved.

Table 7 also describes the components accompanying the active quercetin and kaempferol, which differed from sample to sample. G3 displayed the lowest content of the flavonoids pair among the studied samples. Except for the sample G5, a good agreement was observed by comparing the RP with the NP results obtained with DAD. The discrepancy in G5 can be due to the sugar matrix in the tablet acting as excip-

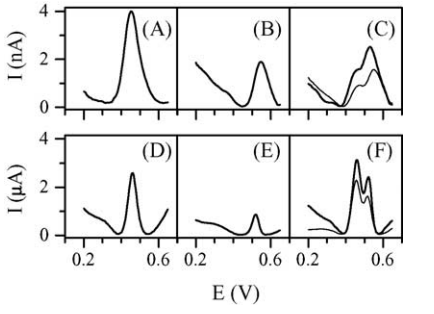

Fig. 4. Differential pulse voltammograms of standard solutions of quercetin (1 μg ml^{-1}) (A, D), kaempferol (0.9 μg ml^{-1}) (B, E) and their mixtures (C, F) on a carbon fibber electrode (A–C) and on the C-PVC electrode (D–F). In mixture (C, F, thick lines) kampferol (1 μg ml^{-1}) was added to quercetin (0.5 μg ml^{-1}) and in mixture (C, F, thin lines) quercetin (0.4 μg ml^{-1}) was added to kaempferol (1 μg ml^{-1}) during the measurement in methanol–$NaClO_4$ (0.1 M) (60:40, v/v).

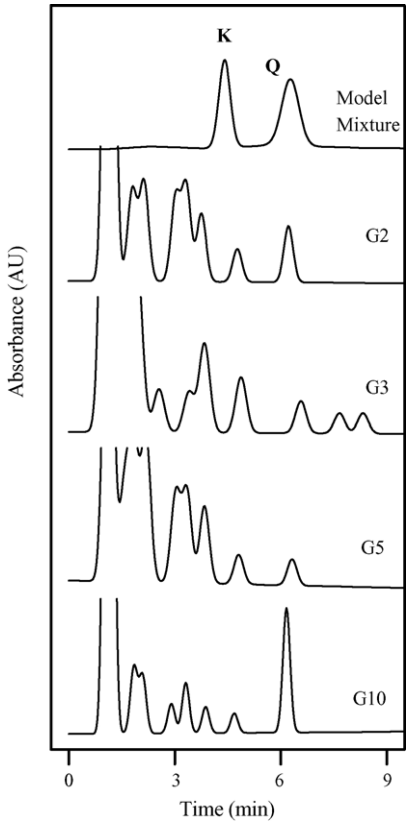

Fig. 5. DAD-NP-HPLC chromatograms of a model mixture of quercetin and kaempferol and of the phytopharmaceutic samples. Column diol. Mobile phase dioxane:hexane (1:1) at 1.0 ml/min. Detection wavelength 255 nm.

Table 7
Comparison of the amounts (mg/tablet) of quercetin (Q) and kaempferol (K) quantified by HPLC in hydrolyzed Mexican samples and by DPV in not hydrolyzed preparations

Sample	Other components of the tablet	Q + K			
		DAD (RP-HPLC)	DAD (NP-HPLC)	ED (RP-HPLC)	Claimed
G2	Calcium carbonate, cellulose	3.4	3.3	4.1	3.8
G3	Ginger, lemon balm	2.5	2.2		Unknown
G5	Sugar, sodium, fat	5.2	1.5	5.5	3.8
G10	Calcium carbonate	3.8	3.8	3.7	3.8
				Q + K	
				ED (DPV)[a]	Claimed
G9	Calcium carbonate			0.3	3.8
G11	Lecithin, calcium carbonate			0.2	3.8

[a] Using a C-PVC electrode.

ient interference in relation with the NP-mobile phase system, since the results of the DAD and ED detection methods in the RP system were similar. Our investigation showed that the studied products contained quercetin and kaempferol in an amount of 2.5–5.2 mg unit^{-1} and a good match in all cases with the manufacturers' claims was observed. Accuracy of the methods was calculated as the percentage of analytes recovered by the assay. The mean values of the percentage recoveries ($n = 3$) in methanol were 98% (R.S.D. = 4.3%) and 97% (R.S.D. = 1.1%) for quercetin and kaempferol, respectively, and were in the ranges reported by other authors [19,20]. Extraction with dioxane showed a lower recovery grade varying from 76 to 87% (R.S.D. = 3.6%).

3.4. Analysis of phytopharmaceuticals subjected to stability testing

The DAD-RP-HPLC method was also used by us [49] to study the stability of the analytes quercetin and kaempferol contained in the phytopharmaceuticals. A qualitative effect even of light and temperature on the chromatograms of the standard solutions could be established, while the DPVs showed no alteration of both the oxidation peaks' maxima and anodic current intensity after 3 days of continuous checking. The chromatograms of the preparations showed the alterations depicted in Fig. 6A. The preparation immersed in 2N CH_3COOH (chromatogram c) resulted in formation of two degradation products; one at shorter and one at longer retention time in relation to quercetin. Similar to the acidic condition, products in small amounts could be observed after immersion in Na_2CO_3 (chromatogram d) and after radiation with UV light (e). Except for the acidic medium (c), during all the four conditions (b, d, e, f) the peak was shifted to shorter retention times, which means a more polar compound in comparison with quercetin was present in the samples. This was the only effect produced by heating. In a weaker acidic medium (Fig. 6B), different preparations showed diverse behavior. In general, the formation of the product at shorter retention time could be observed.

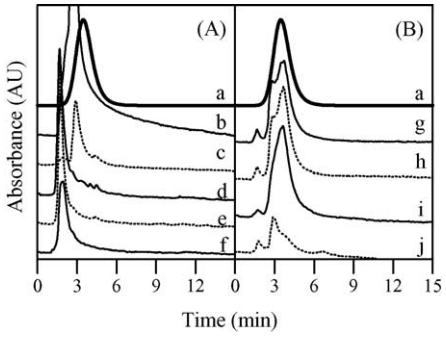

Fig. 6. Chromatograms of the sample G3 (A) after immersion in H_2O_2 (b), 2N CH_3COOH (c), 0.1 mM Na_2CO_3 (d), subjected to radiation at 255 nm (e) and heated at 40 °C (f); and (B) of different phytopharmaceuticals: G10 (g), G9 (h), G5 (i) and G1 (j) after treatment with 0.1N CH_3COOH. Standard blank quercetin solution (a). Column ISCO C18, mobile phase: methanol–acetonitrile–water (25:25:50, v/v/v), flow rate 1 ml/min, injection volume 15 μl, detection at 355 nm.

3.5. Electrochemical analysis of pharmaceutical preparations

The electrochemical method described above was applied to analyze two commercial Ginkgo products G9 and G11 without hydrolysis. As in previous studies of the kaempferol-glucosides [8], in [44] it was stated that the glycosilation of flavonols did not significantly modify their electrochemical behavior. Thus an attempt to analyze electrochemically not hydrolyzed pharmaceutical samples was done. The effort to quantify the quercetin and kaempferol content in these preparations is given in Table 7, where the values obtained differed strongly from the claim. What is certain is that the extraction step was not optimized. Fig. 7A illustrates the DPV of sample G9, where after running the first cycle, one peak at 467 mV could be observed. This suggested that only quercetin was present in the sample. A further possibility was that kaempferol together with other flavonoids in minor concentrations could have been overlapped by the quercetin peak.

In order to explore the oxidation mechanism of quercetin on the C-PVC electrode, the electrolysis solution in Fig. 7A

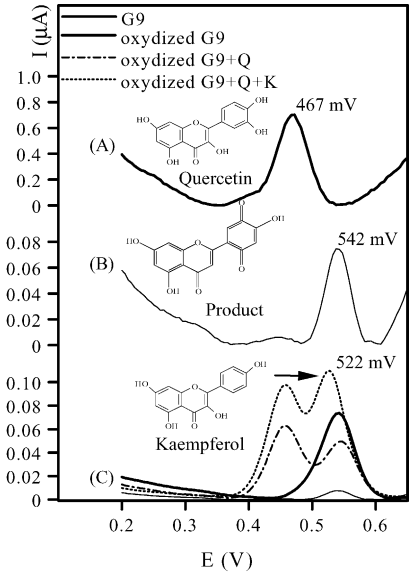

Fig. 7. Differential pulse voltammograms of sample G9 (80 μg ml^{-1}) (A), of an aliquot of the oxidized solution of G9 after 39 cycles (B) and after addition of quercetin and kaempferol standard solutions (C) on the C-PVC electrode in methanol–$NaClO_4$ (0.1 M) (60:40, v/v).

was removed from the cell after 39 cycles. Then a μl aliquot of the latter solution was added to a new blank solution placed in the electrolytic cell. The resulting DPV is depicted in Fig. 7B. The voltammogram presented a minor quercetin peak and a considerable peak at 542 mV, which judging by the precision of the oxidation peaks exhibited by the standard compounds (Table 6), it did not characterize kaempferol. The most probable explanation was then a different compound. After addition of firstly quercetin and secondly kaempferol standards to the solution in Fig. 7B, kaempferol at 522 mV and quercetin at 467 mV could be confirmed (Fig. 7C). The possible explanation is that the peak at 542 mV corresponded to the oxidation product of quercetin shown in Fig. 7B, according to the proposed first-order mechanism [31], where the presence of only one hydroxyl group and a quinone type compound at ring B of the flavonoid basic structure could produce a signal at a slightly higher potential than for kaempferol. The competing postulate of Hendrickson et al. [31] of a zero-order oxidation mechanism of quercetin produces a dihydroxylated B-ring [31,34] after intramolecular rearrangement, which would have produced an oxidation peak near quercetin, but shifted to lower potentials relative to kaempferol, i.e. at $E_a < 522$ mV.

4. Conclusions

In the chromatographic part, the NP-HPLC system consisting of a diol column and dioxane–hexane mixture showed less peak tailing than the current RP-HPLC systems and resolved satisfactory the pair quercetin–kaempferol. The electrochemical response was optimized by the application of DPV prior to the chromatographic work. ED provided lower LODs of quercetin than that obtained by DAD. However, the electrochemical detection was less sensitive in NP-HPLC in comparison with RP-HPLC. Depending on the required levels and laboratory facilities, the DAD-RP-HPLC, DAD-NP-HPLC, ED-RP-HPLC or ED-NP-HPLC can be applied. The DAD-NP-HPLC and ED-RP-HPLC methods exhibiting good precision, sensitivity, LOD, separation factor, resolution and less peak tailing would allow suitable analysis of phytopharmaceuticals after hydrolysis and extraction procedures. The electrochemical study demonstrated the application of DPV by using the novel C-PVC electrode for the determination of quercetin and kaempferol in commercial preparations without hydrolysis step. Despite the separation of the analytes peaks and the much higher sensitivity of this electrode in comparison with the current carbon electrode used in DPV analysis of flavonoids, the extraction has to be optimized in order to obtain reliable quantitative results.

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