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Journal of Chromatography A, 881 (2000) 449–460

JOURNAL OF CHROMATOGRAPHY A

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Spectrophotometric and coulometric detection in the high-performance liquid chromatography of flavonoids and optimization of sample treatment for the determination of quercetin in orange juice

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Abstract

The capabilities of spectrophotometric and electrochemical detection techniques were investigated for the high-performance liquid chromatographic determination of flavonoids. Liquid chromatographic analyses were performed on eleven compounds belonging to three different classes of flavonoids: flavanone glycosides, flavone and flavonol aglycones. Separation of all compounds examined was carried out under reversed-phase conditions on a C_{18} narrow-bore column for UV detection, whereas for electrochemical detection, a C_{18} standard-bore column was used. UV analyses were carried out at 280 nm for flavanones and at 265 nm for flavones and flavonols, whereas controlled-potential coulometric measurements were performed using a porous graphite electrode. Analytical performances of the methods were compared in terms of linearity, limits of detection (LODs) and precision. Linearity over two orders of magnitude and LODs at low-ppm levels (0.06–1 mg/l) were demonstrated for all techniques considered. Instrumental precision in terms of relative standard deviation was found to be between 0 and 5% for the liquid chromatography (LC)–UV system and between 0.6 and 10% for the LC–electrochemical detection (ED) system. The methods developed were applied to the analysis of flavanones and flavonols in a real sample, such as an extract of orange juice. Even though quercetin glycoside is mostly present in orange juice as rutin, other different glycosides of this flavonol could be present; on this basis, the hydrolysis of all glycosides to aglycone allows one to obtain more accurate data on the flavonol concentration in orange juice. To avoid sample degradation and to increase extraction efficiency, quercetin hydrolysis was optimized using a central composite design to investigate the effects of acid concentration and hydrolysis time on extraction recovery.

Keywords: Fruit juices; Food analysis; Experimental design; Statistical analysis; Flavonoids; Quercetin

1. Introduction

Polyphenolic compounds play an important role as natural potent antioxidants, exhibiting various physiological and biological activities, such as anti-inflammatory, anti-allergic and anti-carcinogenic activities, in the human metaboliC system [1,2].

Over the past few years, various liquid chromatographic (LC) methods with UV–Vis absorption [3–6] or diode-array ultraviolet (DAD–UV) [7], fluorescence [8] and, more recently, with mass spectrometric (MS) detection [9,10] have been developed for the analysis of these substances.

As a part of research programs dealing with the

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0021-9673/00/$ – see front matter
PII: S0021-9673(00)00256-9

application of chromatographic and LC–MS techniques in food chemistry [11,12], in this study, flavonoids were considered among natural substances of nutritional interest. In particular, the main point of interest of this work was to evaluate the advantages and disadvantages of spectrophotometric and electrochemical controlled-potential coulometric (ED) detection systems for the LC analysis of flavonoids and to compare these techniques with LC–turboionspray (TIS)–MS. Three classes of flavonoids were considered: flavone and flavonol aglycones (apigenin, chrysin, kaempferol, quercetin and galangin) and flavanone glycosides [eriocitrin, narirutin and hesperidin as 6-*O*-(rhamnopyranosyl)glucopyranosides; neoeriocitrin, naringin and neohesperidin as 2-*O*-(rhamnopyranosyl)glucopyranosides]. Very recently, we devised and validated a method based on the use of LC–MS with a TIS interfacing system for the analysis of the same compounds [13].

In this work, as a first step, two reversed-phase (RP) chromatographic methods on C_{18} narrow-bore columns were developed for flavanones and for the simultaneous separation of flavones and flavonols with UV–Vis detection, whereas LC–ED separations were performed on a standard-bore column. Further, to evaluate and compare the analytical performances of the LC detection techniques proposed, linearity, limits of detection (LODs) and instrumental precision in terms of RSDs were determined.

The three LC detection techniques were demonstrated for the determination of flavonoids present in an orange juice sample.

Quercetin is the compound mostly studied for its demonstrated important anti-carcinogenic and anti-arthritic properties in human metabolism [14]. Even if quercetin glycoside is mostly present as rutin in orange juice [15], other different glycosides could be present. On this basis, hydrolysis of all glycosides to aglycone allows one to obtain more accurate data on flavonol concentrations in food. On the other hand, flavonols are often present in fruits and vegetables as glycosides and, for their analysis as aglycones, an hydrolysis treatment is necessary. Therefore, before the analysis of orange juice samples, an optimization on the quercetin aglycone extraction procedure was carried out. Due to the lability of this compound, the effects of acid concentration and hydrolysis time on the extraction recovery were investigated by performing a statistical data treatment based on a central composite design (CCD). In particular, this optimization strategy allows a direct evaluation of the variables involved in the extraction together with the determination of interactions between the factors considered, providing valuable information on the sample treatment procedure.

2. Experimental

2.1. Chemicals

Quercetin dihydrate, kaempferol and apigenin aglycones were obtained from Fluka (Buchs, Switzerland), chrysin and galangin aglycones were purchased from Sigma–Aldrich (Milan, Italy), neoeriocitrin, eriocitrin, hesperidin, neohesperidin, naringin and narirutin glycosides were obtained from Extrasynthèse (Genay, France).

Water was purified with a Milli-Q water purification system (Millipore, Bedford, MA, USA). Acetonitrile and methanol (HPLC-grade purity) were purchased from Carlo Erba (Milan, Italy). Analytical reagent grade formic acid and hydrochloric acid were from Carlo Erba. Butylated hydroxytoluene (BHT) was supplied by Sigma (Milan, Italy).

2.2. Stock solutions

Stock solutions of eriocitrin, galangin, kaempferol, narirutin, neoeriocitrin, neohesperidin and quercetin were prepared in methanol. Hesperidin was dissolved in a methanolic solution of $2 \cdot 10^{-4}$ *M* sodium hydroxide. Apigenin and chrysin stock solutions were prepared in a methanol–acetonitrile mixture (70:30, v/v). Naringin was dissolved in a formic acid aqueous solution–methanol, pH 2.4 (70:30, v/v) mixture. All stock solutions (1 mg/ml) were stored at 4°C and protected from daylight. Prior to injection, stock solutions were appropriately diluted with methanol unless specified otherwise, before being used as working solutions.

2.3. RPLC–(UV–Vis)

For flavanone analysis, the HPLC separation was performed on a C_{18} narrow-bore column (Luna,

150×2.0 mm, 3 μm; Phenomenex, Torrance, CA, USA) using an isocratic solvent system [aqueous formic acid, pH 2.4 (A)–acetonitrile (B); 80:20, v/v] at a flow-rate of 200 μl/min. For the simultaneous separation of flavones and flavonols, a gradient elution was followed: solvent (B) was delivered by a linear gradient from 50 to 65% in 2 min, followed by an isocratic elution of the solvent mixture A–B (35:65, v/v) for 7 min; the initial mobile-phase composition was restored in 2 min and held for 15 min. The mobile phase was delivered by a Hewlett-Packard HP 1050 solvent delivery pump (Palo Alto, CA, USA) equipped with a Hewlett-Packard HP 1050 autosampler and a variable-wavelength HP series 1050 UV detector. Measurements were performed at 280 nm for flavanones, whereas flavones and flavonols were detected at 265 nm.

For quantitative assay of quercetin in orange juice samples, a wavelength of 370 nm, corresponding to maximum absorbance, was selected. Data acquisition was performed using laboratory-made software.

2.4. Hydrodynamic voltammograms and RPLC–ED

A Perkin-Elmer series LC 200 quaternary pump liquid chromatograph (Perkin-Elmer, Foster City, CA, USA) equipped with a BCS autosampler (BCS, Milan, Italy) was connected to a 5100A ESA Coulochem detector with a dual electrode analytical cell (Bedford, MA, USA). Each cell is made of a porous graphite working electrode together with associated reference and counter electrodes; both of the latter electrodes are covered by a patent. In order to avoid contaminants or a decrease in sensitivity, the detector cells were periodically flushed with a 7-*M* HNO_3 solution.

Initially, hydrodynamic voltammograms were recorded in the 0.1–1 V range (steps of 0.1 V) to determine the optimum working electrode potential of the analytes by flow injection analysis (FIA). Solutions were prepared at 10 mg/l by dilution of stock standard solutions in the solvent mixture used as the mobile phase and were injected in FIA by performing replicate measurements ($n=4$) at each potential value. Hydrodynamic voltammograms were obtained by plotting the measured peak areas against the applied potentials.

On the basis of these findings, the potential applied to the first cell (E_1) was −0.5 V for all of the flavonoids, whereas at the second cell (E_2), a potential of +0.8 V for flavanones and +0.7 V for flavones and flavonols was applied.

HPLC separation was achieved on a C_{18} standard-bore column (150×4.6 mm, 5 μm; Alltech, Milan, Italy). An isocratic solvent system made up of aqueous formic acid–acetonitrile, pH 2.4 (80:20, v/v) was used as the mobile phase at a flow-rate of 0.8 ml/min for flavanone separation. Two different LC separations of flavones and flavonols were carried out under isocratic elution conditions, using a mixture of acetonitrile–aqueous phosphate buffer, pH 2.4 (50:50, v/v) as the mobile phase at a flow-rate of 0.8 ml/min.

Data acquisition and processing were performed on a Maxima 820 system (Waters, Milford, MA, USA).

2.5. Validation of the LC–(UV–Vis) and LC–ED methods

For the LC–(UV–Vis) method, linearity was checked over two orders of magnitude for flavanones in the 1–100 mg/l range and for flavones and flavonols in the 0.5–50 mg/l range. Calibration curves for quantitative assay in the orange juice sample were calculated in the 2.5–100 mg/l range for flavanones and in the 0.5–20 mg/l range for quercetin.

To check the precision of the method, the intra-day ($n=5$) and inter-day ($n=15$) repeatability was studied using concentration levels of 80 mg/l for flavanones and 40 mg/l for flavones and flavonols (1 μl injected).

In the case of the LC–ED method, linearity for flavones and flavonols was explored in the 2.5–250 mg/l range, whereas for flavanones it was checked in the 2–200 mg/l range. Calibration curves for the quantitative assay in the orange juice sample were calculated in the 5–50 mg/l range for flavanones and in the 2.5–50 mg/l range for quercetin. The injection volume varied from 1 to 3 μl.

Precision was evaluated by studying the intra-day ($n=5$) and inter-day ($n=15$) repeatability at concentration levels of 20 mg/l for flavanones and 50

mg/l for flavones and flavonols (1 μl injected).

For both techniques, the limits of detection were calculated as the minimal concentration producing a reproducible peak with a signal-to-noise ratio greater than three; for the LC–ED technique, LODs were also calculated as the minimal concentration corresponding to 3σ/slope of the calibration curve (3σ calculated on the measured average blank). Average blank was evaluated by running operational blank analysis.

The results obtained with the LC–UV method were compared with those obtained under the same chromatographic conditions as in a previous study dealing with the application of the LC–TIS–MS method to flavonoid analysis [13].

2.6. Quercetin extraction and hydrolysis procedure

Sample treatment was as follows: ca. 4 ml of methanol, 1 ml of 12 *M* hydrochloric acid and 12 mg of BHT, as antioxidant, were added to 3.5 g of orange juice to obtain a mixture consisting of 1.5 *M* HCl in a methanol–water solution (50:50, v/v) containing 1500 mg/l BHT. This mixture was mixed and refluxed at 90°C for 1 h. After cooling, methanol was added to a final volume of 10 ml; after sonication for 5 min, a 2-ml portion ml was filtered on a PTFE membrane filter (0.45 μm) before the injection of 1-μl aliquots into the HPLC system.

2.7. Flavanone extraction

Flavanone extraction was obtained in the following way: 3 ml of methanol and 50 μl of neoeriocitrin (4000 ng/μl), as internal standard, were added to 1 g of orange juice. The mixture was stirred for 30 s and heated at 55°C for 15 min to increase hesperidin solubility. After centrifugation at 3150 *g* for 15 min, the supernatant was collected into a 10-ml flask. A second supernatant was collected by adding another aliquot of methanol (3 ml) to solid material. The final extract solution was diluted to 10 ml with water and filtered on a nylon membrane filter (0.45 μm). HPLC injections were performed after adequate sample dilution depending on the detection technique.

Table 1
Factors and levels tested (coded values in parentheses) for the central composite design

Factors	Low level (−1)	Neutral (0)	High level (+1)
HCl concentration (X_1)	1 *M*	1.5 *M*	2 *M*
Hydrolysis time (X_2)	30 min	60 min	90 min

2.8. Statistical analysis

A CCD was chosen to investigate the influence of hydrochloric acid concentration and hydrolysis time on the extraction recovery of quercetin. Each factor was tested at three levels, as shown in Table 1. A *k*-factor two-level CCD requires $2^k+2\ k+C$ experiments, where 2^k points are in the corners of the square representing the experimental domain, 2 *k* points are in the centre of each side of the square and *C* points are the replicates in the centre of the square that are necessary to estimate the variability of the experimental measurements. Therefore, considering two factors and four replicates at the centre point, this design involves 12 experiments, which were performed in random order (Table 2).

All statistical analyses and tests were carried out by using the statistical package SPSS 8.0 for Windows (SPSS, Bologna, Italy).

Table 2
Experimental matrix of the central composite design (coded levels of the factors)

Experiment	Factors	
	X_1 (HCl concentration)	X_2 (Hydrolysis time)
1	−1	−1
2	−1	0
3	−1	+1
4	0	−1
5	0	0
6	0	+1
7	+1	−1
8	+1	0
9	+1	+1
10	0	0
11	0	0
12	0	0

3. Results and discussion

3.1. LC validation methods

3.1.1. LC–UV

Several methods have been described for the determination of flavonoids in food and most of them involve RP-HPLC with standard-bore columns and UV detection [3–6,16]. In this work, we evaluated the performance of this detector in terms of response linearity, LODs and precision using a C_{18} narrow-bore column. Good separation of flavanones was obtained under isocratic elution conditions in less than 13 min, as shown in Fig. 1A. Noticeable results were obtained for flavones and flavonols: in fact, their simultaneous separation required a total analysis time as low as 12 min, while in the literature, these classes are generally analysed separately with longer analysis times [15,17]. Although it is possible to observe a partial overlap of the second and the third peak, corresponding to apigenin and kaempferol, respectively, the resolution is enough to perform quantitative analysis (Fig. 1B). Analogous separation had been obtained using the LC–MS system, even though, in the case of incomplete resolution, the selectivity of the MS detector towards compounds having different molecular masses, such as apigenin and kaempferol, proved helpful in distinguishing these signals, showing a well known advantage of this technique [13].

Good linearity of the LC–UV method was established over two orders of magnitude (r^2=0.999–1.000, n=24) for all flavonoids, as shown in Table 3. Analogously, in the case of TIS–MS detection, and operating under the same chromatographic conditions, linear correlation has been demonstrated over two decades in the 0.2–50 mg/l range for flavanones and in the 1–100 mg/l range for flavones and flavonols [13].

UV detection provided LODs, calculated as S/N= 3, in the low-ng range, i.e., one order of magnitude lower than those reported in a recent paper [7] (Table 4) and comparable to those obtained using analogous chromatographic conditions with the LC–MS systems. The improved sensitivity observed for a concentration-sensitive detector, such as a UV detector, using narrow-bore columns and a low-volume detector cell is a consequence of the reduced peak

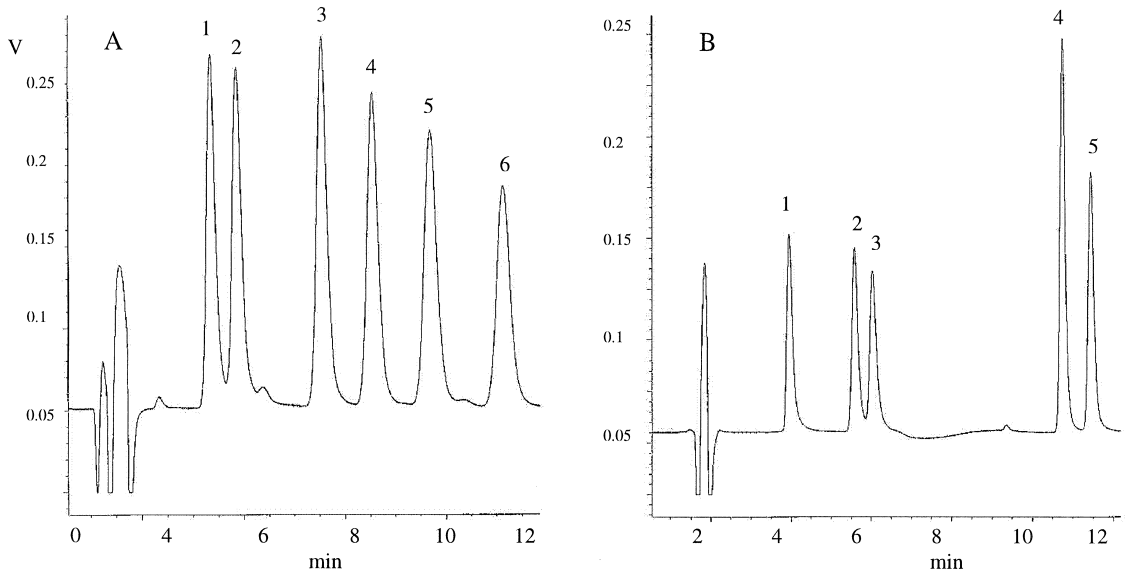

Fig. 1. LC–(UV–Vis) chromatograms of a mixture of (A) flavanones and (B) flavones and flavonols. Peaks: (A) 1, eriocitrin; 2, neoeriocitrin; 3, narirutin; 4, naringin; 5, hesperidin and 6, neohesperidin; (B) 1, quercetin; 2, apigenin; 3, kaempferol; 4, chrysin and 5, galangin. For chromatographic conditions, see Experimental.

Table 3
Linearity of response for flavonoids using LC–UV and LC–ED. Calibration fitting: $y = ax + b$[a]

Analyte	UV[b]			ED[c]	
	$a \times 10^{-2}$	b[d]	r^2 (n=24)	$a \times 10^{-6}$	r^2 (n=21)
Eriocitrin	4.950 (±0.002)	–	1.000	4.06 (±0.03)	1.000
Neoeriocitrin	3.157 (±0.002)	–	1.000	3.78 (±0.04)	1.000
Narirutin	3.701 (±0.002)	–	1.000	2.74 (±0.13)	0.998
Naringin	3.752 (±0.002)	–	1.000	2.58±0.07)	0.998
Hesperidin	3.761 (±0.002)	–	1.000	2.85 (±0.03)	1.000
Neohesperidin	3.284 (±0.002)	−2.19 (±0.01)	0.999	2.84 (±0.03)	1.000
Quercetin	0.731 (±0.001)	–	0.999	0.178(±0.006)	0.982
Apigenin	0.756 (±0.001)	0.347 (±0.002)	0.999	0.172(±0.005)	0.988
Kaempferol	0.876 (±0.000)	–	0.999	0.172(±0.006)	0.980
Chrysin	0.145 (±0.001)	–	1.000	0.128(±0.007)	0.972
Galangin	0.125 (±0.001)	–	1.000	0.057(±0.003)	0.986

[a] y=mean values of peak chromatographic areas (V); x=analyte concentration (μg/ml).
[b] 1–100 μg/ml range for flavanones; 0.5–50 μg/ml range for flavones and flavonols.
[c] 2–200 μg/ml range for flavanones; 2.5–250 μg/ml range for flavones and flavonols.
[d] $p > 0.05$.

volumes of solutes [18]. The intra- and inter-day precision, calculated as RSDs, varied from 0 to 6% (n=5) and from 0.8 to 5% (n=15), respectively. RSDs between 0.6 and 6.3% (n=5) and 1.4–5.3% (n=15) have been found for the intra- and inter-day precision of TIS–MS detection for all flavonoids [13].

3.1.2. RPLC–ED

In the case of the LC–ED technique, a preliminary study on the electrochemical activity of flavonoids was carried out by voltammetric hydrodynamic measurements to find the optimum working potential for the coulometric investigation of the analytes. The voltammograms of three flavonoids, each being a representative of the other analytes belonging to the same class, are shown in Fig. 2. The maximum electrochemical response for flavanone glycosides was found in the +0.8–+0.9 V range, whereas for flavones and flavonols in the +0.7–+0.8 V range. To improve selectivity, the values of potential were set at +0.8 V for flavanones and at +0.7 V for flavones and flavonols; higher potential values can cause a background current increase because of electrooxidation of possible impurities present in the mobile phase, resulting in a decrease in the signal-to-noise ratio.

Table 4
Limits of detection (LODs) of flavonoids using the LC–UV, LC–MS and LC–ED techniques

Analyte	LODs (μg/ml)[a]			
	UV	TIS–MS[b]	ED	ED[c]
Eriocitrin	0.2	0.08	0.5	0.13
Neoeriocitrin	0.2	0.06	0.3	0.39
Narirutin	0.2	0.1	1.0	1.21
Naringin	0.25	0.15	1.0	1.27
Hesperidin	0.3	0.1	0.5	0.78
Neohesperidin	0.3	0.1	0.5	0.80
Quercetin	0.15	0.2	0.3	0.19
Apigenin	0.08	0.4	0.5	0.60
Kaempferol	0.08	0.3	0.5	0.3
Chrysin	0.08	0.3	1.0	1.84
Galangin	0.08	0.4	1.0	0.74

[a] Calculation based on a signal-to-noise ratio of three.
[c] Calculated as 3σ/slope of the calibration curve.
[b] Calculated under selected-ion monitoring conditions.

Concerning RPLC–ED, all peaks of flavanones, except those of eriocitrin and neoeriocitrin, were baseline-resolved on a C_{18} standard-bore column. In the chromatograms illustrating the separation of flavonols and flavones (Fig. 3), it is possible to observe peak widths greater than 1 min, due to interaction of these flavonoids with the free silanols present in the stationary phase.

Linearity was established for the LC–ED technique in the 2–200 mg/l range for flavanones and in the 2.5–250 mg/l range for flavones and flavonols (Table 3); in particular, a good linearity was found for flavanones ($r^2 \geq 0.998$, n=21).

As expected, the internal diameter of the column

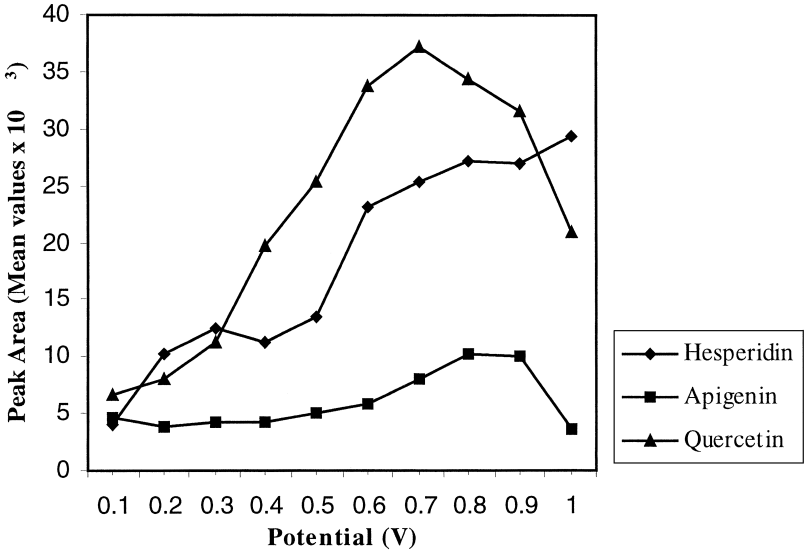

Fig. 2. Hydrodynamic voltammograms of apigenin, hesperidin and quercetin. Amount injected: 10 μg/ml. For chromatographic conditions, see Experimental.

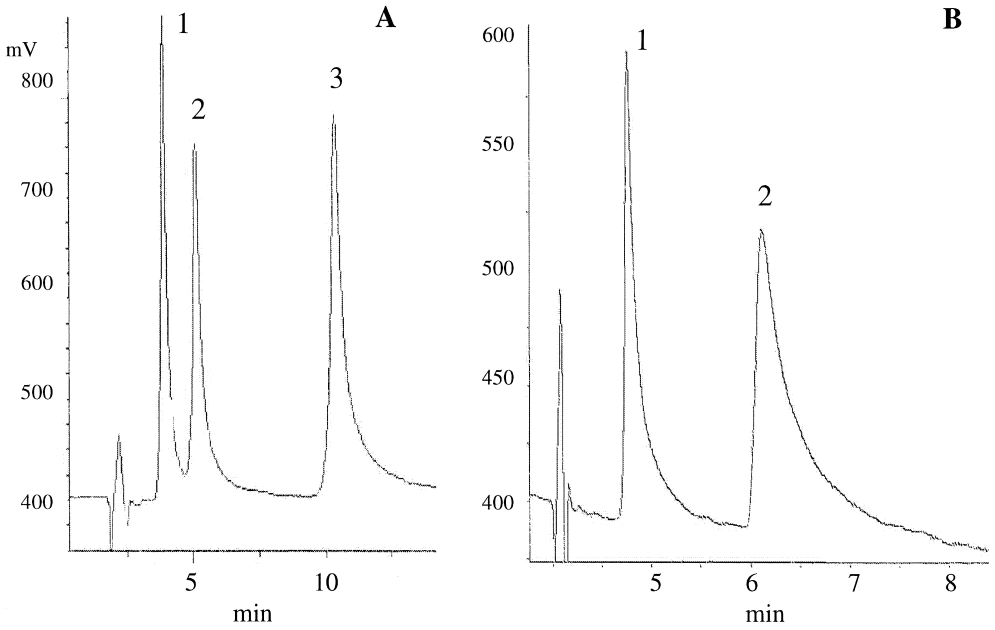

Fig. 3. LC–ED chromatogram of (A) flavonols and (B) flavones obtained using a C_{18} standard-bore column with 25 m*M* phosphate buffer–acetonitrile (50:50, v/v) at a flow-rate of 0.8 ml/min. E_2, +0.7 V. Peaks: (A) 1, quercetin; 2, kaempferol and 3, galangin; (B) 1, apigenin and 2, chrysin.

had an influence on the detectability of the analytes (Table 4). LODs determined at a signal-to-noise ratio greater than three were in agreement with those calculated as 3σ/slope of the calibration curve. The application of the electrochemical detection technique also proved valuable in terms of precision,

which was 5 and 8%, at most, for intra- and inter-day repeatability, respectively (Table 5).

3.2. Optimization of quercetin recovery

For the determination of flavonol aglycones, an hydrolysis procedure is required to break the glycosidic bonds. Depending on the sugar, several parameters can influence the extraction recovery because degradation reactions can occur or hydrolysis can be incomplete. The major factors are the molarity of HCl, the hydrolysis time, the temperature and the composition of the extraction solvent. In fact, the most polar flavonoid glycosides show enhanced solubility in water compared to the corresponding aglycones, which exhibit higher solubility in organic solvents such as methanol. A previous study [19] dealing with flavonol and flavone determination in vegetables reported that the use of a mixture made up of methanol–water (50:50, v/v) lead to good extraction of glycosides from the samples and a good partition of aglycones in a 1.2-*M* HCl solution at a temperature of 90°C. On the basis of the previous report [19], a central composite experimental design was used to investigate the effects of HCl concentration (X_1) and hydrolysis time (X_2) on the extraction recovery of quercetin from orange juice samples and to find the best extraction conditions for this compound.

By applying a multiple regression analysis on the data set (i.e. mean values of chromatographic peak area; $n=3$), it was possible to obtain a mathematical model that took linear, quadratic and cross-product terms into account (Table 6).

The regression model was as follows:

$$Y = b_0 + b_1X_1 + b_2X_2 + b_{11}X_1^2 + b_{22}X_2^2 + b_{12}X_1X_2 \quad (1)$$

In Eq. (1), the b values are the estimates of the polynomial coefficients and the X_i ($i=1,2$) values represent the coded values of the factors (hydrolysis time and hydrochloric concentration). The linear terms, b_iX_i, are responsible for the main effects; the quadratic terms, $b_{ii}X_i^2$, are responsible for the curvature effects and the cross-products bifactorial terms, $b_{ij}X_{ij}$, are responsible for the interaction effects.

The resulting mathematical model (Table 7), obtained by applying a backward variable selection procedure up to (in the final refined model) all coefficients turned out to be significant ($p<0.1$), was as follows:

$$Y = 6700 - 1100X_1^2 - 600X_2^2 - 900X_1X_2$$

In order to assess how well the mathematical model calculated fit experimental data, an analysis of variance (ANOVA) was performed (Table 8). At a 95% significance level, the p value of the F ($S^2_{\text{regression}}/S^2_{\text{residual}}$) ratio was lower than 0.002, demonstrating the goodness-of-fit of the calculated model to the experimental data.

Table 5
Intra- and inter-day precision of the LC–UV and LC–ED methods

Analyte	UV[a]: RSD (%)		ED[b]: RSD (%)	
	Intra-day[c]	Inter-day[d]	Intra-day[c]	Inter-day[d]
Eriocitrin	2	4	0.6	6
Neoeriocitrin	0.0	2	1.0	5
Narirutin	2	1.6	5	1.8
Naringin	2	3	4	5
Hesperidin	0.1	4	1.4	7
Neohesperidin	2	4	1.0	8
Quercetin	0.9	2	0.8	3
Apigenin	1.0	1.7	1.0	4
Kaempferol	0.1	0.8	2	4
Chrysin	3	3	0.4	10
Galangin	3	5	3	3

[a] 80 μg/ml for flavanones; 40 μg/ml for flavones and flavonols.
[b] 20 μg/ml for flavanones; 50 μg/ml for flavones and flavonols.
[c] $n=5$
[d] $n=15$

Table 6
Values and significance of the coefficients of the original multiple regression model

Variable	Coefficient	Standard deviation	Significance[a]
Intercept parameter (b_0)	6700	200	0.000
X_1 (b_1)	−300	200	0.142
X_2 (b_2)	200	200	0.353
X_1^2 (b_{11})	−1100	300	0.007
X_2^2 (b_{22})	−600	300	0.087
X_1X_2 (b_{12})	−900	300	0.010

[a] Significance level >0.1.

Table 7
Values and significance of the coefficient of the final multiple regression model

Variable	Coefficient	Standard deviation	Significance[a]
Intercept parameter (b_0)	6700	200	0.000
X_1^2 (b_{11})	−1100	300	0.007
X_2^2(b_{22})	−900	300	0.010
X_1X_2 (b_{12})	−500	300	0.100

[a] Significance level >0.1.

Table 8
ANOVA results

Model	*d.f.*	Mean of square	*F*-ratio	Significance[a]
Regression	3	3 220 915	11.441	0.002
Residual	9	281 533		
Total	12			

[a] Significance level >0.05.

As can be seen, in the refined model, only the quadratic terms and the cross-product terms were significant. The presence of significant interaction effects between the experimental factors (hydrolysis time and HCl concentration) and of curvature effects means that a traditional approach, i.e. considering one variable at a time, would be inadequate to describe the complex dependence of the response on the experimental conditions. Therefore, it can be inferred that the optimisation of hydrolysis conditions for quercetin glycoside in orange juice requires a simultaneous consideration of the extraction time and the molarity of the acid; optimum conditions to maximise quercetin extraction recovery corresponded to a HCl concentration of 1.5 *M* and a hydrolysis time of 1 *h*.

A good reproducibility of the method, evaluated at the central point of the experimental design, was proved by an RSD value of 1.4%.

The three-dimensional plot of the modelled response surface of quercetin is shown in Fig. 4.

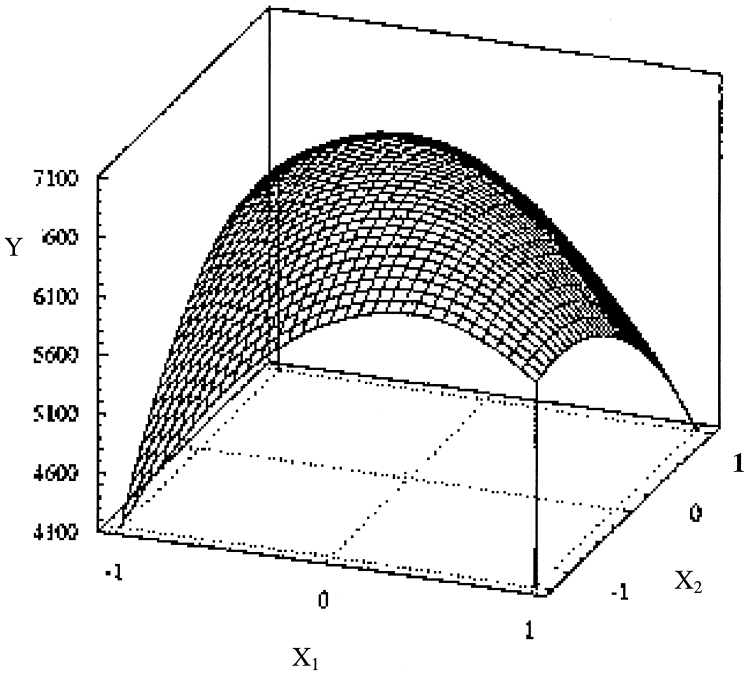

Fig. 4. Response surface of the extraction recovery of quercetin in orange juice as a function of coded HCl concentration (X_1) and hydrolysis time (X_2).

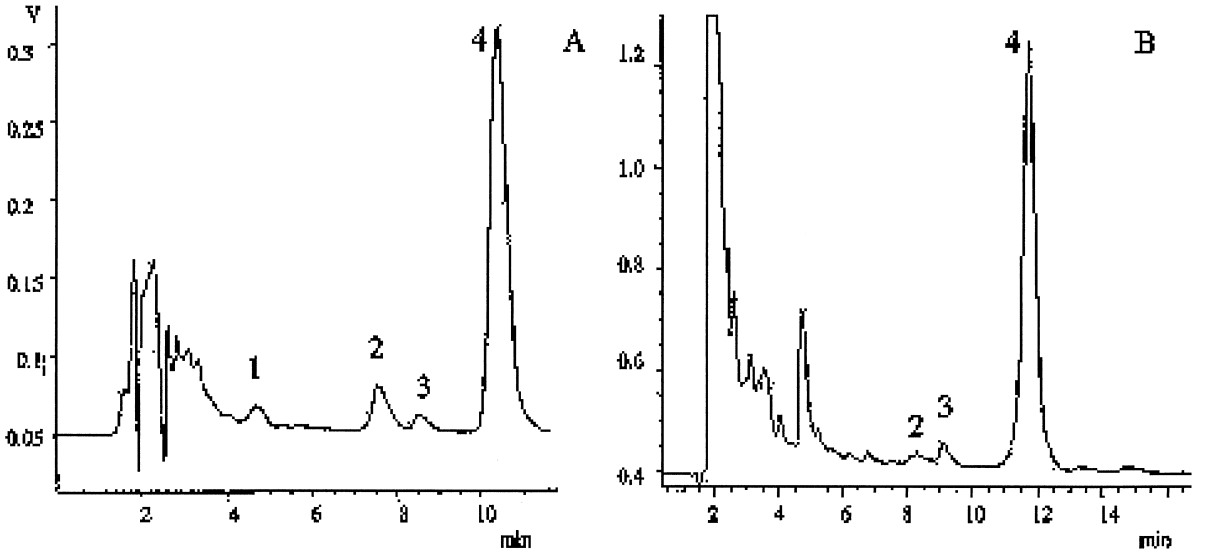

Fig. 5. (A) LC–UV and (B) LC–ED chromatograms of flavanones in an orange juice sample. Peaks: 1, eriocitrin; 2, narirutin; 3, naringin and 4, hesperidin.

3.3. Flavonoid determination in orange juice samples

In order to verify the applicability of the analytical methods proposed, a qualitative and quantitative assay of flavonoids was carried out on an orange juice sample. Fig. 5 shows the LC–UV and LC–ED chromatograms of the orange juice extract for the analysis of flavanones. On the basis of retention times, it was possible to identify the following

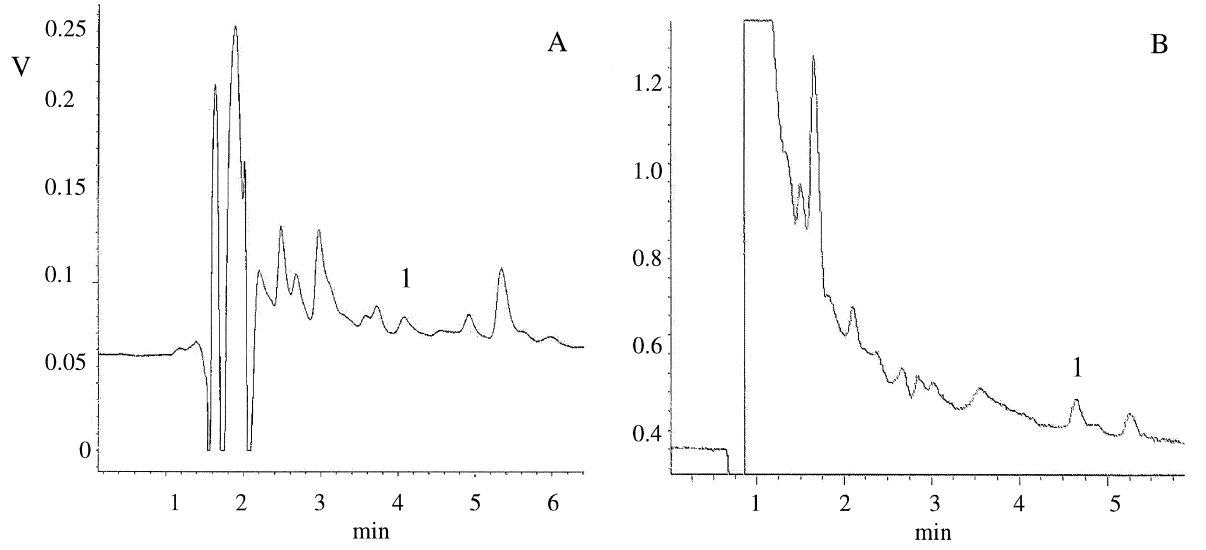

Fig. 6. (A) LC–UV–Vis and (B) LC–ED chromatograms of quercetin (1) in an orange juice sample.

Table 9
Concentration of flavonoids (mg/100 g) determined in an orange juice sample by the LC–UV, LC–ED and LC–MS techniques

Analyte	Concentration (mean±SD, $n=3$) mg/100 g		
	LC–UV	LC–ED	LC–TIS–MS[a]
Eriocitrin	1.0±0.2	n.d.[b]	0.9±0.3
Narirutin	5.10±0.17	5.0±1.6	5.4±0.2
Naringin	2.6±0.3	n.d.[b]	0.14±0.00
Hesperidin	73±7	79.3±0.5	78.9±0.4
Quercetin	1.34±0.15	1.18±0.11	0.90±0.03

[a] For chromatographic conditions, see Ref. [13].
[b] Not detected.

analytes, i.e. eriocitrin, narirutin, naringin and hesperidin. As aspected, among the flavonols, only quercetin was detected (Fig. 6). To confirm the identification of analytes, LC–TIS–MS analysis was carried out on the same samples. Hesperidin was found to be the most abundant flavonoid present in orange juice, as confirmed by other studies [20]. By comparing the quantitative analysis results for the content of flavonoids in the sample analysed, the accuracy of the methods developed was confirmed for flavonoid analysis (Table 9).

Since flavonoids are known to stick to membrane filters, two different kinds of membranes were tested: nylon and PTFE filters. Experimental data showed that the use of a nylon filter causes a loss of flavonoids, whereas no retention effect was observed with the PTFE membranes. For the quercetin recovery data, we referred to values reported in the literature and obtained under analogous conditions for acid concentration and hydrolysis time in various vegetables and fruits, in which quercetin yield greater than 85% was reported [19]. Mean recovery of flavanones from orange juice was calculated to be in the 98±2% range, and this value was obtained by spiking samples with a standard solution of neoeriocitrin; the absence of this compound, which usually is not found in orange, was verified in the sample under investigation. The higher concentration found for naringin using the LC–UV system compared with the values obtained using the LC–TIS–MS and the LC–ED methods could be explained on the basis of a possible spectrophotometric matrix interference, confirming the much greater selectivity of MS and ED detection.

4. Conclusions

The three methods compared afford a fast qualitative, quantitative and reproducible determination of flavonoids in real samples such as orange juices. In particular, the LC–UV and LC–MS methods showed high precision and sensitivity for the quantitation of flavonoids at sub-ng levels.

Quantitative data found for orange juice sample analysis using the LC–ED and LC–MS techniques demonstrated the high selectivity of these two systems due to the specificity of the applied potentials for coulometric measurements and the ability to select characteristic ions for the MS detector, in contrast to the lower specificity of UV detection caused by spectophotometric interferences.

The experimental design set-up for the optimization of the hydrolysis parameters of quercetin evidenced the significant interaction effects of the different terms (acid concentration and hydrolysis time) on the extraction recovery and allowed the determination of the best hydrolysis conditions. These findings show the usefulness of the mathematical method and how the traditional approach, which considers different factors one-at-a-time, can fail in the evaluation of the sample treatment conditions because of the possible presence of crossing effects.

Acknowledgements

This work was financially supported by Ministero dell'Università e della Ricerca Scientifica e Tecnologica (MURST) and by Consiglio Nazionale delle Ricerche (CNR), Italy.

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