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Journal of Pharmaceutical and Biomedical Analysis
35 (2004) 349–363
JOURNAL OF PHARMACEUTICAL AND BIOMEDICAL ANALYSIS
www.elsevier.com/locate/jpba

Study of the extraction procedure by experimental design and validation of a LC method for determination of flavonoids in *Citrus bergamia* juice

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Received 1 July 2003; received in revised form 2 October 2003; accepted 2 October 2003

Abstract

A reversed-phase high-performance liquid chromatographic (HPLC) separation with photo-diode array detection was developed for the simultaneous determination of flavonoids extracted from *Citrus bergamia* juice. It employs a C_{18} reversed-phase column and a linear gradient elution system with methanol/water with 5% acetic acid (v/v), as mobile phase.

The method was validated in terms of detection limits (LOD), quantitation limits (LOQ), linearity, precision and accuracy.

Limits of detection ranged from a low of 0.007 mg ml^{-1} (narirutin) to a high of 0.018 mg ml^{-1} (didymin). The limits of quantitation were between a low of 0.011 mg ml^{-1} (7-OH flavanone) and a high of 0.024 mg ml^{-1} (didymin).

An excellent linear response was observed over the range specified for all analytes, as confirmed by the correlation coefficient with ranged from 0.9982 and 0.9999.

The intra-day R.S.D.% ranged from 0.11 to 3.64%. The intermediate precision R.S.D.% were not higher than 7.62%. The accuracy of the method was confirmed with an average recovery ranging, except for neoeriocitrin, between 88.07% and 102.45%.

Since the extraction conditions can affect analyte recovery, a suitable optimization strategy of the procedure was needed. The experimental parameters optimized were extraction time, temperature, and solvents. A multivariate approach was used to provide direct evaluation of the selected variables and related interactions. The D-optimal design was constructed by applying the exchange algorithm. All experimental results were computed by NEMROD-W software. This methodology led us to obtain the best recovery for all the flavonoids in the least number of experiments.

Keywords: *Citrus bergamia*; Experimental design; HPLC; Method validation; Flavonoids

1. Introduction

Flavonoids are a widely distributed group of polyphenolic compounds characterised by a common benzo-γ-pirone structure, with health-related properties, which are especially based on their antioxidant activity [1,2]. These properties include anticancer, antiviral, anti-inflammatory activities, effects on capillary fragility and ability to inhibit human platelet aggregation [3,4]. Flavonoids are present in a wide variety of edible plants. Most *Citrus* species accumulate substantial quantities of flavonoids during the development of their different organs [5,6]. Three types of flavonoids (flavanones, flavones, flavonols) occur

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doi:10.1016/S0731-7085(03)00585-5

in *Citrus* and more than 60 individual flavonoids have been identified [7]. Whereas polymethoxylated flavones (PMfs) are generally found in large amounts in the peel of some *Citrus*, flavanones, are the most abundant and are specific of the juice [8]. Recently, dietary *Citrus* flavonoids have been associated with reduced risk of coronary heart disease in epidemiological studies which placed a new perspective on these food components; they have been suggested as one of the possible cancer-preventing agents and their influence on the metabolism of acid arachidonic and histamine release has been reported [9–13]. There have been reports on the HPLC of *Citrus* flavonoids, but none of these was about flavonoids analysis in *Citrus bergamia* juice [14–20].

So far this typical fruit of Reggio Calabria province has been used only for the extraction of the essential oil from the peel; in fact oils are the most profitable products of the industrial processing of Bergamot. *C. bergamia* juice obtained from the endocarp after essential oils extraction is considered just a secondary and discarded product in the working diagram of this *Citrus* fruit [21].

On these grounds it seemed interesting to investigate all possible uses of the juice in order to take advantage of the large amount of this discarded product.

Considering the possible potentialities of its principal component we aimed our studies on flavonoids investigation in bergamot juice. The presence of these polyphenolic compounds with their health-related properties could lead to a complete and rational use of *C. bergamia*.

As a first step, an improved and sensitive analytical method was developed for the separation and determination of flavonoids in extracts from *Citrus* juice, using HPLC with photodiode-array detection.

Since the extraction conditions can affect analyte recovery, a suitable optimization strategy of the procedure was needed. The experimental parameters optimized were extraction time, temperature, and solvents. A multivariate approach was used to provide direct evaluation of the selected variables and related interactions [22]. In particular the extraction procedure was investigated by performing a statistical data treatment based on a D-optimal design. This experimental design methodology led us to reduce the number of analyses by choosing “informative” experiments in such a way as to obtain information which is uniformly spread over the region of interest. The results obtained on these selected points allow to the knowledge of the qualities of all the extraction procedures by using predictive mathematical models. The validity of this forecast is independent from experimental results and depends only on the form of the postulated model and on the choice of experiments [23,24]. This methodology led us to obtain good recovery of all flavonoids determined with respect to a reduced number of experiments.

In a further step, the method was validated in terms of detection limits, quantitation limits, linearity, precision and accuracy.

Application of this validated LC method provides novel information on the flavonoids composition in *C. bergamia* juice (eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, didymin, 7-OH flavanone) and could be an excellent approach to the quantitative analysis of the identified flavonoids in different matrices.

2. Experimental section

2.1. Chemicals and reagents

Eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, didymin and 7-hydroxyflavanone were purchased from Extrasynthese (Genay, France).

Water was distilled, deionised and filtered through 0.22 μm Millipore® GSWP filters (Bedford USA).

Methanol and acetic acid, both high-performance liquid chromatography grade purity were provided from Merck (Darmstadt, Germany).

Ethyl acetate and acetonitrile, both analysis grade, were provided from Merck (Darmstadt, Germany). Hexane, diethyl ether were purchased from Carlo Erba (Milano, Italy). *C. bergamia* juice was provided from Malara s.r.l. (Reggio Calabria, Italy). All the preparations (solutions and extracts) were filtered through 0.45 μm membrans Millipore® and degassed in an ultrasonic bath before use.

2.2. Apparatus

The HPLC apparatus was a Perkin–Elmer chromatographic system (Series 410 liquid chromatograph) equipped with a septumless injector (Rheodyne

7125-075) with a 6 μl sample loop, and column heater (Perkin–Elmer TC 931). A diode array detector (DAD) (Perkin–Elmer LC 235) was used. Peak area integration was performed using a chromatographic data system (Perkin–Elmer LCI-100 Laboratory Computing Integrator).

Chromatographic separations were performed on a RP-18 Perkin–Elmer (25cm × 4.6 mm i.d., particle size 5 μm) column.

C. bergamia juice was lyophilized by instrument Basi & C. BVF.6/R.

The extraction procedures were performed by using a Haake C25 thermostated bath equipped with a Haake F6 controller which allows variation of temperature with an accuracy of ±0.01 °C.

The experimental design and statistical analysis of the data were performed by NEMROD-W Software (LPRAI, Marseille, France) [25].

2.3. Sample treatment

C. bergamia var.Risso fruits from trees of different cultivars (*fantastico, femminello, castagnaro*) were collected between November 2001 to February 2002 in the plantation of Melito (Reggio Calabria). The fresh fruits were treated by specific procedure by Malara s.r.l. and the juice obtained was immediately lyophilised.

2.4. Extraction procedure

In order to determine the most convenient method for flavonoids extraction, several procedures, described below, were assayed. In all cases, 250 mg of lyophilised material were accurately weighed into a glass stoppered vessel. A 5 ml volume of solvent A (ether or hexane) was added to the sample and shaken briefly. The mixture was extracted under controlled temperature (25 or 40 °C), using a magnetic stirring plate for a fixed extraction time (20 or 40 min). After stirring, the organic supernatant was eliminated and the procedure was repeated once more. The residue was extracted with 5 ml of solvent B (ethyl acetate, methanol or acetonitrile), using the same temperature and time conditions. The extraction solvent was decanted and the remaining solid residue was extracted another four times, following the same procedure. At the end, the five collected extracts were evaporated to dryness; the residue was redissolved in 2 ml of methanol and filtered through a 0.45 μm filter, prior injection into the chromatographic system.

2.5. Experimental design

The performance of the extraction procedure depends not only on the type of solvent, but also other parameters can have an important effect on analytes recovery. An experimental design was applied in order to investigate the effect of four process variables. The selected factors were temperature (X_1), extraction time (X_2), solvent A (X_3) and solvent B (X_4). The variables X_1, X_2 are continuous quantitative factors, whereas the variables X_3, X_4 are qualitative ones. To be homogeneous and to simplify the mathematical treatment, X_1 and X_2 are considered as quantitative discrete factors fixed at two definite levels. As regards qualitative factors, the first one (X_3) was fixed at two levels, whilst the other one (X_4) at three levels. The considered experimental responses (Yi) were the found amounts of each flavonoid (mg ml^{-1}).

A model suitable for the description of the selected variables was postulated to represent the variations of the experimental responses Y_i, as follows:

$$Y_i = b_0 + b_A X_1 + b_B X_2 + b_C X_3 + b_{D\alpha} X_4 + b_{D\beta} X_4 + b_{AB} X_1 X_2 + b_{BD\alpha} X_2 X_4 + b_{DB\beta} X_2 X_4$$

where b_0 is the average experimental response, the coefficients b_A to b_D represent the estimated main effects of the corresponding factors, while the coefficients b_{AB} and b_{BD} are the interaction terms.

Few interaction terms (X_1X_2 and X_2X_4) were considered useful for description of the analytical process; this assumption was supported by previous experiments.

The influence of different process factors on the extraction procedure could be studied by using classical designs such as factorial, central composite designs. It is also possible to select irregularly shaped designs based on optimality criteria such as D-optimality, A-efficiency and variance function. The D-criterion is based on the minimization of the determinant (D) of the dispersion matrix; A-optimality is based on the minimization of the trace of the dispersion matrix; the smallest variance function leads to the best prediction of the experimental response [26].

A complete asymmetric factorial matrix for one three-level and three two-level factors ($2^3 \times 3^1$) requires 24 experiments, or 48 if each of them is repeated once. But carrying out 48 experiments is, of course, unnecessary to solve a model with only nine coefficients. Furthermore, for the flavonoids determination, the goal was to improve existing methodology to obtain maximum recovery with a minimum amount of time.

Starting from the complete asymmetric factorial matrix, optimality criteria allow us to select a subset of candidate points which represent a compromise between a minimal number of experiments and enough information to precisely estimate the model coefficients. The D-optimal design was constructed by applying the exchange algorithm [26].

2.6. Stock solutions

Stock solutions of eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, didymin and 7-OH flavanone were prepared in methanol at a concentration of 0.8 mg ml^{-1}.

A methanolic solution containing 0.8 mg ml^{-1} of cathechin as internal standard, was also prepared. Working standards were stored at 4 °C and protected from daylight.

2.7. HPLC conditions

The analysis was monitored at 280 nm and the absorption spectra of compounds were recorded between 210–350 nm. For the simultaneous separation of flavonoids, a two-solvent gradient system was used. Solvent A was 5% (v/v) acetic acid in water, solvent B was methanol. The gradient programme consisted of three periods: (1) 0–10 min, 80/20% (v/v) A/B, isocratic; (2) 10–12 min, 80–74% (v/v) A in B, linear gradient; (3) 12–77 min, 74–31% (v/v) A in B, linear gradient. The initial mobile phase composition was restored in 15 min and held for 20 min. The column was operated at 25 °C and the flow rate was 1 ml min^{-1}. Identification of compounds was performed by comparing their t_R with those of standards and confirmed with characteristic spectra using the photodiode array detector. This procedure also confirmed the purity of the peak. Concentrations of the compounds were calculated from integrated peak areas of the sample and corresponding standard.

2.8. Validation procedure

The detection limit (LOD) and the quantitation limit (LOQ) were expressed as signals (y_d and y_q, respectively) on the basis of the mean value ($\bar{y}_b$) and the standard deviation (s_b) of the blank responses, as follows:

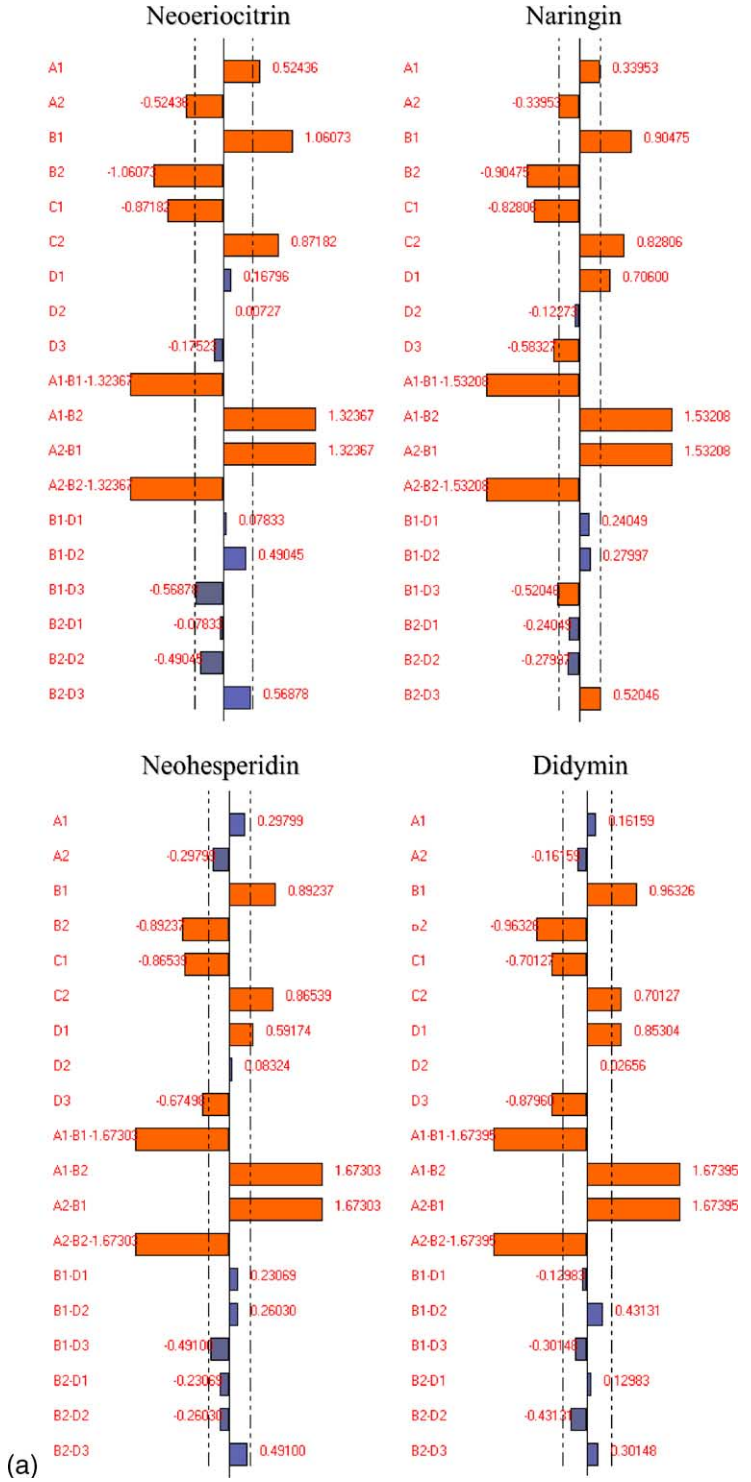
$$y_d = \bar{y}_b + 2ts_b, \qquad y_q = \bar{y}_b + 10s_b.$$

where t is the constant from the Student (one sided) t-test, that depends on the confidence level and on the degrees of freedom ($\nu = n - 1$, n = number of measurements). A 95% confidence level was chosen. For $\bar{y}_b$ and s_b determination, ten blank measurements were performed ($n = 10$). The concentration values for the detection limit (LOD) and the quantitation limit (LOQ) were obtained by using a calibration curve calculated for each analyte in the 0.02–0.24 mg ml^{-1} range.

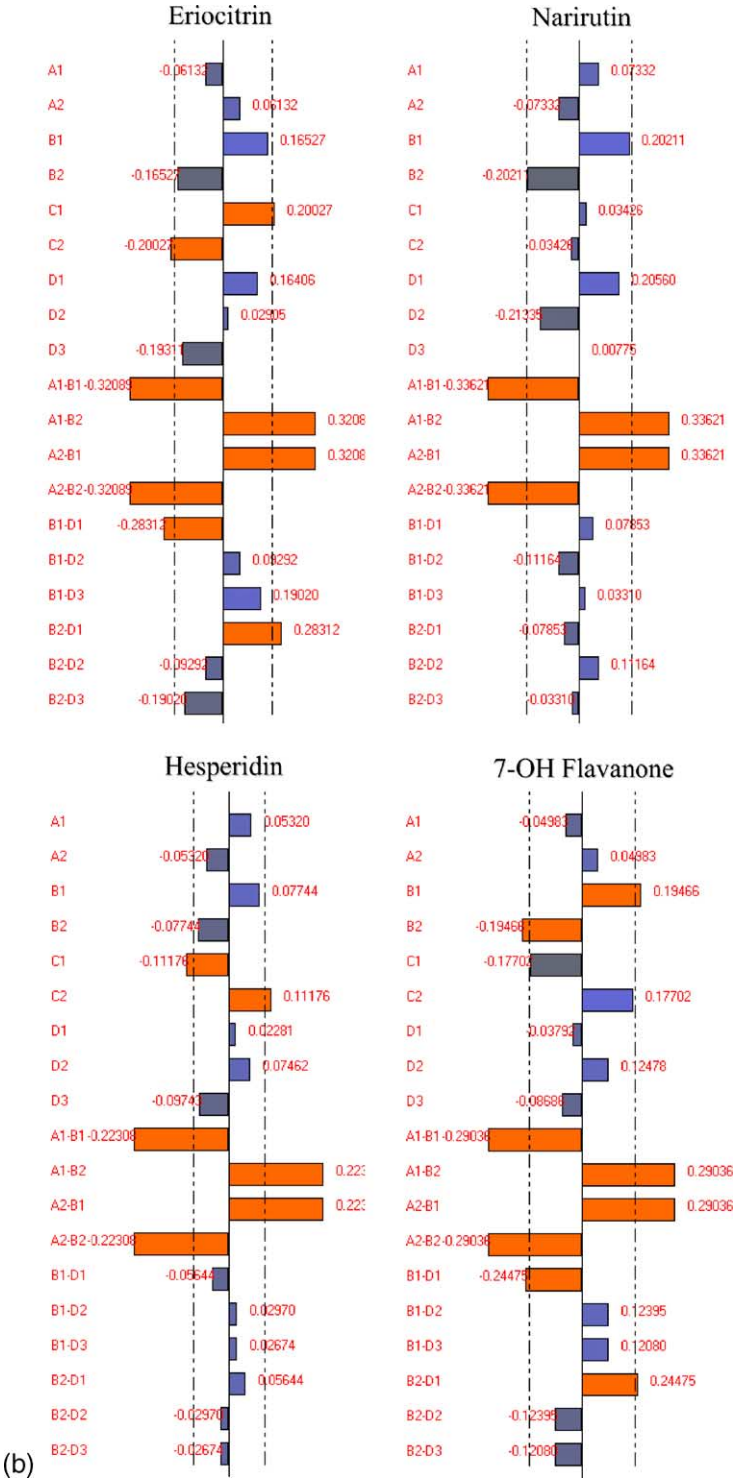
The linearity of the method was studied in the 0.02–0.8 mg ml^{-1} range. Six concentration levels (0.8, 0.32, 0.24, 0.16, 0.08, 0.02 mg ml^{-1}) of the eight standard flavonoids were chosen. Five determinations ($n = 5$) were carried out for each solution. Quantitative analysis was accomplished using the internal standard method. The correlation graphs were constructed by plotting the peak areas obtained versus the injected amounts.

The intra-day repeatability and the intermediate precision on two different days were calculated at three concentration levels (0.08, 0.32, 0.8 mg ml^{-1}) for each analyte and expressed as R.S.D. (%). Three determinations ($n = 3$) were carried out for each

Fig. 1. (a) Graphical analysis of the factor effects for neoeriocitrin, naringin, neohesperidin, didymin. The histograms show the partial effects of each factor on the experimental responses when this factor is set at level 1, 2 or 3. Each level is named with an arbitrary symbol (A_1, A_2, B_1, ..., etc.). (b) Graphical analysis of the factor effects for eriocitrin, narirutin, hesperidin, 7-OH flavanone. The histograms show the partial effects of each factor on the experimental responses when this factor is set at level 1, 2 or 3. Each level is named with an arbitrary symbol (A_1, A_2, B_1, ..., etc.).


Neoeriocitrin
A1 0.52436
A2 -0.52436
B1 1.06073
B2 -1.06073
C1 -0.87182
C2 0.87182
D1 0.16796
D2 0.00727
D3 -0.17523
A1-B1 -1.32367
A1-B2 1.32367
A2-B1 1.32367
A2-B2 -1.32367
B1-D1 0.07833
B1-D2 0.49045
B1-D3 -0.56878
B2-D1 -0.07833
B2-D2 -0.49045
B2-D3 0.56878
Naringin
A1 0.33953
A2 -0.33953
B1 0.90475
B2 -0.90475
C1 -0.82806
C2 0.82806
D1 0.70600
D2 -0.12273
D3 -0.58327
A1-B1 -1.53208
A1-B2 1.53208
A2-B1 1.53208
A2-B2 -1.53208
B1-D1 0.24049
B1-D2 0.27997
B1-D3 -0.52046
B2-D1 -0.24049
B2-D2 -0.27997
B2-D3 0.52046
Neohesperidin
A1 0.29799
A2 -0.29799
B1 0.89237
B2 -0.89237
C1 -0.86539
C2 0.86539
D1 0.59174
D2 0.08324
D3 -0.67498
A1-B1 -1.67303
A1-B2 1.67303
A2-B1 1.67303
A2-B2 -1.67303
B1-D1 0.23069
B1-D2 0.26030
B1-D3 -0.49100
B2-D1 -0.23069
B2-D2 -0.26030
B2-D3 0.49100
Didymin
A1 0.16159
A2 -0.16159
B1 0.96326
B2 -0.96326
C1 -0.70127
C2 0.70127
D1 0.85304
D2 0.02656
D3 -0.87960
A1-B1 -1.67395
A1-B2 1.67395
A2-B1 1.67395
A2-B2 -1.67395
B1-D1 -0.12983
B1-D2 0.43131
B1-D3 -0.30148
B2-D1 0.12983
B2-D2 -0.43131
B2-D3 0.30148

(a)



(b)

Fig. 1. (*Continued*),

solution. The accuracy of the method was confirmed by analysing the mixture prepared by adding suitable amounts of standard flavonoids at three concentration levels (0.036, 0.052, 0.1 mg ml^{-1}) to *C. bergamia* extract, containing known amounts of analysed flavonoids. Three determinations ($n = 3$) were carried out for each solution. Recovery of each flavonoid was calculated as follows:

$$\left[\frac{\text{Amount measured} - \text{Amount pure extract}}{\text{Amount added}}\right] \times 100$$

3. Results and discussion

3.1. Experimental design

The D-optimal design was constructed by applying the exchange algorithm and consisted of 13 experiments.

The appropriate selection of the experimental domain for each factor, described in Table 1, was made from prior experience and knowledge of the analytical process. Selection of maximum and minimum values for the factors is critical for generation of useful data. Practical considerations, such as low stability of flavonoids at high temperature and analysis time, place limitations on value selection. As regards the choice of the appropriate extraction solvent, it was considered a suitable polarity range ($p' = 0.1$–5.8).

All experiments were carried out in randomized order to minimize the effects of uncontrolled factors that may introduce a bias on the measurements. In order to estimate the experimental error variance, each analysis was replicated. All experimental results were computed by NEMROD-W software. D-optimal design, expressed in real variables U_i, and the corresponding experimental responses are shown in Table 2.

Table 1
Experimental domain for the selected factors

	Factors	N. levels	Levels[a]
U_1	Temperature	2	A_1: 25 °C A_2: 40 °C
U_2	Extraction Time	2	B_1: 20 min B_2: 40 min
U_3	Solvent A	2	C_1: Ether C_2: Hexane
U_4	Solvent B	3	D_1: Ethyl acetate D_2: Methanol D_3: Acetonitrile

[a] Each level is named with an arbitrary symbol (A_1, A_2, B_1, ..., etc.).

The coefficients of the polynomial models were estimated by the least square regression and are reported in Table 3. Classical statistical tools, as analysis of variance (ANOVA) and residual analysis, were employed to validate the mathematical models. ANOVA results are given in Table 4. A significant relationship between the chosen parameters and the experimental responses is proved, confirming that the selected coefficients give real information about the studied phenomenon. The models are highly significant and fitted accurately to the experimental data.

The interpretation of data can be obtained by means of a graphical analysis, in which effects due to the selected parameters and interactions are represented. The histograms reported in Fig. 1a and b show the partial effects of each factor on experimental responses, when it is set at level 1, 2 or 3 and the corresponding interaction terms. The length of each bar graph is proportional to the effect value. The coefficients that exceed the reference lines, corresponding to 95% confidence interval, are significant. As results from Fig. 1a and b, the recovery of the studied compounds can vary not only with different types of solvents A and B employed during extraction procedure, but also with the assumed values for the other two variables, temperature and extraction time. In particular, effects due to the temperature can be strongly affected by extraction time. As regards the flavonoids present in higher amount in *C. bergamia* extract (naringin, neohesperidin, didymin, neoeriocitrin) the graphical analysis of the factors shows a positive effect on flavonoids recovery when the hexane (solvent A) and ethyl acetate (solvent B) are employed, respectively, in the first and the second phase of extraction procedure; furthermore the interaction between temperature and extraction time is significant, evidencing better results when temperature is maximized and extraction time is minimized (Fig. 1a). Similar effects of solvent A, temperature and extraction time are noticed for the trace flavonoids (eriocitrin, narirutin, hesperidin, 7-OH flavanone). Only a negative effect on the eriocitrin recovery is noted when hexane is employed as solvent A.

Table 2
D-optimal design: experimental plain and content of extracted flavonoids from *Citrus bergamia* juice from various extraction procedures selected

No.	T (°C)	Time (min)	Solv. A	Solv. B	Eriocitrin (mg ml^{-1})[a]	Neoeriocitrin (mg ml^{-1})[a]	Narirutin (mg ml^{-1})[a]	Naringin (mg ml^{-1})[a]	Hesperidin (mg ml^{-1})[a]	Neohesperidin (mg ml^{-1})[a]	Didymin (mg ml^{-1})[a]	7-OH flavanone (mg ml^{-1})[a]
1	25	20	Ether	E.A.	0.0279	0.1280	0.0431	0.1154	0.0186	0.1242	0.1374	0.0050
2	40	20	Ether	E.A.	0.0401	0.1487	0.0468	0.1384	0.0265	0.1519	0.1694	0.0122
3	25	20	Hexane	E.A.	0.0214	0.1340	0.0354	0.1265	0.0208	0.1400	0.1410	0.0008
4	25	40	Hexane	E.A.	0.0306	0.1380	0.0413	0.1309	0.0243	0.1449	0.1592	0.0126
5	40	40	Hexane	E.A.	0.0262	0.1157	0.0339	0.1100	0.0199	0.1221	0.1314	0.0067
6	40	20	Ether	MeOH	0.0366	0.1357	0.0412	0.1288	0.0227	0.1420	0.1540	0.0123
7	40	40	Ether	MeOH	0.0222	0.0828	0.0261	0.0752	0.0151	0.0916	0.0890	0.0068
8	25	20	Hexane	MeOH	0.0234	0.1397	0.0450	0.1225	0.0222	0.1355	0.1383	0.0084
9	25	40	Hexane	MeOH	0.0320	0.1427	0.0407	0.1328	0.0247	0.1471	0.1515	0.0093
10	25	20	Ether	ACN	0.0292	0.1164	0.0352	0.1014	0.0196	0.1133	0.1144	0.0079
11	25	40	Ether	ACN	0.0238	0.1292	0.0337	0.1181	0.0201	0.1299	0.1329	0.0057
12	40	20	Hexane	ACN	0.0249	0.1376	0.0382	0.1287	0.0229	0.1355	0.1515	0.0118
13	40	40	Hexane	ACN	0.0334	0.1146	0.0379	0.1027	0.0186	0.1139	0.1116	0.0075

[a] mg/250 mg lyophilised juice.

Table 3
Estimates of the model coefficients and corresponding standard deviations (S.D.) for the eight measured responses

Coefficients	Y_1, eriocitrin (mg ml^{-1})	Y_2, neoeriocitrin (mg ml^{-1})	Y_3, narirutin (mg ml^{-1})	Y_4, naringin (mg ml^{-1})	Y_5, hesperidin (mg ml^{-1})	Y_6, neohesperidin (mg ml^{-1})	Y_7, didymin (mg ml^{-1})	Y_8, 7-OH flavanone (mg ml^{-1})
b_0 ± S.D.	2.629[a] ± 0.079	15.289[a] ± 0.195	3.699[a] ± 0.092	15.075[a] ± 0.160	2.628[a] ± 0.040	17.315[a] ± 0.176	15.369[a] ± 0.206	1.868[a] ± 0.077
b_A ± S.D.	−0.061 ± 0.079	0.524[a] ± 0.194	0.073 ± 0.091	0.339[a] ± 0.158	0.053 ± 0.039	0.298 ± 0.174	0.161 ± 0.204	−0.050 ± 0.077
b_B ± S.D.	0.165 ± 0.084	1.061[a] ± 0.207	0.202 ± 0.097	0.905[a] ± 0.169	0.077 ± 0.042	0.892[a] ± 0.187	0.963[a] ± 0.219	0.195[a] ± 0.082
b_C ± S.D.	0.200[a] ± 0.087	−0.872[a] ± 0.215	0.034 ± 0.101	−0.828[a] ± 0.176	−0.112[a] ± 0.044	−0.865[a] ± 0.194	−0.701[a] ± 0.227	−0.177 ± 0.085
$b_{D\alpha}$ ± S.D.	0.164 ± 0.108	0.168 ± 0.266	0.205 ± 0.125	0.706[a] ± 0.217	0.023 ± 0.054	0.592[a] ± 0.240	0.853[a] ± 0.281	−0.038 ± 0.105
$b_{D\beta}$ ± S.D.	0.029 ± 0.120	0.007 ± 0.296	−0.213 ± 0.139	−0.123 ± 0.242	0.075 ± 0.060	0.083 ± 0.267	0.026 ± 0.312	0.125 ± 0.117
b_{AB} ± S.D.	−0.321[a] ± 0.079	−1.324[a] ± 0.195	−0.336[a] ± 0.092	−1.532[a] ± 0.156	−0.223[a] ± 0.040	−1.673[a] ± 0.176	−1.674[a] ± 0.206	−0.290[a] ± 0.077
$b_{BD\alpha}$ ± S.D.	−0.283[a] ± 0.111	0.078 ± 0.273	0.078 ± 0.128	0.240 ± 0.223	−0.056 ± 0.055	0.231 ± 0.246	−0.130 ± 0.288	−0.245[a] ± 0.108
$b_{BD\beta}$ ± S.D.	0.093 ± 0.12	0.490 ± 0.295	−0.112 ± 0.139	0.280 ± 0.242	0.030 ± 0.060	0.260 ± 0.266	0.431 ± 0.312	0.124 ± 0.117

[a] Significant coefficient.

Table 4
Analysis of variance for the eight mesured responses (content of each extracted flavonoid)

Source of variation	Sum of squares	Degrees of freedom	Mean square	*F*-ratio	Significance
Eriocitrin (mg ml^{-1})					
Regression	5.717	8	0.715	5.119	0.338**
Residuals	2.094	15	0.140		
Lack of fit	0.402	4	0.100	0.653	63.9
Pure Error	1.692	11	0.154		
Total	7.811	23			
Neoeriocitrin (mg ml^{-1})					
Regression	64.794	8	8.099	9.552	0.0138***
Residuals	12.719	15	0.848		
Lack of fit	5.266	4	1.316	1.943	17.3
Pure Error	7.453	11	0.678		
Total	77.513	23			
Narirutin (mg ml^{-1})					
Regression	4.208	8	0.526	2.802	4.07*
Residuals	2.815	15	0.188		
Lack of fit	1.276	4	0.319	2.279	12.6
Pure Error	1.539	11	0.140		
Total	7.023	23			
Naringin (mg ml^{-1})					
Regression	76.349	8	9.544	16.805	<0.01***
Residuals	8.519	15	0.568		
Lack of fit	3.953	4	0.988	2.381	11.5
Pure Error	4.565	11	0.415		
Total	84.867	23			
Hesperidin (mg ml^{-1})					
Regression	1.637	8	0.205	5.818	0.183**
Residuals	0.527	15	0.035		
Lack of fit	0.247	4	0.062	2.427	11.0
Pure Error	0.280	11	0.025		
Total	2.164	23			
Neohesperidin (mg ml^{-1})					
Regression	86.061	8	10.758	15.626	<0.01***
Residuals	10.327	15	0.688		
Lack of fit	4.875	4	1.219	2.459	10.7
Pure Error	5.451	11	0.496		
Total	96.388	23			
Didymin (mg ml^{-1})					
Regression	91.816	8	11.477	12.144	<0.01***
Residuals	14.175	15	0.945		
Lack of fit	5.068	4	1.267	1.530	26.0
Pure Error	9.108	11	0.828		
Total	105.992	23			
7-OH Flavanone (mg ml^{-1})					
Regression	4.474	8	0.559	4.218	0.812**
Residuals	1.989	15	0.133		
Lack of fit	1.008	4	0.252	2.825	7.7
Pure Error	0.981	11	0.089		
Total	6.463	23			

* Significance level <0.05.
** Significance level <0.01.
*** Significance level <0.001.

In any case, all factors have a slightly influence on trace flavonoids recovery (Fig. 1b).

The experimental parameters are set considering the fixed aim: to obtain highest recoveries of trace flavonoids, an optimal condition can be obtained by using ether and ethyl acetate as extraction solvents; to improve total flavonoid content extracted by *C. bergamia* juice, hexane and ethyl acetate give better results. In both cases, temperature $\geq 40\,^{\circ}C$ and extraction time ≤ 20 min values will be fixed as well. The maximum levels of flavonoids extracted with optimizated procedure are listed in Table 5.

3.2. Chromatographic determination and method validation

A sensible chromatographic separation of eight flavonoids extracted from *C. bergamia* juice was developed on a reversed-phase C_{18} column under linear gradient system, using an organic aqueous mobile phase (for the elution system see Experimental section). As shown in the UV chromatogram (Fig. 2) baseline resolution was obtained for all the analytes. The first compounds eluted were flavanones rutinosides, such as eriocitrin, narirutin, hesperidin and didymin, immediately followed by the relative neohesperidosides, such as neoeriocitrin, naringin, neohesperidin. The relative structures of the investigated flavonoids are given in Fig. 3. Eriocitrin, neoeriocitrin, narirutin, naringin, hesperidin, neohesperidin, didymin, 7-OH-flavanone were identified in extract by comparing their retention times and UV spectra with standards and by subsequent enrichment of the sample. In order to determine the authenticity of our standards, each one was dissolved in methanol and their UV maxima were determined. The reported maxima were well correlated with those obtained by photo-diode-array detector during the experimental runs. In addition, application of peak purity software to the DAD data indicated no impurities present in the chromatographic peaks of interest.

Quantitative assay of compounds identified in the extract was carried out by using the LC-UV method developed and a suitable calibration curve, obtained by applying the internal standard method (Table 6). The flavonoid content of the *C. bergamia* juice was measured by using the selected optimal extraction conditions. Table 5 gives the results of the HPLC

Table 5
Maximum levels of flavonoids extracted from *C. bergamia* with optimised extraction procedure

T (°C)	Time (min)	Solv. A	Solv. B	Eriocitrin (mg ml^{-1})[a]	Neoeriocitrin (mg ml^{-1})[a]	Narirutin (mg ml^{-1})[a]	Naringin (mg ml^{-1})[a]	Hesperidin (mg ml^{-1})[a]	Neohesperidin (mg ml^{-1})[a]	Didymin (mg ml^{-1})[a]	7-OH Flavanone (mg ml^{-1})[a]
40	20	Hexane	E.A.	0.0370	0.1511	0.0456	0.1504	0.0305	0.1623	0.1757	0.0112

[a] mg/250 mg lyophilised juice.

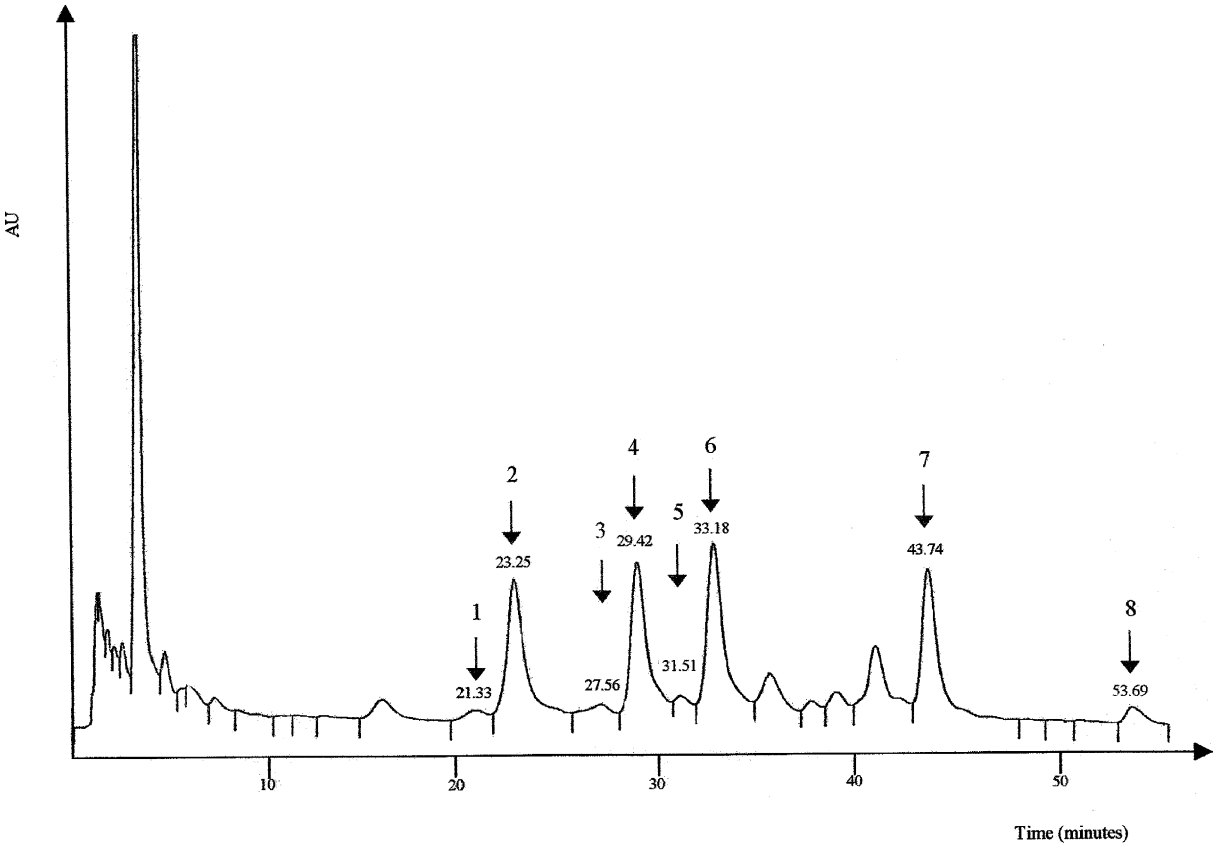


Fig. 2. LC-UV chromatogram of *C. bergamia* juice extract. In increasing retention order: (1) Eriocitrin, (2) Neoeriocitrin, (3) Narirutin, (4) Naringin, (5) Hesperidin, (6) Neohesperidin, (7) Didymin, (8) 7-Hydroxyflavanone. HPLC conditions, column: $C_{18;}$ mobile phase: water/acid acetic (95/5 v/v) (A) and methanol (B) with a linear gradient system; temperature: 25°C; flow-rate: 1 ml min^{-1}; detection wavelength: 280 nm.

analysis expressed as milligram of flavonoid per 250 mg lyophilised juice.

In a further step, the LC method was validated in terms of detection limits (LOD), quantitation limits (LOQ), linearity, precision and accuracy, in accordance with ICH guidelines [27].

In order to develop an analytical method suitable for quantitative determination of trace flavonoids in

Table 6
HPLC-UV linearity of the investigated flavonoids

Analyte	Linear range (mg ml^{-1})	Slope ± S.E.	Intercept ± S.E.	r^2 ($n = 5$)
Eriocitrin	0.02–0.8	4.2104 ± 0.05	−0.0079 ± 0.02	0.9996
Neoeriocitrin	0.02–0.8	5.1821 ± 0.15	−0.0038 ± 0.05	0.9982
Narirutin	0.02–0.8	4.3522 ± 0.08	−0.0098 ± 0.03	0.9992
Naringin	0.02–0.8	5.3649 ± 0.03	0.0377 ± 0.01	0.9998
Hesperidin	0.02–0.8	4.8669 ± 0.02	0.0195 ± 0.01	0.9999
NeoHesperidin	0.02–0.8	5.7583 ± 0.01	−0.0003 ± 0.006	0.9999
Didymin	0.02–0.8	4.9539 ± 0.01	−0.0088 ± 0.01	0.9999
7-OH Flavanone	0.02–0.8	8.6337 ± 0.02	0.0093 ± 0.01	0.9999

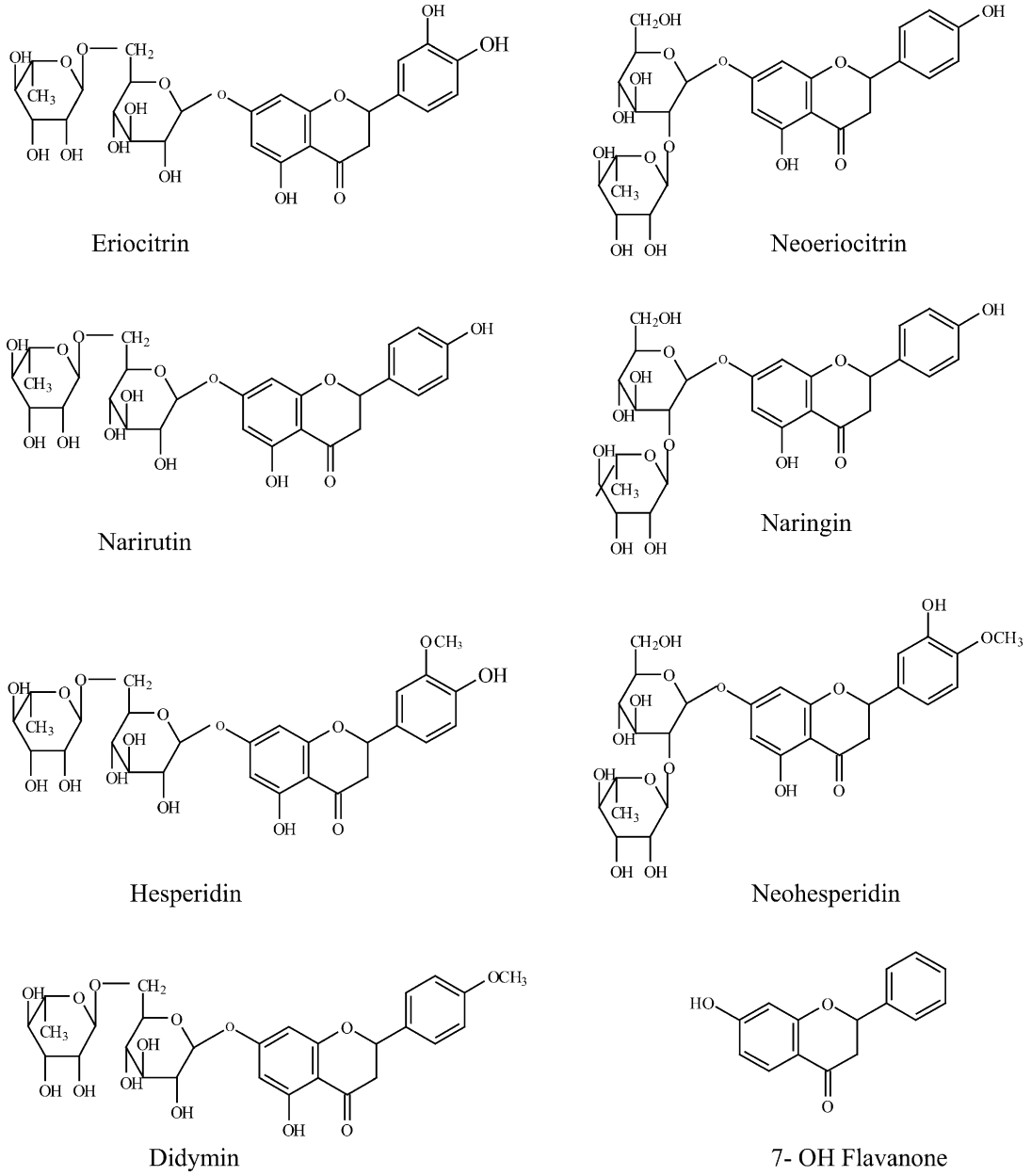


Fig. 3. Structures of flavonoids investigated.

C. bergamia juice, LOD and LOQ were statistically determined for each analyte. According to ICH guidelines, several approaches for determining LOD and LOQ are possible. In this work, the signals, corresponding, respectively, to the detection limit (y_d) and the quantitation limit (y_q) were expressed on the basis of the mean value ($\bar{y}_b$) and the standard deviation (s_b) of the blank signals, as described in the experimental section. A signal value usually cannot be used for direct comparison of different methods, because it depends on various instrument settings. A conversion from the signal domain (y_d, y_q) to the concentration

Table 7
Detection limits (LOD) and quantitation limits (LOQ) of the investigated flavonoids

Analyte	LOD (mg ml^{-1})	LOQ (mg ml^{-1})
Eriocitrin	0.014	0.021
Neoeriocitrin	0.018	0.023
Narirutin	0.007	0.014
Naringin	0.013	0.018
Hesperidin	0.010	0.016
NeoHesperidin	0.014	0.019
Didymin	0.018	0.024
7-OH Flavanone	0.008	0.011

domain (mg ml^{-1}) was thus performed in order to obtain the lowest concentration of flavonoids that can be detected (LOD) or determined (LOQ) with acceptable precision and accuracy under the stated operational conditions of the method. For this purpose, a calibration curve for each analyte was constructed in the 0.02–0.24 mg ml^{-1} range, close to the concentration values expected (data not shown). LOD and LOQ values were calculated for the eight flavonoids, as reported in Table 7.

In order to check the dynamic range of linearity between flavonoid concentration and peak area, six concentration levels (0.8, 0.32, 0.24, 0.16, 0.08, 0.02 mg ml^{-1}) of the eight flavonoids were considered starting from a concentration value close to LOQ of each analyte. As shown in Table 6, an excellent linear response was observed over the range specified for all analytes, as confirmed by the correlation coefficient which ranged from 0.9982 and 0.9999. All flavonoids were in accordance with Beer's law in the concentration investigated.

The HPLC-UV method precision was calculated in terms of intra-day repeatability and intermediate precision. Intra-day repeatability was evaluated by performing three repetitive analyses for each concentration levels (0.08, 0.32, 0.8 mg ml^{-1}) which gave an R.S.D. between 0.11–3.64% showing a good

Table 8
HPLC-UV intra-day repeatability and intermediate precision of investigated flavonoids

Analyte	Concentration level (mg ml^{-1})	Intra-day R.S.D. (%)	Intermediate precision R.S.D. (%)
Eriocitrin	0.08	2.73	6.33
	0.32	0.64	5.70
	0.8	0.45	7.62
Neoeriocitrin	0.08	2.80	3.59
	0.32	3.64	3.74
	0.8	2.35	6.62
Narirutin	0.08	0.92	5.36
	0.32	2.73	3.66
	0.8	0.30	3.93
Naringin	0.08	0.85	1.75
	0.32	0.98	4.78
	0.8	0.71	6.56
Hesperidin	0.08	0.09	6.32
	0.32	1.17	7.30
	0.8	0.83	4.70
Neohesperidin	0.08	3.02	6.53
	0.32	1.72	7.17
	0.8	1.79	6.82
Didymin	0.08	0.70	5.87
	0.32	1.48	7.22
	0.8	0.59	5.07
7-OH Flavanone	0.08	0.72	1.66
	0.32	0.61	6.29
	0.8	0.11	1.59

Table 9
Recoveries of flavonoids from *C. bergamia* juice

Analyte	Amount added ($mg\,ml^{-1}$)	Recovery (%) ± S.D.	Mean ± S.D.	R.S.D. (%)
Eriocitrin	0.036	102.47 ± 0.34	98.71 ± 3.50	3.55
	0.052	98.13 ± 0.35		
	0.1	95.53 ± 0.68		
Neoeriocitrin	0.036	67.75 ± 0.37	70.11 ± 2.20	3.13
	0.052	70.5 ± 0.72		
	0.1	72.10 ± 0.10		
Narirutin	0.036	102.77 ± 0.36	102.45 ± 1.91	1.86
	0.052	104.18 ± 0.46		
	0.1	100.4 ± 0.19		
Naringin	0.036	93.46 ± 0.10	93.42 ± 0.14	0.15
	0.052	93.55 ± 0.13		
	0.1	93.27 ± 0.05		
Hesperidin	0.036	93.4 ± 0.75	96.88 ± 3.03	3.13
	0.052	98.27 ± 0.50		
	0.1	98.97 ± 0.87		
Neohesperidin	0.036	86.48 ± 0.29	88.07 ± 1.47	1.67
	0.052	89.39 ± 0.40		
	0.1	88.36 ± 0.37		
Didymin	0.036	89.65 ± 0.33	91.58 ±2.25	2.46
	0.052	94.06 ± 0.31		
	0.1	91.05 ± 0.24		
7-OH Flavanone	0.036	98.04 ± 0.17	100.83 ± 2.41	2.39
	0.052	102.18 ± 0.70		
	0.1	102.28 ± 0.42		

precision (Table 8). The intermediate precision was evaluated over two different days at the same concentration levels. R.S.D. values were not higher than 7.62% (Table 8).

The criterion of accuracy is the relationship between the amounts of added standards and the amounts detected by the HPLC-UV assay. Three different concentrations of markers: 0.036, 0.052, and 0.1 $mg\,ml^{-1}$ were added to sample solution of which the marker contents were determined. All samples were subjected to HPLC analysis. As shown in Table 9, the recovery values are expressed as the percentage of assayed concentration relative to the calculated concentration. The recoveries of target compounds, except for neoeriocitrin, were between 88.07 and 102.45%.

In conclusion, the sensible methodologies developed allowed quantitative extraction and determination of the flavonoids in *C. bergamia* juice. The HPLC method showed good analytical characteristics for quantitative determination of this type of compounds (precision of calibration graph slopes, low detection limits as well as good accuracy). The optimization of extraction procedure by experimental design methodology led us to obtain the best recovery for all the flavonoids in the least number of experiments. Furthermore, these results could be an excellent approach to the true quantitative flavonoid composition in different matrices.

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