Isolation of Antioxidant Compounds from Orange Juice by Using Countercurrent Supercritical Fluid Extraction (CC−SFE)

Francisco J. Señoráns,[†] Alejandro Ruiz-Rodriguez,[†] Sofía Cavero,[†] Alejandro Cifuentes,[‡] Elena Ibañez,[†] and Guillermo Reglero*[,†]

Área de Tecnología de Alimentos, Facultad de Ciencias, Universidad Autónoma de Madrid, 28049 Cantoblanco, Madrid, Spain, and Departamento de Caracterización de Alimentos, Instituto de Fermentaciones Industriales (CSIC), Juan de la Cierva 3, 28006 Madrid, Spain

Antioxidants from orange juice are isolated by the use of countercurrent supercritical fluid extraction (CC−SFE) and characterized by reversed-phase liquid chromatography (RPLC) coupled to mass spectrometry (MS) and diode-array detection (DAD). A pilot-scale SFE plant equipped with a packed column has been employed for countercurrent extraction and fractionation of raw orange juice with carbon dioxide. Several experiments have been performed in order to study the effect of the countercurrent conditions on the content of antioxidative compounds. In this study, the main variable that has been considered is the solvent-to-feed ratio (*S*/*F*) because it plays an essential role in the extraction efficiency. The values tested covered a wide range of sample and solvent (CO_2) flow rates. In each experimental run, two different extracted fractions and the residual nonextracted juice were obtained and characterized. Different flavonoids have been identified in the fractions obtained after CC−SFE. The possibility of using this process for antioxidant compounds enrichment is discussed.

Keywords: *Antioxidants; countercurrent; orange juice; supercritical fluid extraction*

INTRODUCTION

The citrus industry has an extraordinary economic and social importance in the Mediterranean Basin, especially in Spain which is the main citrus producer in Europe (*1*). At present there is a renewed interest in product and process innovation related to both the citrus and citrus juice industries. In Europe, research relative to the citrus juice industry is focused on juice quality improvement, production of valuable byproducts, the extraction, recovery and characterization of essential oils, and the use and upgrading of waste material (*2*).

Citrus products contain significant amounts of flavonoids, a widely distributed group of polyphenolic compounds with inferred health-related properties, which are based in their antioxidant activity. These properties include anticancer, antiviral, and antiinflammatory activities (*3*). In vitro and animal studies have demonstrated that flavonoids have antioxidant and antimutagenic activities, and some studies suggest that flavonoids may reduce the risk of cardiovascular disease and stroke (*4*).

The presence of flavonoids in citrus fruits (*5*) occurs in citrus peel and seeds (*6*). Usually these compounds have been isolated by extraction with organic solvents (*7*). Supercritical carbon dioxide has been widely used in conventional citrus processing applications, e.g., for citrus oil fractionation (*8*), citrus oil extraction (*9, 10*), orange juice debittering (*11, 12*), and deterpenation (by using countercurrent supercritical fluid extraction (CC−SFE)) (*13, 14*). To our knowledge, only one work has been directed toward the use of supercritical carbon dioxide extraction of citrus-related antioxidative components (*15*).

The objective of the present work was to study a new continuous process based on the use of CC−SFE for extracting antioxidant-enriched fractions from orange juice. The use of a fractionation column with a continuous sample feeding and countercurrent extraction with carbon dioxide implies the necessity for optimization of the main variables involved in the CC−SFE process, i.e., sample flow rate and solvent flow rate that determine the solvent-to-feed ratio (*S*/*F*) (*16*).

The *S*/*F* ratio plays an essential role in the extraction efficiency, and the values tested in this study covered a wide range of sample and solvent (CO_2) flow rates. For each experimental run, two different extracted fractions and the residual nonextracted juice were obtained and characterized. The isolated fractions are usually complex matrixes where active substances can be found; to evaluate the content of the fraction in such compounds, a separation−identification step is needed.

Some investigators have demonstrated the use of HPLC, with confirmation by mass spectrometry (MS) and diode-array detection (DAD), to identify nonvolatile components in lemon peel (*17*). On the other hand, HPLC has been widely used for characterization of SFE extracts from different samples (see e.g., *18, 19*). In a previous work we have demonstrated the suitability of the use of reversed-phase liquid chromatography (RPLC) coupled to MS and DAD to fully identify the antioxidant fraction of rosemary extracted by SFE (*20*). Therefore, this methodology has been employed to characterize the fractions obtained after CC−SFE of orange juice.

* To whom correspondence should be addressed. Fax: +34-91-3978255. E-mail: Guillermo.Reglero@uam.es.

[†] Universidad Autónoma de Madrid.

[‡] Instituto de Fermentaciones Industriales (CSIC).

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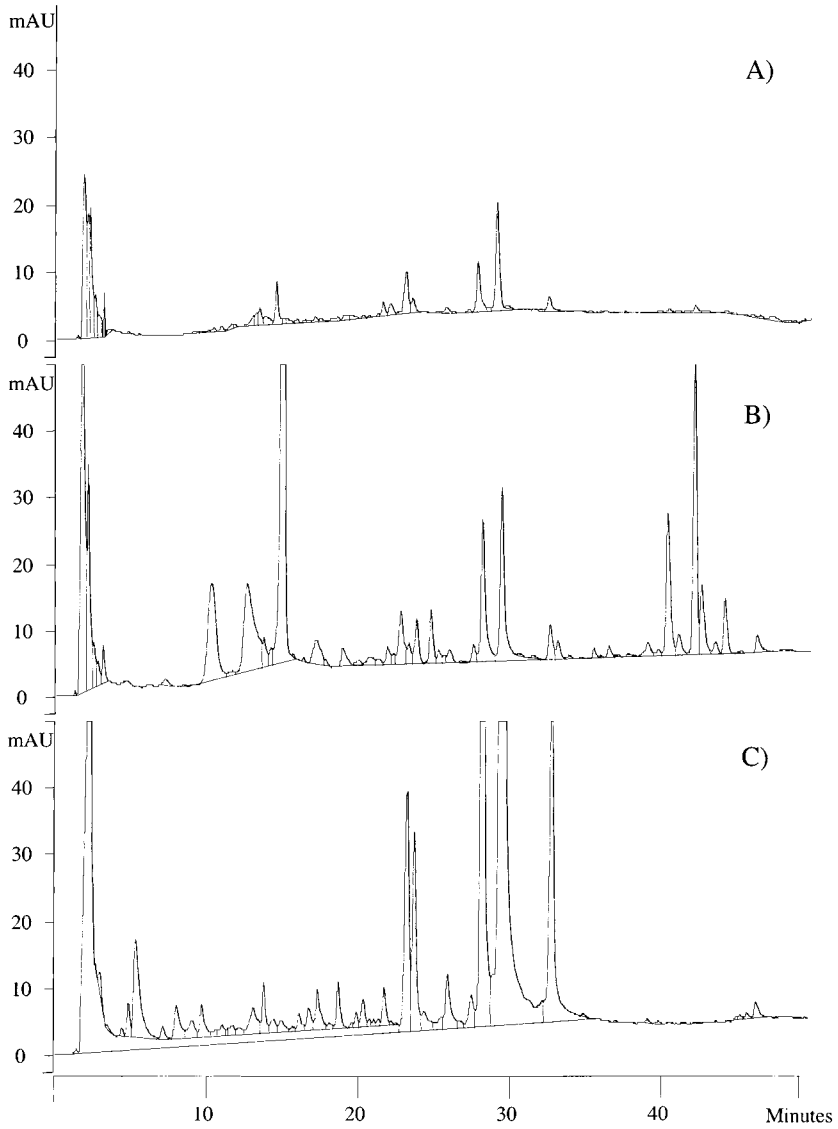

Figure 1. Liquid-chromatographic profiles obtained by DAD at 280 nm, using a *S*/*F* ratio equal to 7 for fraction 1 (A); fraction 2 (B); and raffinate (C). See Table 1 for peak assignments.

MATERIALS AND METHODS

Sample and Reagents. Oranges used in this work belong to the species sweet oranges (*Citrus sinensis*) variety *Valencia-Late*. The orange juice was freshly squeezed and filtered to remove pulp prior to use.

All solvents were purchased from Lab Scan (Dublin, Ireland). Milli-Q purified water was used (Millipore, Bedford, MA). CO_2 (N-38 quality) was kindly donated by AL Air Liquide España S. A. (Madrid, Spain).

Instrumentation and Extraction Method. The countercurrent (CC) SFE system employed in this study has been previously described (*16*). The liquid sample introduction was carried out through the middle point of the packed column, located over the inlet of the CO_2, creating a countercurrent between the flow of sample (downward) and the CO_2 flow (upward).

The variable selected for the countercurrent supercritical fluid extraction process was the *S*/*F* ratio that was tested at three different levels: 3, 7, and 11. For all the conditions tested, the CO_2 flow rate was kept constant at 2400 mL/h while the sample flow rate was modified (220, 340, and 800 mL/h) to obtain the desired solvent-to-feed ratios.

Extraction and fractionation conditions were kept constant throughout the experiments: extraction pressure was chosen at 160 bar, and the cascade fractionation was achieved by setting pressures in separators 1 and 2 equal to 80 and 20 bar, respectively. These values were selected to be able to test the countercurrent conditions over a wide range with minimum experimental problems. Extraction temperature was maintained at 40 °C to avoid sample degradation, while temperatures in separators 1 and 2 were fixed at 35 °C and 25 °C, respectively. The total extraction time was 20 min.

HPLC−DAD and HPLC−MS Analysis of the Extracts. Analysis of the extract components were carried out in an HPLC apparatus (Varian ProStar series), with an injection loop of 20 μL, equipped with a Spherisorb ODS2 column (5-μm particle, 250 × 4.6 mm). The mobile phase consisted of mixtures of two solvents, A (methanol) and B (water), utilizing a step gradient, changing from 99% B to 5% B at a flow rate of 1 mL/min over 50 min. Detection was accomplished using a ProStar 330 photodiode array detector, at a wavelength of 280 nm. A personal computer system incorporating Varian software was used for data acquisition and processing.

Identification of compounds was confirmed by using a quadrupole 1100 MSD (Hewlett-Packard) with an electrospray interface (ESI). In the HPLC−ESI−MS method, the eluted compounds were mixed with nitrogen in the heated nebulizer interface and polarity was tuned to positive. Adequate calibration of ESI parameters (needle potential, gas temperature, and nebulizer pressure) was required to optimize the response and to obtain a high sensitivity to the molecular ion. The optimized values were the following: needle potential 4000 V, gas

Table 1. Characteristic Parameters of the Compounds Detected in the Extracts Analyzed by LC–DAD–MS

peak no.	compound	retention time (min)	mass ions (ES+) M+	major fragment ion	UV absorbance maxima (nm)	fractions[a]
1	benzoic acid 1 (BA1)	14.7	290	121	230, 283	Exp-1-*F*1, *R*, Exp-2-*F*1, *F*2, *R*, Exp-3-*F*1, *F*2, *R*
2	flavanone 1	23.1	–	–	278	Exp-3-*F*1, *R*
3	flavanone 2	23.6	–	–	281	Exp-3-*F*1, *R*
4	narirutin	28.0	580	273	283	Exp-1-*F*1, *F*2, *R*, Exp-2- *R*, Exp-3-*F*1, *F*2, *R*
5	hesperidin	29.3	610	303	283	all
6	naringin	32.6	580	273	283	Exp-1-*F*1, *F*2, *R*, Exp-2- *R*, Exp-3-*F*1, *F*2, *R*
7	sinensetin	40.2	372	350	239, 331	Exp-1-*F*2, Exp-3-*F*2
8	nobiletin	41.9	402	380	251, 338	Exp-1-*F*2, Exp-3-*F*2
9	3,5,6,7,8,3′,4′-hepta-methoxy flavone	42.5	432	410	264, 322	Exp-1-*F*2, Exp-3-*F*2

[a] Fractions correspond to those where the compound had been detected.

temperature 335 °C, drying gas 12.0 mL/min, and nebulizer pressure 50 psig.

RESULTS AND DISCUSSION

In the present work, different conditions were selected to study the effect of the countercurrent conditions in the antioxidant extraction from orange juice in a CC–SFE at pilot plant scale. In a previous work, in which ethanol and aroma extraction from alcoholic beverages was studied (*20*), we concluded that the sample flow rate was the main parameter controlling the efficiency and the selectivity of the system, therefore, the *S*/*F* ratio was optimized in this study. To achieve the different ratios, a constant solvent flow rate was considered at 2400 mL/h and the sample flow rate was changed at three different levels (800, 340, and 220 mL/h) to provide a wide range of *S*/*F* ratios (3, 7, and 11).

Initially, an intermediate extraction pressure was chosen at 160 bar while extraction temperature was fixed at 40 °C (extraction density of 0.8 g/mL). As described in Materials and Methods, further fractionation was achieved by setting the pressures for separators 1 and 2 equal to 80 and 20 bar, respectively. Such separation conditions provide a density of about 0.5 g/mL in the first separator, whereas a total decompression stage was achieved in the second separator. Three different products were obtained after extraction and fractionation of the orange juice: those in separators 1 (*F*1) and 2 (*F*2) and the raffinate (*R*) which is the byproduct of the extracted samples collected at the bottom of the column.

Characterization of Extracts by HPLC–DAD–MS. To obtain semiquantitative data, the primary detection wavelength used was 280 nm. Simultaneously, spectral data were obtained over the range of 200 to 420 nm by using a diode array detector. Such data can be very useful in identifying compounds of interest. Figure 1 shows chromatographic profiles, obtained using DAD at 280 nm, of the three fractions obtained under the extraction conditions described above, and using an intermediate *S*/*F* ratio (equal to 7), designated (A) fraction 1, (B) fraction 2, and (C) raffinate.

Specific compounds were characterized for their retention time, UV spectra, and mass spectra. Table 1 shows retention time, molecular ion (MH+), and UV maximum absorbance for all the compounds detected in the samples. Additional data about the major fragments obtained using electrospray with positive ionization are also presented for all the compounds found in the sample in substantial amounts. Table 1 also includes both the experiments and fractions where the compounds had been detected.

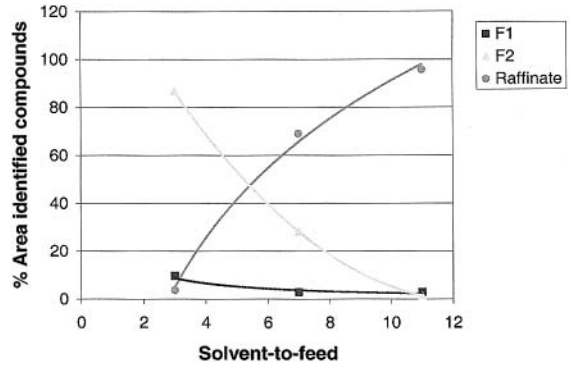

Figure 2. Graph representing the log (enrichment) as a function of the *S*/*F* ratio. The enrichment was calculated as the ratio of the total area of the chromatogram of the extract in the selected fraction (*F*1, *F*2, and *F*1 + *F*2 (total)) and the total area of the chromatogram of the raffinate (*R*).

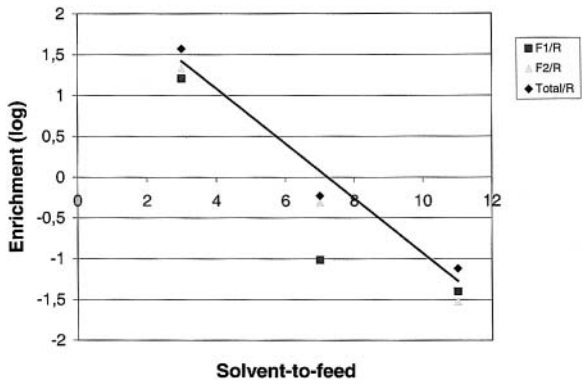

Figure 3. Graph representing the % total area of the identified compounds vs solvent-to-feed ratio.

Among the compounds found in orange juice were flavanones, including hesperidin, narirutin, and naringin. The compounds extracted in the present work have spectra typical of flavanones, with a maximum at around 285 nm and a shoulder absorbance in the region from 320 to 350 nm. It is well-known that these flavanones play an important role in human nutrition, and they are also used in the determination of citrus quality and origin (*1*). The profile of flavanones obtained in the present work is similar to the one described previously for the same variety of orange (*1*), although in our study we also detected the presence of naringin.

The flavones found in the present work, such as sinensetin, nobiletin, and 3,5,6,7,8,3′,4′-heptamethoxy flavone, have also been described previously as some of the major compounds found in sweet orange (*21*). The other compounds shown in Table 1, such as benzoic acid 1 (BA1) and flavanone 1 and 2, could not be completely

Table 2. Relative Percentage (Normalized Areas (%)) of the Compounds Identified by LC−DAD−MS and Selected To Semi-quantitatively Describe the Composition of the Extracts Obtained at Different *S*/*F* Ratios

compound	Exp-1-*F*1 (%)	Exp-1-*F*2 (%)	Exp-1- *R* (%)	Exp-2-*F*1 (%)	Exp-2-*F*2 (%)	Exp-2- *R* (%)	Exp-3-*F*1 (%)	Exp-3-*F*2 (%)	Exp-3- *R* (%)
benzoic acid 1 (BA1)	1.2	79.2	0.9	10.6	63.0	0.7	73.8	11.5	49.3
flavanone 1	–	–	–	18.1	–	4.6	–	–	–
flavanone 2	–	–	–	5.6	–	3.4	–	–	–
narirutin	19.5	3.8	27.1	19.6	6.7	15.3	–	–	12.7
hesperidin	72.2	7.6	64.0	39.9	9.2	67.4	26.2	88.5	33.6
naringin	7.1	2.4	8.0	6.1	–	8.6	–	–	4.4
sinensetin	–	2.3	–	–	6.5	–	–	–	–
nobiletin	–	3.7	–	–	11.3	–	–	–	–
3,5,6,7,8,3′,4′-hepta-methoxy flavone	–	1.0	–	–	3.3	–	–	–	–

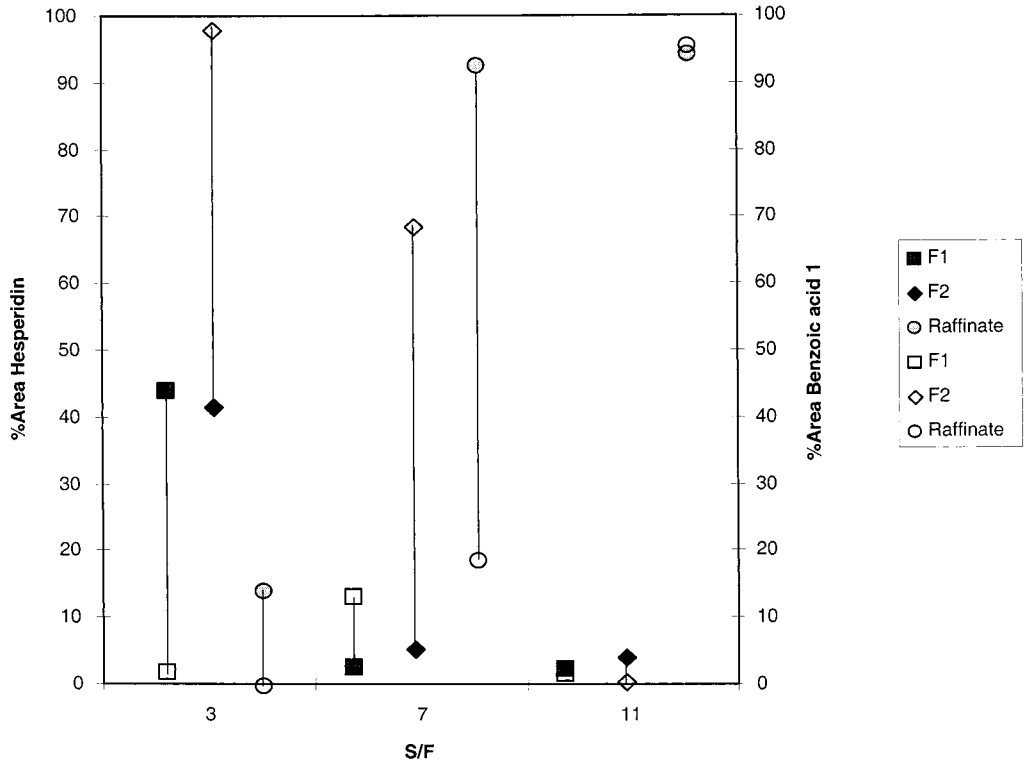

Figure 4. Distribution of hesperidin and benzoic acid 1 (% of area of the compound in each separator fraction and in raffinate) vs solvent-to-feed ratio. Solid symbols represent hesperidin content and empty symbols represent benzoic acid 1 (BA1) content. Lines show the difference between values of hesperidin and benzoic acid 1.

identified. Nevertheless, it was possible, on the basis of data of the UV spectra, to identify the family of compounds to which they belong. Thus, for BA1, the large and symmetric UV absorption maximum obtained at 283 nm (see Table 1) can be assigned to a 4-substitute benzoic acid (e.g., *p*-hydroxybenzoic acid) and/or substituent groups in 3- and 5-position of this compound (e.g., gallic acid). The major MS fragment obtained at $m/z = 121$ probably is the ion $HO-C_6H_4-CO+$ derived from *p*-hydroxybenzoic acid. Moreover, the second UV maximum obtained at 230 nm can be assigned to a potential glycosylated derivative of benzoic acid (e.g., via an ester bond). These types of benzoic acids and their corresponding glucosides have been already reported in *Citrus sinensis* (*21*).

Effect of the Solvent-to-Feed Ratio on the Composition of the Extracts. The first response discussed provides overall information on the extracts after extraction, corresponding to the total area of the chromatograms obtained after HPLC−DAD analysis (recorded at 280 nm). The enrichment factors have been calculated as the ratio of the total area of the chromatogram of the extract in the selected fraction to the total area of the chromatogram of the raffinate (*R*). Enrichment results are shown in Figure 2 as a function of the *S*/*F* ratio; data corresponding to the enrichment achieved in separators 1 and 2 individually and the total (considering both separators together) are also presented. A high correlation (96%) was obtained for log(total enrichment) vs *S*/*F* using a linear regression ($y = -0.3365x + 2.4304$; $R^2 = 0.963$).

The values of enrichment were higher (higher concentration of compounds extracted in both separators, compared to those in the raffinate) at the lowest *S*/*F* ratio (at highest sample flow rate). When working with 800 mL/h of orange juice, an enrichment of 37 (ratio between extract and raffinate) can be achieved. This is in agreement with the results obtained previously for the extraction of alcoholic beverages (*16*). When using low sample flow rates, an enrichment is produced in the raffinate (ratio between raffinate and extract = 17). By using this response, a selective enrichment can be observed toward the extracts or the raffinate as a function of the *S*/*F* ratios selected.

If, instead of considering the total enrichment, we consider only the area of the identified compounds

(Table 1), the results obtained are quite similar in terms of enrichment. Nevertheless, this result gives the possibility of studying also the distribution of the different compounds among the different fractions (*F*1, *F*2, and *R*). Figure 3 shows the % total area of the identified compounds vs solvent-to-feed ratio. When low *S*/*F* ratios are used, a maximum extraction of flavonoids is obtained in separator 2, with a very low percentage recovered in the raffinate. The opposite is found at *S*/*F* equal to 11 where almost 96% of the compounds identified are found in the raffinate.

To perform the study of the semiquantitative composition of the extracts, the compounds shown in Table 1 were selected and their relative percentage (referred to the total area of the selected components) is shown in Table 2. Therefore, this table provides valuable information about the composition of the extracts. Unfortunately, the lack of standards for most of the components and their unknown molecular absorption coefficients did not allow quantitation of their contents.

Sinensetin (pentamethoxy flavone), nobiletin (hexamethoxy flavone), and the heptamethoxy flavone were found in fraction 2. Nevertheless, flavanones 1 and 2 are usually found in fraction 1 but they can also appear in the raffinate if extraction is performed at *S*/*F* equal to 7.

Compounds such as hesperidin, narirutin, naringin, and the benzoic acid 1 (BA1) were found in almost all fractions but at different compositions depending on the solvent-to-feed ratio used.

In terms of relative composition of the extracts, the largest differences observed occurred in experiment 3 at the highest *S*/*F* ratio (11). In this run, only two compounds were extracted in fractions 1 and 2 (hesperidin and BA1), while a completely different pattern was found in the raffinate. The two experiments performed at lower *S*/*F* ratios (3 and 7) followed a similar pattern and the compositions of each fraction were similar.

One trend that can be observed in Table 2, when comparing the relative percentages of hesperidin and BA1, is the different distribution of each of them in the separators and raffinate, respectively. To study only the distribution and not the global composition of the extracts, a comparison of the areas of both compounds in the different fractions was performed. With this information, an idea about the selectivity that can be obtained in the CC−SFE system used as a function of the different *S*/*F* ratios can be obtained. Figure 4 shows the distribution of hesperidin and benzoic acid 1 (in terms of % of area of the compound in each separator and raffinate) vs the solvent-to-feed ratio used. Solid symbols represent hesperidin content and empty symbols represent BA1 content. A very important difference in distribution of the two compounds can be observed between experiment 1 ($S/F = 3$) and experiment 3 ($S/F = 11$). The selectivity was calculated according to the equation described by Brunetti (*22*) using the weight fraction of the component in the extract referred to the weight of the compound in the raffinate. A very high selectivity can be observed for the extraction of benzoic acid 1 in relation to that of hesperidin. Results show that the compound belonging to the benzoic acid family, with much lower molecular weight than hesperidin and different chemical structure, can be selectively extracted in separator 2 (when $S/F = 3$) with values of selectivity of around 70 (graphically this can be seen as the difference between the solid and empty symbols of the same shape, represented as a line in Figure 4). This observed high selectivity continues when using a *S*/*F* equal to 7, although important differences occur between the types of compounds found in each of the fractions. Almost no selectivity can be observed when $S/F = 11$ is used.

As shown in Table 2, other compounds with chemical structures similar to that of hesperidin, such as narirutin, are extracted. For this pair of compounds no selectivity would be expected; this was confirmed in the present work where selectivity values of only 1.5 could be achieved.

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