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ISOCRATIC LC-DAD-FLD METHOD FOR THE DETERMINATION OF FLAVONOIDS IN PAPRIKA SAMPLES BY USING A RAPID RESOLUTION COLUMN AND POST-COLUMN pH CHANGE

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Abstract

The determination of flavonoid compounds in paprika samples has been performed by liquid chromatography in series diode array and fluorescence detection (LC-DAD-FLD), by means of a pH change to basic medium just before FLD detection. The validation of the method was performed through the establishment of the external standard calibration curves and the analytical figures of merit. Limits of detection ranging from 0.006 to 0.02 mg L^{-1} and 0.007 to 0.09 mg L^{-1} were achieved using DAD and FLD detection, respectively. The experimental conditions to carry out the hydrolysis procedure to obtain flavonoid aglycones from flavonoid glycosides have been optimized applying an experimental design and the response surface methodology. The final conditions selected were 2.5 M HCl during 45 min at 85 ºC. The repeatability of this procedure was assayed and relative standard deviation (RSD) values for concentration of quercetin and luteolin compounds were lower than 2 %. The quantification of quercetin, luteolin and kaempferol compounds was carried out in less than 6 minutes in paprika samples by means of the external standard calibration. The analytes were extracted with methanol and the extracts were previously subjected to a cleanup procedure to extend the use of the chromatographic column.

Keywords: flavonoids, liquid chromatography, fluorescence detection, paprika, acid hydrolysis, Protected Designation of Origin

1. Introduction

Peppers are an important agricultural crop with numerous varieties cultivated around the world, not only due to its economic importance, but also for the nutritional value of the fruits [1]. Five main domesticated pepper species are grown commercially including *Capsicum annuum, C. baccatum, C. chinense, C. frutescens and C. pubescens* [2]. The predominant phenolics found in pepper fruits are capsaicinoids such as capsaicin, dihydrocapsaicin and nordihydrocapsaicin, and flavonoids, glycosides of quercetin and luteolin being the major flavonoids found in pepper [1]. Paprika, the dehydrated and milled fruit of certain varieties of red peppers (*Capsicum annuum L.*), is one of the most widely used food colorants for culinary and industrial purposes [3,4]. For these reasons, it is interesting the study of this product, according its flavonoid content, which present important properties for the health, such as, antioxidant activity, vascular protection, and due to their anti-hepatotoxic, anti-allergic, anti-proliferative, anti-osteoporotic, and anti-inflammatory properties. Moreover, these compounds are potent regulators for cell cycle progression, which may be involved in the prevention of carcinogenesis [5, 6].

Flavonoids are a large family of low molecular weight polyphenolic compounds with diphenylpropanes (C6C3C6) skeletons. The four major classes are the 4-oxoflavonoids (flavones, flavonols, etc.), anthocyanins, isoflavones, and the flavan-3-ol derivatives (catechin and tannins) [7,8]. Flavonols and flavones are flavonoids of particular importance as they were found to contain antioxidant and free radical scavenging activity in foods [9]. These compounds are distributed in medicinal plants, vegetables, fruit juices and beverages (tea, coffee, wines…) [10-12]. The role of flavonoids is related with the basic structure (hydroxylation, methoxylation), the degree of polymerization and the type of conjugation (glycosylation, malonylation, sulphonation) [9].

Quantitative determination of individual flavonoid glycosides in plant materials is difficult, due to their large number. Therefore, the glycosides are normally hydrolysed and the resulting aglycones are identified and quantified [13]. Methods for acid hydrolysis of flavonoids from peppers have been published by Nuutila et al. [13], Bae et al. [2] and Shim et al. [14]. Usually,

hydrolysis of flavonoid glycosides requires high concentrations (1 – 2 M) of mineral acids during long times at high temperatures.

Numerous analytical methods for the detection of flavonoid compounds have been reported to the date. The most common method for the identification and quantification of flavonoids involves an HPLC system combined with a UV detector or diode array detector (DAD) [2,9,15-19]. Most of them use conventional columns and gradient elution. Moreover, liquid chromatography (LC) coupled to mass spectrometry (MS) or nuclear magnetic resonance (NMR), and capillary electrophoresis coupled to UV detection, have been employed to determine flavonoids in tea, onions, peppers, etc. [20-22].

According to all of this, the objectives of this study were optimizing a hydrolysis procedure and developing an isocratic chromatography method, with DAD and FLD detection, to quantify the most abundant flavonoid compounds in paprika samples (myricetin, quercetin, luteolin, kaempferol and apigenin) by means of using a rapid resolution C18 column in order to develop a quicker analysis procedure.

2. Materials and methods

2.1. Chemical reagents and samples

Apigenin, luteolin, myricetin and kaempferol standards were purchased from Extrasynthese (Genay Cedex, France). HPLC-grade methanol solvent and quercetin standard were obtained from Sigma (Sigma-Aldrich Química, S.A., Madrid). Stock solutions of 100 mg L^{-1} were prepared in MeOH and stored at 4 ºC in the dark until use. High-purity water was obtained from a Milli-Q water system (Millipore S.A.S., Molsheim, France). Sodium hydroxide pellets pharma grade, and hydrochloric acid, 37%, were obtained from Panreac (Panreac Química, S.A.U., Barcelona). Sep-Pak Plus C18 cartridges of 360 mg were obtained from Waters (Waters Corp., Milford, MA, USA).

Samples of paprika belonging to different origins, the Spanish Protected Designation of Origin (PDO) "*Pimentón de La Vera*" and other different producers, were obtained from Regulatory Council of the Designation of Origin "*Pimentón de La Vera*" and from local market, respectively. The origin of the samples which are not belonging to the Spanish PDO is not available although in their label it is reported that they have been packaged in Spain. It should be noted that this product is obtained from dried peppers whose stem and seeds are eliminated in later stages before milling.

2.2. Instrumentation and software

The chromatographic studies were performed with an Agilent Model 1100 LC instrument (Agilent Technologies, Palo Alto, CA, USA), equipped with degasser, quaternary pump, column oven, autosampler Agilent 1260 infinity, UV-visible-diode-array detector (DAD) and fluorescence-detector (FLD). The OpenLAB LC ChemStation software (Version A.01.04) was used to control the instrument, data acquisition and data analysis. To carry out a post-column derivatization, a Kontron 420 HPLC pump (Kontron instrument AG) was employed. The analytical column employed was a rapid resolution Zorbax Eclipse XDB-C18 column (4.6 mm x 50 mm x 1.8 μm) (Agilent Technologies). Calibration curves and analytical figures of merit were obtained by means of the homemade ACOC program [23]. The software package The Unscrambler® v6. 11 (CAMO A/S Olav Tryggvasonsgt, N-7011, Trondheim, Norway) was used for the experimental design. The software package SPSS v.19 (IBM, Statistical Package for Social Sciences) was used for the statistical treatment.

2.3. Chromatographic conditions

The mobile phase consisted in H_3PO_4 (0.03 M) in water (solvent A) and methanol (solvent B). The isocratic elution employed for the analysis of flavonoid compounds was 50:50 (solvent A: solvent B). The flow rate was set constant at 1.0 mL min^{-1} and the injection volume was 10 μL. The DAD detection was performed at 360 nm (for luteolin and apigenin) and at 370 nm (for quercetin, myricetin and kaempferol), and the FLD detection was at 420 nm for the excitation

104 wavelength, and 550 nm (kaempferol), 520 nm (luteolin) and 560 nm (myricetin and quercetin),
105 for the emission wavelength.

106 In order to carry out a post-column derivatization, a 0.03 M NaOH solution was used and the flow
107 rate of the auxiliary Kontron 420 HPLC pump was 2.0 mL min^{-1}. Derivatization was performed
108 just before sample reached the FLD detector.

109 **2.4. Calibration curves**

110 To obtain the calibration curves, standard solutions containing mixtures of flavonoid compounds
111 (quercetin, myricetin, apigenin, luteolin and kaempferol) were prepared in methanol: water
112 (50:50, v/v), taking the corresponding volumes of more concentrated stock solutions in methanol.
113 The concentrations employed were between 0.05 – 15 mg L^{-1} except in the luteolin fluorescence
114 as this analyte did not exhibit fluorescence below 0.5 mg L^{-1} . The peak area values in the different
115 detection conditions were measured using the Chemstation package.

116 **2.5. Treatment of the sample**

117 The analytes were extracted from precisely weighed aliquots of 0.5 g of paprika samples with 20
118 mL of MeOH for 30 min in an ultrasonic bath. The extract solution was centrifuged and the
119 supernatant liquid evaporated to dryness. The residue was suspended in 50 mL of water and
120 loaded on a C18 cartridge (Solid Phase Extraction), previously conditioned with 8 mL of
121 acetonitrile and 8 mL of water. The cartridge was successively washed with 20 mL of water and
122 20 mL of 10% aqueous methanol to eliminate other compounds present in paprika matrix. The
123 analytes were eluted with 2.5 mL of 85% (v/v) MeOH. Finally, they were diluted to a final volume
124 of 3.0 mL with 85% (v/v) MeOH.

125 An aliquot of 200 µL of the obtained solution was subjected to hydrolysis in a water bath at 85
126 ºC for 45 min with a 2.5 M final concentration of HCl in the 3.0 mL volumetric flask, to obtain
127 flavonoids. Finally, the samples were in methanol: water (50:50, v/v) by means of dilution of
128 samples with corresponding volumes of methanol and water.

129 **3. Results and discussion**

3.1. Optimization of chromatographic conditions

Firstly, the optimization of the percentage of the solvent A (0.03 M H_3PO_4) and B (MeOH) in the mobile phase was performed, varying percentage of methanol between 40 – 60 %. Final conditions selected (50:50, v/v) offered a good resolutions of the five analytes (myricetin, quercetin, luteolin, kaempferol and apigenin), which are present in foods (paprika, peppers, onions…), in a time lower than 6 minutes, which is an improvement respect to the conventional columns used in the literature [1,2,24]. In addition, peaks resolution is higher than 2 in all cases. At the same time, the optimal wavelengths for the detection of each analyte were selected from the observation of their UV-Vis spectra. These spectra have been included in the supplementary material (Fig. S1A).

On the other hand, previous studies have shown that some flavonol compounds, myricetin, quercetin and kaempferol, exhibit fluorescence in strong basic medium due to a resonant form which results from the second deprotonation of these compounds [25]. In the Fig. 1 the emission spectra of these three analytes at a pH of 9.5, exciting at 420 nm, can be observed. These signals disappeared when the pH was lower than 8.5. Subsequently, the optimization of eluate modification post-column was carried out in order to get a pH value providing a good fluorescence signal. However, these compounds are oxidized in this medium, therefore it was decided derivatizing just before the FLD detector. This methodology based on a change to basic pH after the DAD detection has been employed for the first time in this study.

For this, a NaOH solution was inserted in the flow rate after the DAD detector with the aid of a high-pressure additional pump equipped with a pulse suppressor, and the concentration was varied between 0.03 – 0.7 mol L^{-1}, with a flow rate of 1.5 mL min^{-1}. The highest signal was obtained with a NaOH solution of 0.1 mol L^{-1}. Higher concentrations produce a decrease of the signal that may be due to the fact that, when the pH increases, oxidation of flavonoid compounds occurs more quickly. For these reason, the repeatability was only studied utilizing NaOH concentrations of 0.1 mol L^{-1} and 0.03 mol L^{-1}, and the results were better in the case of 0.03 mol L^{-1} (1.3 – 5.3 % RSD, n= 6). In addition, the flow rate was varied between 1.5 – 2.5 mL min^{-1}.

157 The higher signals were for a flow rate of 2.0 mL min^{-1} and repeatability was also better in the
158 case of a flow rate of 2.0 mL min^{-1} (1.3 – 3.5 % RSD, n= 6). Thus, the final conditions selected
159 were a NaOH concentration of 0.03 M and a flow rate of 2.0 mL min^{-1}. The optimal excitation
160 and emission wavelengths were chosen with these conditions. It were selected 420 nm for the
161 excitation and 520 nm (luteolin), 550 nm (kaempferol) and 560 nm (quercetin and myricetin) for
162 the emission. The excitation wavelength was selected with the aim of increasing the signal of the
163 less fluorescent compound, luteolin. Apigenin did not present fluorescence emission. Fig. 2 shows
164 the chromatograms corresponding to a standard solution before and after the derivatization step
165 and in both detection modes and, at the supplementary material, Fig. S1B shows the excitation-
166 emission of myricetin, quercetin, luteolin and kaempferol.

167 **3.2. Analytical parameters**

168 For assessing the method quality, the calibration curves of each compound were constructed
169 according to the procedure described in the section *Calibration curves*, and the analytical figures
170 of merit were calculated employing the peak areas (PA) in both, DAD and FLD, detectors (Table
171 1).

172 The evaluation of the precision was performed by carrying out the analysis of several standard
173 solutions containing 3.00 mg L^{-1} of each flavonoid compound in the same day (intra-day
174 precision, n = 8), and different days during 6 days (inter-day precision). The precision was also
175 examined for several standard solutions containing 0.05 mg L^{-1} of each flavonoid compound in
176 the same day (intra-day precision, n = 8) and different days during 6 days (inter-day precision).
177 The RSD values of PA and retention times were determined for each compound. Data obtained
178 in this study are shown in Table 2. In all cases, the precision was better than 8.0 %.

179 **3.3. Real samples analysis**

180 **3.3.1. Optimization of the acid hydrolysis procedure**

As it is known, flavonoids are present in peppers, which gets the paprika, in several glycosidic forms [2,7] and, because of that, a hydrolysis step is necessary before quantifying them as aglycones.

In order to optimize the variables that could affect the efficacy of the acid hydrolysis process, a Box-Behnken experimental design was used and the effect of the variables affecting the acid hydrolysis and their possible interactions were examined. This design allows interpreting the results using the Response Surface Methodology (RSM). The optimized variables were the temperature (80 – 95 ºC), time (30 – 90 min) and HCl concentration (1.0 – 3.0 M). The experiments were performed in triplicate to obtain the relative standard deviation of each experiment. Samples of 0.5 g of paprika were treated as it is indicated in the section 2.5 and the hydrolysis conditions were varied according to the experimental design. The optimization was followed injecting the extracts in the chromatographic system and using the DAD signal.

To get the response surface which enables the interpretation of the results, a response function (RF) is necessary. In this case, it was observed that the only important peaks were those of quercetin and luteolin and that repeatability was a factor very important, so the RF selected was:

RF = Mean Peak Area of quercetin and luteolin / mean RSD of both analytes

The results obtained were interpreted with the RSM using The Unscrambler® v6. 11 software package, and assuming a quadratic model. The application of the analysis of variance test (ANOVA) indicates the significant influence variables ("p-values" < 0.05). The model has a R^2 equal to 0.893, which indicates that the quadratic model is appropriate. The response surfaces, for each pair of variables, are shown in Fig. 3. It can be observed that the best results are obtained when concentration of acid is higher than 2.0 M and that the hydrolysis time and the temperature have very little influence on the response function.

As a result, according to these observations, the following conditions were selected: 2.5 M HCl concentration, 85 ºC and 45 min of hydrolysis time. These conditions were tested by extracting the glycosides from paprika and hydrolysing them. The extraction was carried out six times. Good

207 signals were obtained for both main analytes (quercetin and luteolin) and the results offered good
208 precision values (RSD, 1.8%). In the Fig. 4, a chromatogram of a paprika sample before
209 hydrolysis and after hydrolysis is shown.

210 Additionally, a rutine (3-glycoside of quercetin) standard of 8 mg L^{-1} was prepared in 85% (v/v)
211 aqueous methanol and an aliquot of 200 μL was hydrolized by means of the optimized procedure.
212 Finally, the samples were diluted to 3.0 mL with the corresponding volumes of water and
213 methanol and injected in the chromatographic system to calculate their equivalent concentration
214 of quercetin. This procedure was performed in duplicate. The results showed a percentage of
215 hydrolysis of 98 ± 1 %.

216 **3.3.2. Optimization of Solid Phase Extraction**

217 In order to protect and extend the life time of the chromatographic column, a solid phase
218 extraction procedure was optimized for the cleanup of the paprika extracts, before their
219 introduction in the chromatographic system. Sep-pak C18 cartridges of 360 mg, previously
220 conditioned passing 8 mL of acetonitrile and 8 mL of water, were utilized and the flavonoids, as
221 well as their glycosides, were retained in them by passing the aqueous extract obtained from
222 paprika samples as described in section 2.5. Samples were brought to slightly acid pH with HCl
223 (0.1 M) to guarantee the protonation of the analytes and their retention in the cartridge. Firstly, a
224 cleanup step was chosen. After checking that these compounds were not eluted with 20 mL of
225 water and 20 mL of 10% (v/v) aqueous methanol, these conditions were used to wash the column
226 after the passing of the sample solution.

227 To select the appropriate eluent for extracting the analytes from the cartridge, 65, 75 and 85%
228 (v/v) aqueous methanol mixtures were used. The paprika samples were fortified with a known
229 amount of quercetin, luteolin and kaempferol and their retention on the cartridge was evaluated
230 by using recovery assays. The analytes were eluted with a constant volume of 2.5 mL and it was
231 observed that the recoveries increased when the percentage of aqueous methanol did it. As a
232 result, 85% (v/v) aqueous methanol was used as eluent and the extract was diluted to 3.0 mL in a
233 volumetric flask with 85% (v/v) aqueous methanol.

234 Moreover, to check that the cleanup and concentration procedures was efficient, a paprika sample
235 was analyzed with and without using the developed solid phase extraction procedure, for four
236 times, and the results were very similar, as can be observed in the Table 3.

237 It can be concluded that the previous cleanup step can be avoided. However, it was decided using
238 it in order to increase the utilization time of the chromatographic column, as well as to facilitate
239 its rinse and the subsequent conditioning after each analysis. This way, the procedure allowed to
240 analyze several samples without clean up the column until the final of the day.

241 **3.3.3. Quantification of flavonoid compounds**

242 After the optimization of the conditions for the cleanup and hydrolysis, a recovery study of the
243 extraction procedure was performed. For this, two consecutive extractions were carried out as it
244 is described in the section 2.5. The experiences were performed in triplicate with three different
245 randomly selected paprika samples and, in different days. The extraction procedure offered a
246 recovery of 85 ± 3 % and 79 ± 4 % for quercetin and luteolin, respectively, in the first extraction.
247 Hence, it was decided to carry out one single extraction in order to save time.

248 On the other hand, the repeatability of the complete procedure was examined and good precision
249 results were obtained. For quercetin, the RSD, were 2.3 % and 2.0 % for DAD and FLD detection,
250 respectively, for luteolin the results were 5.6 % and 4.8 %, respectively and, for kaempferol, the
251 RSD for FLD detection was 9.0 %. This high value can be due to the low concentration of
252 kaempferol in the samples.

253 Then, the chromatographic method was employed to analyse different paprika samples. The
254 external standard methodology was used. In the Table 4, the results obtained in that analysis are
255 reported, as well as the standard deviation for each compound in both detection modes. The
256 samples were divided in two groups, according they belong or not to the PDO "*Pimentón de La*
257 *Ver*a". In Spain, La Vera (Extremadura) is one of the main geographical areas where paprika is
258 cultivated and produced [26]. This product is recognized under Protected Designation of Origin
259 (PDO) by the European Union. La Vera paprika is obtained from peppers which are dried by

means of a characteristic drying system. Thus, La Vera peppers are smoked-dried and the rest of peppers produced in other Spanish areas or in other countries are sun dried or hot air dried [27]. For these reason, the samples are divided in two groups.

Regarding the different groups established, there was no significant difference in quercetin concentration between PDO samples and no PDO samples, at the 5% confidence level, according to a t-student test carried out by means of SPSS software. In the case of kaempferol, the results showed the same conclusion; the mean concentrations did not significantly differ. However, the difference was significant in luteolin concentration between PDO samples and no PDO samples, at the 5% confidence level. In spite of this, we consider that these differences are not sufficient to be used to group the samples according their origin.

On the other hand, and comparing these results with those reported for paprika or paprika peppers from other countries, according to the literature, the main flavonoid present in peppers or paprika is quercetin [2,7,28]. However, in Spanish paprika samples the results obtained are different because both, quercetin and luteolin concentrations, are very similar in these samples. Mean concentrations of quercetin were 130 mg kg^{-1} and 190 mg kg^{-1}, for PDO and no PDO samples, respectively, and mean luteolin concentrations were 160 mg kg^{-1} and 110 mg kg^{-1} for PDO and no PDO samples, respectively. For kaempferol, the mean concentrations were 4 and 5 mg kg^{-1}, respectively. The total content of flavonoids is lower than the results obtained by Zaki et al. [28] and Perucka et al. [29], and more similar to concentrations found by Bae et al. [2] in paprika peppers.

The differences found in Spanish paprika samples could be due to the variety of the peppers used to obtain the powder, because the concentration of these compounds can be different according their variety or location of production [30].

4. Conclusions

The utilization of a rapid resolution chromatographic column has allowed determining the main five flavonoids present in paprika in less than 6 minutes, utilizing reversed phase isocratic mode

with DAD and FLD detection. The FLD detection of these compounds has been employed for the first time, based on a change to basic pH after the DAD detection. On the other hand, response surface methodology (RSM) together with experimental design have been utilized for the optimization of the procedure for the hydrolysis of flavonoid glycosides present in the extracts of these samples. The optimal conditions selected were 2.5 M HCl, 85 ºC and 45 min. This procedure has been utilized in Spanish paprika samples, and quercetin, luteolin and kaempferol have been quantified. The repeatability of the procedure to extract and hydrolyze the flavonoid glycosides has been examined obtaining good results. Different proportion of quercetin/luteolin concentrations has been found in Spanish paprika, in comparison with other reported values in the literature, which can be due to the location and variety of the peppers employed to obtain the paprika.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure captions

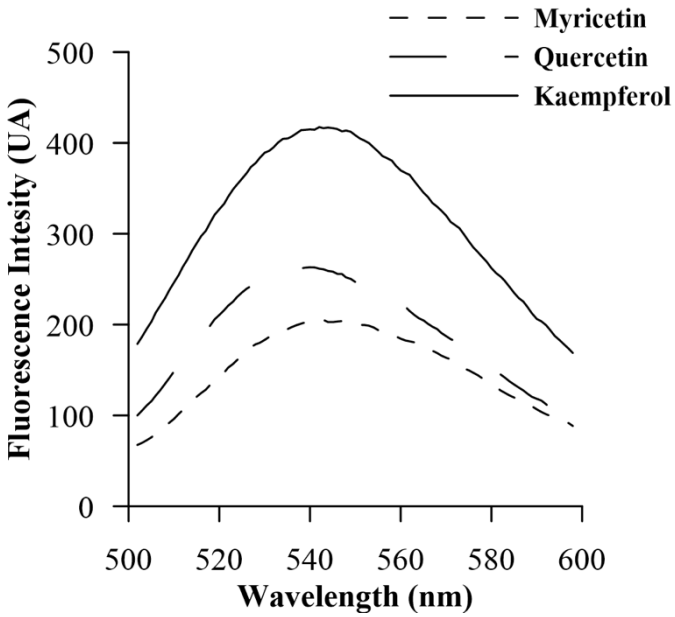

Fig. 1 Emission spectra of flavonol compounds (quercetin and myricetin $5 \cdot 10^{-6}$ M and kaempferol $3.5 \cdot 10^{-6}$ M) in basic medium (pH = 9.5) at an excitation wavelength of 420 nm.

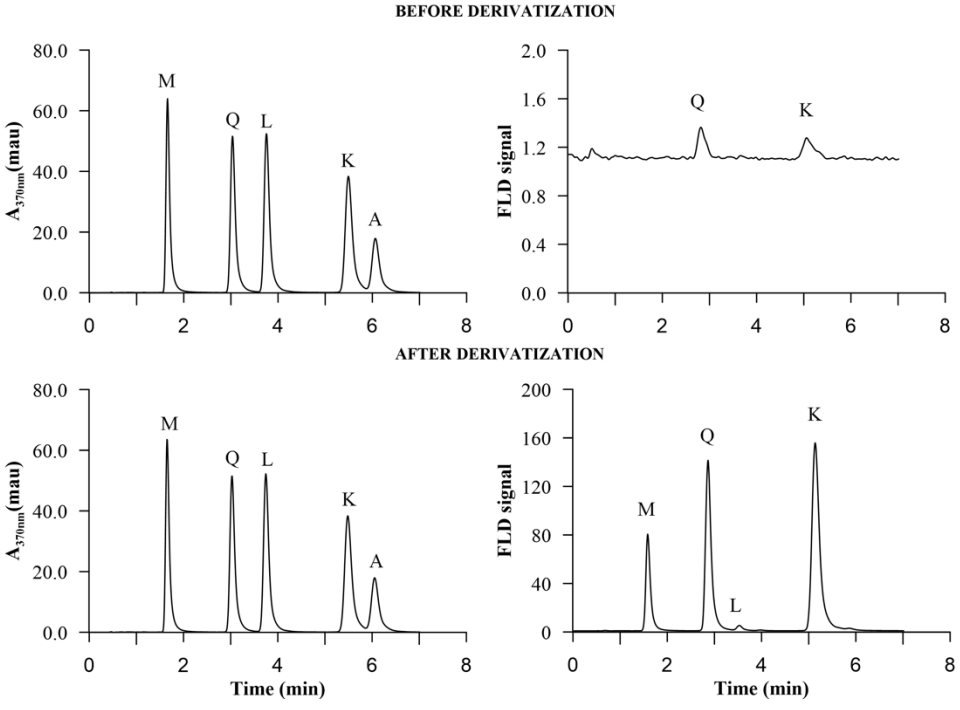

Fig. 2 Chromatograms corresponding to a flavonoid stock solution of 5.0 mg L^{-1} of each compound (M: myricetin, Q: quercetin, L: luteolin, K: kaempferol and A: apigenin) in the DAD

detector (370 nm), on the left, and in the FLD detector (λ_{exc}/ λ_{em} 420/560 nm), on the right, and before (above) and after (below) derivatization.

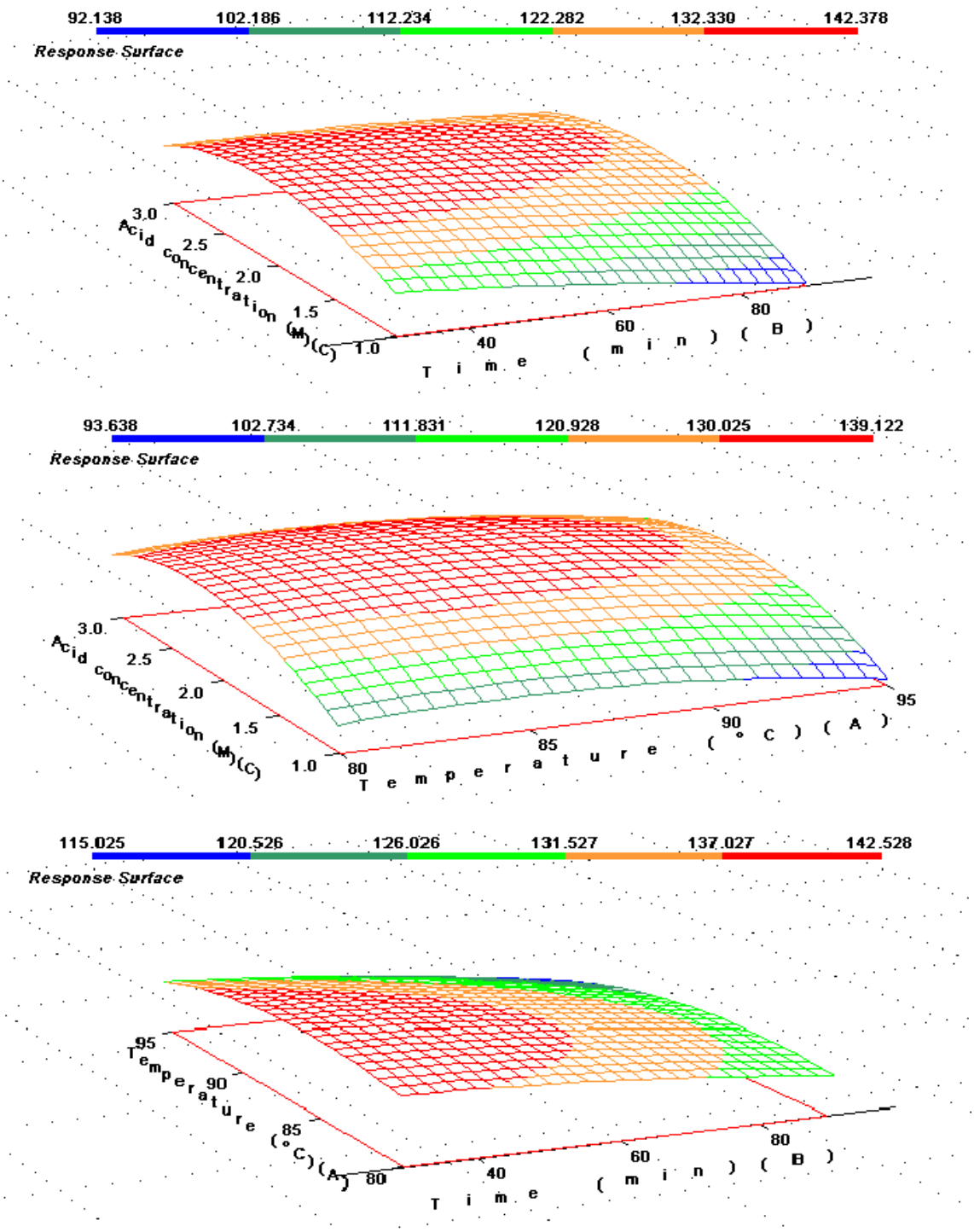

Fig. 3 Estimated response surfaces for each pair of variables. Above, acid concentration (M) versus time (min), in the centre, temperature (ºC) versus acid concentration (M) and, below, temperature (ºC) versus time (min).

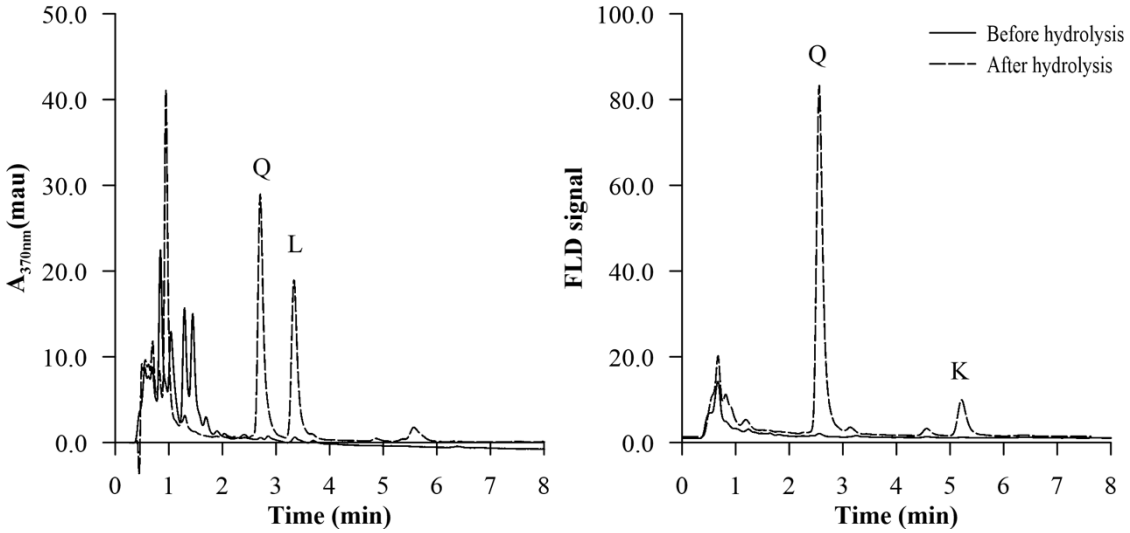

Fig. 4 Chromatograms corresponding to a real paprika sample before (solid line) and after (dash line) hydrolysis in both modes of detection, DAD (370 nm) on the left and FLD (λ_{ex}/ λ_{em} 420/560 nm) on the right.

Table 1 Analytical figures of merit

Analyte	Analytical signal (PA)	Lineal range (mg L^{-1})	Intercept ± SD	Slope ± SD (L mg^{-1})	Determination coefficient (R^2)	Linearity (%)	LOD[a] (mg L^{-1})	LOQ[b] (mg L^{-1})
Myricetin	$\lambda_{max.}$ 370 nm	0.05 – 15	3 ± 4	69.1 ± 0.5	0.999	99.3	0.006	0.02
	λ_{exc}/ λ_{em} 420/560 nm	0.05 – 15	-3 ± 10	124 ± 1	0.998	98.9	0.007	0.02
Quercetin	$\lambda_{max.}$ 370 nm	0.05 – 10	4 ± 6	71.4 ± 0.8	0.998	98.9	0.02	0.05
	λ_{exc}/ λ_{em} 420/560 nm	0.05 – 5	-7 ± 8	255 ± 3	0.999	98.9	0.01	0.04
Luteolin	$\lambda_{max.}$ 360 nm	0.05 – 15	2 ± 5	81.5 ± 0.6	0.999	99.2	0.008	0.03
	λ_{exc}/ λ_{em} 420/520 nm	0.5 – 15	-2 ± 2	14.8 ± 0.2	0.996	98.4	0.09[c]	0.30[c]
Kaempferol	$\lambda_{max.}$ 370 nm	0.05 – 15	6 ± 5	76.9 ± 0.6	0.999	99.2	0.02	0.05
	λ_{exc}/ λ_{em} 420/550 nm	0.05 – 3	-6 ± 4	373 ± 2	0.999	99.4	0.009	0.03
Apigenin	$\lambda_{max.}$ 360 nm	0.05 – 15	1 ± 2	41.2 ± 0.3	0.999	99.3	0.01	0.04
-	-	-	-	-	-	-	-	-

PA: Peak Area
SD: Standard Deviation
[a]LOD: Limit of detection, calculated as SD of a standard of 0.05 mg L^{-1} (n = 11)·3/Slope
[b]LOQ: Limit of quantification, calculated as SD of a standard of 0.05 mg L^{-1} (n = 11)·10/Slope
[c]In this case, the standard solution was 0.5 mg L^{-1}

Table 2. Relative Standard Deviation (%)

Analyte	Intra-day[a]				Intra-day[b]			
	DAD signals (n = 8)		FLD signals (n = 8)		DAD signals (n = 8)		FLD signals (n = 8)	
	tR	PA	tR	PA	tR	PA	tR	PA
Myricetin	0.20	1.7	0.34	1.8	0.16	4.5	0.17	4.3
Quercetin	0.27	2.0	0.28	1.2	0.12	3.7	0.14	2.3
Luteolin	0.29	0.79	0.29	5.2	0.15	2.6	-	-
Kaempferol	0.27	1.0	0.28	1.8	0.17	5.7	0.36	5.9
Apigenin	0.28	2.5	-	-	0.11	5.4	-	-

Analyte	Inter-day[a]				Inter-day[b]			
	DAD signals (n = 6)		FLD signals (n = 6)		DAD signals (n = 6)		FLD signals (n = 6)	
	tR	PA	tR	PA	tR	PA	tR	PA
Myricetin	0.89	6.8	0.78	7.1	0.97	4.9	0.82	6.9
Quercetin	1.1	4.4	0.75	7.0	1.2	7.5	1.0	8.5
Luteolin	1.5	1.4	1.1	7.7	1.4	5.4	-	-
Kaempferol	1.2	1.0	1.3	6.8	1.4	6.2	1.3	6.5
Apigenin	0.86	2.3	-	-	0.72	4.6	-	

[a]Standard solutions containing 3 mg L^{-1} of each analyte
[b]Standard solutions containing 0.05 mg L^{-1} of each analyte
tR: time retention
PA: peak area

Table 3. Peak area (PA) obtained for different experiments.

	Analyte				
	PA Quercetin ± SD		PA Luteolin ± SD		PA Kaempferol ± SD
	DAD signal	FLD signal	DAD signal	FLD signal	FLD signal
Cartridge	182 (± 4)	182 (± 4)	124 (± 7)	145 (± 7)	8.0 (± 0.7)
Without cartridge	152 (± 5)	177 (± 4)	124 (± 7)	154 (± 6)	7.8 (± 0.3)

Table 4. Results of the analysis of flavonoids by HPLC-DAD-FLD in real paprika samples.

	DAD Signal			FLD Signal		
PDO	**(Analytes concentration (mg kg^{-1*})± SD)·10^{-1}**			**(Analytes concentration (mg kg^{-1*})± SD)·10^{-1}**		
	Quercetin	**Luteolin**	**Kaempferol**	**Quercetin**	**Luteolin**	**Kaempferol**
1	14 ± 2	17 ± 1	n.q	14.0 ± 0.2	19 ± 1	0.3 ± 0.2
3	11 ± 2	15 ± 1	n.q	12.3 ± 0.2	17 ± 1	0.4 ± 0.2
3	8 ± 2	24 ±1	n.q	8.8 ± 0.2	23 ± 1	0.3 ± 0.2
4	14 ± 2	20 ± 1	n.q	15.2 ± 0.2	25 ± 1	0.3 ± 0.2
5	12 ± 2	15 ± 1	n.q	12.3 ± 0.2	20 ± 1	0.4 ± 0.2
6	19 ± 2	10 ±1	n.q	19.5 ± 0.2	14 ± 1	0.5 ± 0.2
7	25 ± 2	19 ± 1	n.q	27.2 ± 0.2	10 ± 1	0.5 ± 0.2
8	12 ± 2	16 ± 1	n.q	12.6 ± 0.2	20 ± 1	0.4 ± 0.2
9	8 ± 2	10 ± 1	n.q	9.0 ± 0.2	16 ± 1	0.7 ± 0.2
10	6 ± 2	11 ± 1	n.q	6.6 ± 0.2	16 ± 1	0.2 ± 0.2
11	9 ± 2	8 ± 1	n.q	10.1 ± 0.2	16 ± 1	0.3 ± 0.2
12	23 ± 2	26 ± 1	n.q	24.3 ± 0.2	29 ± 1	0.5 ± 0.2
13	10 ± 2	12 ± 1	n.q	10.7 ± 0.2	17 ± 1	0.8 ± 0.2
14	10 ± 2	19 ± 1	n.q	10.7 ± 0.2	19 ± 1	0.3 ± 0.2
15	18 ± 2	19 ± 1	n.q	18.8 ± 0.2	22 ± 1	0.6 ± 0.2
16	10 ± 2	15 ± 1	n.q	11.3 ± 0.2	16 ± 1	0.3 ± 0.2
17	11 ± 2	20 ± 1	n.q	11.0 ± 0.2	24 ± 1	0.4 ± 0.2
Mean	13	16		14.0	19	0.4
NO PDO	**(Analytes concentration (mg kg^{-1*})± SD)·10^{-1}**			**(Analytes concentration (mg kg^{-1*})± SD)·10^{-1}**		
	Quercetin	**Luteolin**	**Kaempferol**	**Quercetin**	**Luteolin**	**Kaempferol**
1	11 ± 2	13 ± 1	n.q	11.6 ± 0.2	14 ± 1	0.5 ± 0.2
3	10 ± 2	9 ± 1	n.q	10.1 ± 0.2	13 ± 1	0.4 ± 0.2
3	20 ± 2	8 ± 1	n.q	24.4 ± 0.2	23 ± 1	0.5 ± 0.2
4	25 ± 2	13 ± 1	n.q	25.2 ± 0.2	19 ± 1	0.5 ± 0.2
5	26 ± 2	11 ± 1	n.q	27.2 ± 0.2	29 ± 1	0.5 ± 0.2
6	13 ± 2	11 ± 1	n.q	14.6 ± 0.2	20 ± 1	0.4 ± 0.2
7	27 ± 2	10 ± 1	n.q	29.1 ± 0.2	27 ± 1	0.4 ± 0.2
8	31 ± 2	17 ± 1	n.q	32.6 ± 0.2	24 ± 1	1.0 ± 0.2
9	20 ± 2	14 ± 1	n.q	22.1 ± 0.2	21 ± 1	0.8 ± 0.2
10	8 ± 2	9 ± 1	n.q	8.6 ± 0.2	16 ± 1	0.3 ± 0.2
11	13 ± 2	8 ± 1	n.q	14.6 ± 0.2	28 ± 1	0.5 ± 0.2
12	16 ± 2	16 ± 1	n.q	16.8 ± 0.2	25 ± 1	0.8 ± 0.2
13	21 ± 2	13 ± 1	n.q	21.9 ± 0.2	13 ± 1	0.4 ± 0.2
14	58 ± 2	18 ± 1	n.q	69.0 ± 0.2	26 ± 1	0.6 ± 0.3
15	15 ± 2	8 ± 1	n.q	14.6 ± 0.2	21 ± 1	0.3 ± 0.2
16	9 ± 2	8 ± 1	n.q	8.8 ± 0.2	19 ± 1	0.4 ± 0.2
17	10 ± 2	6 ± 1	n.q	11.4 ± 0.2	17 ± 1	0.3 ± 0.2
18	11 ± 2	9 ± 1	n.q	11.8 ± 0.2	26 ± 1	0.4 ± 0.2
19	13 ± 2	8 ± 1	n.q	13.2 ± 0.2	17 ± 1	0.5 ± 0.2
Mean	19	11		20.4	21	0.5

***kg is referred to powder paprika; n.q.: not quantifiable**