

1 Factors affecting sample extraction in the liquid chromatographic  
2 determination of organic acids in papaya and pineapple

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4 Yurena Hernández, M. Gloria Lobo, Mónica González\*

5 *Post-harvest and Food Technology Laboratory, Department of Tropical Fruit Crops, Instituto Canario de*  
6 *Investigaciones Agrarias, Apdo. 60, 38200 La Laguna, Spain*

7  
8 **Abstract**

9 A solvent extraction method was developed for the extraction of organic acids (oxalic, citric, tartaric,  
10 L-malic, quinic, succinic and fumaric acids) in papaya and pineapple. Central composite design “2<sup>n</sup> + star”  
11 was used in order to optimise the following extraction parameters: number of extractions, composition of the  
12 extractant mixture, extraction time and extraction temperature. Optimal conditions for extraction were  
13 determined by experimental design using response surface methodology. The results suggest that the  
14 extractant composition is statistically the most significant factor and that the optimum values for the variables  
15 are: 3 (number de extractions), water as extractant, 60 min (extraction time) and 65 °C (extraction  
16 temperature). The separation and determination of the organic acids was carried out by liquid chromatography  
17 with UV-VIS detection.

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19 *Keywords:* Food analysis; Tropical fruits; Solvent extraction; Experimental design; LC with UV-VIS  
20 detection.

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22 \* Corresponding author. Tel.: +34-922-476310; fax: +34-922-476303.

23 *E-mail address:* [mgonzal@icia.es](mailto:mgonzal@icia.es) (Mónica González)

## 24 **1. Introduction**

25       Organic acids are widely distributed in fruits and originate from biochemical  
26 processes or from the activity of some microorganisms such as yeasts and bacteria. These  
27 carboxylic acids determine pH and total acidity of fruits, inhibit the action of enzymes and  
28 are chelating agents of metals and therefore impede chemical precipitation and oxidation.  
29 Moreover, the non-volatile organic acids influence fruit sensorial properties (flavour, colour  
30 and aroma). Therefore, the determination of organic acids allows different fruit cultivars  
31 (Usenik, Fabcic & Stampar, 2008) and the effect of pre-harvest factors (Keutgen &  
32 Pawelzik, 2007) on the organoleptic quality of the fruit to be evaluated. It also allows the  
33 development (Saradhuldhath & Paull, 2007) or ripening (Sturm, Koron & Stampar, 2003) of  
34 the fruit to be monitored, as well as provides a way to check and control the fermentation  
35 processes (Avenoza, Busto, Canal & Peregrina, 2006). Moreover, their analysis is a  
36 powerful tool to characterise the authenticity of fruit products (Saavedra, Rupérez &  
37 Barbas, 2001) or to control post-harvest (Beirao-Da-Costa, Steiner, Correia, Leitao, Empis  
38 & Moldao-Martins, 2008) or technological processes (Silva, Andrade, Mendes, Seabra &  
39 Ferrerira, 2002) and storage (Marsh, Attanayake, Walker, Gunson, Boldingh & MacRae,  
40 2004) based on their relative stability.

41       A variety of analytical methods for determining organic acids in fruits and fruit juices  
42 have been reported to date. The individual determination of organic acids in these food  
43 matrices is usually carried out using a spectrophotometric detector (Luque-Pérez, Ríos &  
44 Valcárcel, 1998) but the resolution of complex mixtures requires the use of chemometric  
45 techniques or near infrared spectroscopy (Chen, Zhang & Matsunaga, 2006). The non-  
46 specificity of classical electrochemical methods applied to determine organic acids has  
47 been surpassed by their combination with enzymatic biosensors (Kim, 2006). However,

48 because the organic acids are present in mixtures, the preferred choice for organic acids  
49 determination is the use of separation techniques: capillary electrophoresis (Saavedra et al.,  
50 2001; Mato, Suárez-Luque & Huidobro, 2007), gas chromatography (GC) and liquid  
51 chromatography (LC). In spite of its high sensitivity and selectivity, GC has been less used  
52 for the determination of organic acids than LC, because these compounds need to be  
53 derivatised (Chan, Chenchin & Vonnahme, 1973; González-Aguilar, Buta & Wang, 2003).  
54 Most of the liquid chromatographic methods to determine organic acids have been carried  
55 out by ion-exclusion (Cano, Torija, Marín & Cámara, 1994; Bartolomé, Rupérez & Fúster,  
56 1995; Bartolomé, Rupérez & Fúster, 1996; Saradhulhat et al., 2007).

57 To ensure that analysis by LC is effective, it is very important to optimise the sample  
58 extraction when analysing organic acids in complex samples such as fruits, because of their  
59 diverse matrices. Moreover, fruits contain large amounts of potentially interfering  
60 compounds. For these reasons considerable caution should be exercised in the employment  
61 of methods that have been developed for the analysis of specific plant tissue types. To  
62 provide clean extracts, fruits require a pre-treatment that includes organic solvent extraction  
63 and cleanup processes by solid-phase extraction. A critical step in the analytical  
64 determination of organic acids in tropical fruits such as papaya and pineapple, which has  
65 not been studied in detail, is the solvent extraction of these compounds as a function of  
66 these vegetable matrices. Several solvents have been used to extract organic acids from  
67 fruits: water (Sturm et al., 2003; Usenik et al., 2008), methanol (Cano et al., 1994;  
68 Bartolomé et al., 1995; Bartolomé et al., 1996; Silva et al., 2002) and mixtures  
69 ethanol:water (Pérez, Olías, Espada, Olías & Sanz, 1997; Holcroft & Kader, 1999;  
70 Saradhulhat et al., 2007). Other variables that influence the organic acids extraction are  
71 time and temperature. So, it has been used extraction times between 1 and 30 min (Cano et

72 al., 1994; Bartolomé et al., 1995; Pérez et al., 1997; Holcroft et al., 1999; Usenik et al.,  
73 2008) and extraction temperatures between room temperature and 65 °C (Cano et al., 1994;  
74 Bartolomé et al., 1995; Silva et al., 2002; Usenik et al., 2008).

75 Experimental designs are used to select influential factors, optimise conditions and  
76 assess the impact of those factors. In traditional strategies, only one variable is changed  
77 while all the others remain constant. This approach does not allow the study of changes in  
78 the response that may occur when two or more factors are modified simultaneously.  
79 Experimental design is an alternative to this strategy because it allows a large number of  
80 factors to be screened simultaneously and provides less ambiguous data. Furthermore,  
81 experimental designs combined with response surface methodology help to visualise  
82 relationships between responses and factor levels which allows researchers to locate the  
83 region of highest response values.

84 The objective of this research was to establish the optimal conditions for extracting  
85 organic acids from papaya and pineapple using solvent extraction before determining of  
86 these compounds via LC. The use of central composite designs to optimise four variables  
87 (number of extractions, composition of the extractant mixture, extraction time and  
88 extraction temperature) determines an optimal set of operational conditions.

89

## 90 **2. Materials and methods**

### 91 *2.1. Plant material*

92 Papaya (*Carica papaya* L., cv. “Baixinho do Santa Amalia”) was harvested from  
93 fields located in Tejina in northwest Tenerife (Canary Islands, Spain) and pineapple  
94 (*Ananas comosus* L., cv. “Red Spanish”) from Frontera in El Hierro (Canary Islands).  
95 Papaya was harvested at physiological maturity stage (mature-green) and allowed to ripen

96 (full-ripeness or consumption stage) at 18 °C and 80-90% relative humidity; however,  
97 pineapple (which is a non-climacteric fruit) was collected at full-ripeness.

98 The assay was performed using nine homogeneous units of each fruit in a similar  
99 ripening stage, characterised by peel and pulp colour, firmness, total soluble solids (TSS),  
100 pH and titratable acidity. Lightness, hue angle and chromaticity of papaya peel, at full  
101 ripeness, were  $63 \pm 2$ ,  $80 \pm 1$  and  $60 \pm 2$ , respectively. These colour attributes in pineapple  
102 peel ( $51 \pm 2$ ,  $72 \pm 3$  and  $34 \pm 1$ , respectively) were lower than in papaya. The colours of  
103 papaya and pineapple pulp were characterised by a lightness of  $60 \pm 2$  and  $75 \pm 5$ , a hue  
104 angle of  $70 \pm 2$  and  $109 \pm 2$  and a chromaticity of  $46 \pm 2$  and  $16 \pm 2$ , respectively. Pulp  
105 firmness, measured as penetration force, was  $4.3 \pm 0.2$  N and  $15 \pm 2$  N for papaya and  
106 pineapple, respectively. TSS were similar for both fruits:  $13 \pm 1$  °Brix for papaya and  $15 \pm$   
107  $1$  °Brix for pineapple. However, acid content was higher for pineapple (pH  $3.5 \pm 0.1$ ; and  
108 titratable acidity  $1,175 \pm 83$  mg citric acid/100 g) than for papaya (pH  $5.6 \pm 0.1$ ; and  
109 titratable acidity  $90 \pm 3$  mg citric acid/100 g).

110 For organic acids determination, fruits pulp was sliced, frozen into liquid nitrogen  
111 and stored at  $-80$  °C until the analyses were carried out.

112

## 113 2.2. Solvent extraction method

114 Four grams of accurately weighed of frozen pulverised fruit samples were mixed  
115 with 8 ml of extractant [concentration of ethanol (Panreac, Madrid, Spain) and water varied  
116 depending on the particular experiment; ranging between 0 and 100%]. The mixture was  
117 homogenised with a Politron PT 6000 (Kinematica AG, Lucerne, Switzerland) high speed  
118 blender at 12,000 g for 1 min. Then organic acids present in the fruits were extracted  
119 (extraction time varied depending on the particular experiment; ranging between 5 and 60

120 min) in a water bath (extraction temperature varied depending on the particular experiment;  
121 ranging between 25 and 100 °C). Extracts were centrifuged at 5,000 g for 30 min in a Jouan  
122 CR 312 centrifuge (Thermo Electron Corporation, Madrid, Spain). Depending on the  
123 experiment, this procedure was repeated (ranging between 1 and 3 times); the resulting  
124 supernatants were mixed together and a final volume of 25-ml was achieved. An aliquot of  
125 4 ml of the extract was evaporated to dryness at 45°C (approximately 20 h) in a Heto VR 1  
126 evaporator (Allerod, Denmark). The residue was re-dissolved in 4 ml of water milli-Q and  
127 passed through a 300 mg Alltech (Laarne, Belgium) Sep-pack RP-C18 column to eliminate  
128 possible interferences. The eluate was collected, an aliquot of 1 ml was filtered through a  
129 0.45 µm nylon membrane and subsequently used to analyse organic acids. After each  
130 determination, the adsorbent was cleaned with 6 ml of water to remove interfering  
131 compounds adsorbed; finally, the adsorbent was conditioned with 2 ml of ethanol.

132

### 133 *2.3. Chromatographic determination of organic acids*

134 The liquid chromatographic method used for the determination of organic acids  
135 (oxalic, citric, tartaric, pyruvic, L-malic, quinic, succinic and fumaric acid) consisted of an  
136 isocratic elution procedure with UV-visible detection. The analyses were carried out on a  
137 Shimadzu modular chromatographic system (Kyoto, Japan) equipped with a LC-10AD  
138 pump, a SPD-10AV UV-VIS detector and controlled via Class LC-10 data acquisition  
139 software (also from Shimadzu). The injection valve was a Rheodyne 7725i (Cotati, USA)  
140 with an injection loop of 20 µl. Organic acid separation was carried out on a Shodex  
141 (Showa Kenko, Tokyo, Japan) RSpack KC-811 column (5 µm particle size, 250 x 4.6 mm

142 i.d.), using an isocratic 0.1% orthophosphoric acid (Panreac) mobile phase at a flow rate of  
143 0.8 ml/min. Detection wavelength for the UV-visible detector was set at 210 nm.

144 Organic acid peaks were identified by comparing their UV-VIS spectral  
145 characteristics and retention times with those from commercial standards supplied by  
146 Sigma (Madrid, Spain). The spectra (detection wavelengths from 200 to 700 nm) were  
147 recorded for the peaks identified as a particular organic acid by retention time, using a  
148 Shimadzu SPD-M6A UV-VIS diode array detector. For each fruit type the efficiency of  
149 peak separation was checked by the peak purity test carried out at maximum absorbance.  
150 Stock standard solutions containing 1 mg/ml of each organic acid were prepared in milli-Q  
151 water and stored in glass stoppered bottles at 4 °C in the dark. Solutions of variable  
152 concentrations were prepared by diluting the stock standard solution in milli-Q water.

153

#### 154 *2.4. Experimental design*

155 Statgraphics Plus version 4.1 (Statistical Graphics, Rockville, USA) was employed  
156 to generate design, regression analysis and to obtain the response surface plots. A central  
157 composite design (CCD) “ $2^4$  + star” projected on a face-centred star design with two centre  
158 points was chosen to evaluate the combined effects of four independent variables on the  
159 organic acid extraction from two tropical fruits (papaya and pineapple). The variables  
160 (number of extractions, ethanol concentration in the extractant mixture, extraction time and  
161 extraction temperature) were set at three separate coded levels (see Section 2.2.). The  
162 design consisted of 26 randomised runs, doing each experiment in triplicate ( $n = 78$ ). The  
163 unknown function was assumed to be approximated by a second-order polynomial equation  
164 such as:

165 
$$y = \beta_0 + \sum_{i=1}^k \beta_i \cdot x_i + \sum_{i=1}^k \beta_{ii} \cdot x_i^2 + \sum_i \sum_j \beta_{ij} \cdot x_i \cdot x_j + \varepsilon$$

166 
$$(i < j)$$

167 where  $y$  is the organic acid content;  $\beta_0$  (centre point of system),  $\beta_i$ ,  $\beta_{ii}$  and  $\beta_{ij}$  (coefficients  
168 of variables for linear, quadratic and interaction regression effects) are the different  
169 constant coefficients of the model;  $x_i$  and  $x_j$  are levels of independent variables; and  $\varepsilon$  is the  
170 error of the model. An analysis of variance (ANOVA) table was generated to determine  
171 individual linear, quadratic and interaction regression coefficients. The significances of  
172 polynomial relations were examined statistically by computing the  $F$ -value at a probability  
173 ( $p$ ) of 0.050. The regression coefficients were then used to make statistical analysis and to  
174 generate contour maps of the regression models.

175

### 176 **3. Results and discussion**

#### 177 *3.1. Chromatographic conditions to analyse organic acids and analytical features*

178 Initially, the chromatographic conditions described by Cano et al. (1994) and  
179 Bartolomé et al. (1995) were followed to analyse organic acids (oxalic, citric, tartaric,  
180 pyruvic, L-malic, quinic, succinic and fumaric acid) in papaya and pineapple fruits.  
181 However, due to the differences on the stationary phase used for the separation of the  
182 organic acids, some chemical, hydrodynamic, and physical variables had to be optimised to  
183 improve the resolution between the different organic acids. Good results were obtained  
184 using a mixture of water and orthophosphoric acid as the mobile phase. The concentration  
185 of orthophosphoric acid was studied over the range 0-1% and a concentration of 0.1% was  
186 found to be optimal. The flow-rate significantly influenced organic acids retention time and  
187 resolution; the best flow value was 0.8 ml/min (optimised between 0.2 and 1.8 ml/min)



188 because of the better resolution achieved. The oven temperature was studied over the range  
189 25-60 °C. The control of temperature was not considered necessary because it did not  
190 improve the separation of the different analytes.

191 Calibration graphs for organic acids were constructed by plotting the peak area  
192 against the organic acid concentration at seven concentration levels (analysed in triplicate).  
193 Quality parameters for the chromatographic determination of the different organic acids are  
194 reported in Table 1. Detection limit, defined as the minimum concentration capable of  
195 giving a chromatographic signal three times higher than background noise, is also listed in  
196 Table 1. The repeatability of the chromatographic procedure, expressed as relative standard  
197 deviation (RSD), was checked on eleven consecutive injections of a standard solution  
198 containing 100 mg/l of the different organic acids analysed.

199

### 200 *3.2. Optimisation of the organic acid extraction*

201 In accordance with our previous experience in the treatment of papaya and pineapple  
202 fruits, four variable factors that can potentially affect extraction efficiency were chosen:  
203 number of extractions, ethanol concentration in the extractant mixture, extraction time and  
204 extraction temperature. Although organic acids have been extracted from papaya and  
205 pineapple, the analytical methods used were not optimised for this specific fruits (Chan,  
206 Chang, Stafford & Brekke, 1971; Cano et al., 1994; Bartolomé et al., 1995; Bartolomé et  
207 al., 1996). In order to optimise organic acids extraction specifically from papaya and  
208 pineapple, the minimum and maximum levels (Table 2 and Table 3) given to each factor  
209 were chosen based on the experience of other authors in the pre-treatment of different types  
210 of plant materials (Cano et al., 1994; Holcroft et al., 1999; Silva et al., 2002; Sturm et al.,

211 2003; Usenik et al., 2008). Other factors implicated in the extraction were kept constant:  
212 amount of fruit, volume of extractant and final volume of the extract.

213 Table 2 and Table 3 show the design matrix, which include the factors that influence  
214 organic acid extraction and the amounts [expressed as mg/100 g fresh weight (FW)] of the  
215 different organic acids obtained in the different experiments carried out in papaya and  
216 pineapple, respectively. The sequential listing of the experimental design parameters  
217 represents the statistically randomised order in which the experimental treatments were  
218 undertaken. All organic acids analysed, except pyruvic acid, were identified in papaya  
219 (Table 2); however in pineapple five organic acids were identified: oxalic acid, citric acid,  
220 L-malic acid, quinic acid and succinic acid (Table 3). Chan et al. (1971) identified and  
221 quantified citric and malic acids (as the main acids) and  $\alpha$ -ketoglutaric acid in type Solo  
222 papaya. Cano et al. (1994) described the presence of oxalic, citric, galacturonic, ascorbic,  
223 L-malic, quinic, succinic, fumaric and D-malic acid in papaya cv. "Sunrise". Regarding to  
224 pineapple cv. "Red Spanish", Bartolomé et al. (1996) identified oxalic, citric, L-malic,  
225 quinic and succinic acids but only quantified oxalic, citric and L-malic acid. Citric and L-  
226 malic acid have been identified and quantified in cultivar "Smooth Cayenne" by Cano et al.  
227 (1994), Bartolomé et al. (1995), Bartolomé et al. (1996), Brat, Hoang, Soler, Reynes &  
228 Brillouet (2004) and Saradhulhat et al. (2007). Moreover, Cano et al. (1994), Bartolomé et  
229 al. (1996) and Brat et al. (2004) quantified oxalic acid in this pineapple cultivar. Bartolomé  
230 et al. (1996) also quantified quinic and succinic acid.

231 It should be noted that the modification of the experimental conditions used for the  
232 extraction did not affect all of the organic acids equally (Table 2 and Table 3). In fact,  
233 different chromatographic profiles were obtained for different extraction conditions. The

234 comparison of the experimental values of organic acids content with the predicted values  
235 shown that the two sets of values, for each organic acid in the two fruits analysed, were  
236 very close, indicating that the experimental model was valid. The coefficients of  
237 determination ( $R^2$ ) indicated that the model (predicted values) explains between 95 and  
238 99% of the variability observed in organic acids contents (experimental values). The  
239 standard error of the estimates shown a standard deviation of the residuals between 1.9 and  
240 7.6 and the Durbin-Watson statistic tests indicated that, since the  $p$  value was greater than  
241 0.050 for all the acids, there was no indication of serial autocorrelation in the residuals.

242 ANOVA was used to estimate the statistical significance of the factors that had the  
243 greatest effect on the extraction and interactions between them (Table 4). In papaya, the  
244 extractant composition and the number of extractions influenced the extraction efficiency  
245 of all acids contained in this fruit. Extraction time was statistically significant for oxalic,  
246 tartaric, quinic and fumaric acids and extraction temperature for all the acids mentioned  
247 previously plus succinic acid. So, neither time nor temperature affected the extraction  
248 efficiency of citric acid and L-malic acid from papaya. In pineapple, the extractant  
249 composition and the extraction temperature influenced the extraction efficiency of all acids  
250 contained in this fruit. The number of extractions was not statistically significant for quinic  
251 and succinic acids and time did not affect the extraction of citric acid. Analysis of the  
252 experimental results shown that in the majority of cases the most notable effect was caused  
253 by the extractant composition (Table 4). So, its contribution was higher than 40% for citric  
254 acid, L-malic acid, succinic acid and fumaric acid in papaya and for oxalic acid and quinic  
255 acid in pineapple. Among the factors studied, the number of extractions had the second  
256 greatest impact on how efficiently organic acids were extracted from papaya (Table 4). It  
257 was especially relevant in the extraction of oxalic acid, L-malic acid and fumaric acid, with

258 a contribution to the extraction efficiency of 25-30%. However, in pineapple, extraction  
 259 temperature was the second most important factor (Table 4) for oxalic acid (next to the  
 260 interaction between the extraction temperature and the extractant composition), citric acid,  
 261 quinic acid and succinic acid (factor contribution, 17-24%). This fact could indicate that  
 262 pineapple matrix makes more difficult the extraction of organic acids (mostly stored in the  
 263 vacuoles of cells) from pineapple than from papaya.

264 Significant equation models (Table 4) were obtained by applying statistical analysis  
 265 to the organic acid contents of papaya:

266 Oxalic acid =  $3.8 + 3.4 \cdot n_{\text{ext}} + 0.085 \cdot \% \text{ EtOH} - 0.13 \cdot t_{\text{ext}} + 0.074 \cdot T_{\text{ext}} + 0.033$   
 267  $\cdot n_{\text{ext}} \cdot t_{\text{ext}} + 0.00056 \cdot t_{\text{ext}} \cdot T_{\text{ext}} - 0.89 \cdot n_{\text{ext}}^2 - 0.0012 \cdot \% \text{ EtOH}^2 + 0.0011 \cdot$   
 268  $t_{\text{ext}}^2 - 0.00062 \cdot T_{\text{ext}}^2$

269 Citric acid =  $964 + 578 \cdot n_{\text{ext}} - 0.44 \cdot \% \text{ EtOH} - 1.7 \cdot n_{\text{ext}} \cdot \% \text{ EtOH} + 0.028 \cdot$   
 270  $\text{EtOH} \cdot T_{\text{ext}}$

271 Tartaric acid =  $163 - 29 \cdot n_{\text{ext}} - 0.26 \cdot \% \text{ EtOH} + 0.62 \cdot t_{\text{ext}} - 0.58 \cdot T_{\text{ext}} - 0.072 \cdot$   
 272  $n_{\text{ext}} \cdot \% \text{ EtOH} + 0.12 \cdot n_{\text{ext}} \cdot t_{\text{ext}} + 0.13 \cdot n_{\text{ext}} \cdot T_{\text{ext}} - 0.0071 \cdot \% \text{ EtOH} \cdot t_{\text{ext}} -$   
 273  $0.0021 \cdot \% \text{ EtOH} \cdot T_{\text{ext}} - 0.0033 \cdot t_{\text{ext}} \cdot T_{\text{ext}} + 6.0 \cdot n_{\text{ext}}^2 + 0.0066 \cdot \% \text{ EtOH}^2 -$   
 274  $0.010 \cdot t_{\text{ext}}^2$

275 L-malic acid =  $52 + 109 \cdot n_{\text{ext}} - 0.61 \cdot \% \text{ EtOH} - 0.13 \cdot n_{\text{ext}} \cdot \% \text{ EtOH} + 0.16 \cdot n_{\text{ext}}$   
 276  $\cdot t_{\text{ext}} - 0.12 \cdot n_{\text{ext}} \cdot T_{\text{ext}} + 0.0042 \cdot t_{\text{ext}} \cdot T_{\text{ext}} - 22 \cdot n_{\text{ext}}^2 + 0.0047 \cdot \% \text{ EtOH}^2 -$   
 277  $0.010 \cdot T_{\text{ext}}^2$

278 Quinic acid =  $27 - 40 \cdot n_{\text{ext}} + 1.2 \cdot \% \text{ EtOH} - 0.37 \cdot t_{\text{ext}} - 0.061 \cdot T_{\text{ext}} - 0.13 \cdot n_{\text{ext}} \cdot$   
 279  $\% \text{ EtOH} + 0.25 \cdot n_{\text{ext}} \cdot t_{\text{ext}} + 0.19 \cdot n_{\text{ext}} \cdot T_{\text{ext}} - 0.0046 \cdot \% \text{ EtOH} \cdot t_{\text{ext}} - 0.0034 \cdot$   
 280  $\% \text{ EtOH} \cdot T_{\text{ext}} + 0.0066 \cdot t_{\text{ext}} \cdot T_{\text{ext}} + 8.6 \cdot n_{\text{ext}}^2 - 0.0075 \cdot \% \text{ EtOH}^2$

281 Succinic acid =  $1227 + 330 \cdot n_{\text{ext}} - 23 \cdot \% \text{ EtOH} - 8.1 \cdot T_{\text{ext}} + 3.0 \cdot n_{\text{ext}} \cdot t_{\text{ext}} +$   
 282  $0.092 \cdot \% \text{ EtOH} \cdot T_{\text{ext}} + 0.15 \cdot \% \text{ EtOH}^2$

283 Fumaric acid =  $10 + 2.0 \cdot n_{\text{ext}} - 0.026 \cdot \% \text{ EtOH} + 0.014 \cdot t_{\text{ext}} - 0.089 \cdot T_{\text{ext}} -$   
 284  $0.0094 \cdot n_{\text{ext}} \cdot \% \text{ EtOH} + 0.011 \cdot n_{\text{ext}} \cdot t_{\text{ext}} + 0.0052 \cdot n_{\text{ext}} \cdot T_{\text{ext}} + 0.00030 \cdot \%$   
 285  $\text{EtOH} \cdot t_{\text{ext}} - 0.00011 \cdot \% \text{ EtOH} \cdot T_{\text{ext}} - 0.00036 \cdot t_{\text{ext}} \cdot T_{\text{ext}} - 0.00082 \cdot t_{\text{ext}}^2 +$   
 286  $0.00067 \cdot T_{\text{ext}}^2$

287 For pineapple, the response equations for the organic acid contents were:

288 Oxalic acid =  $-3.4 + 3.8 \cdot n_{\text{ext}} - 0.076 \cdot \% \text{ EtOH} + 0.076 \cdot t_{\text{ext}} + 0.097 \cdot T_{\text{ext}} -$   
 289  $0.0066 \cdot n_{\text{ext}} \cdot \% \text{ EtOH} - 0.00034 \cdot \% \text{ EtOH} \cdot t_{\text{ext}} - 0.00057 \cdot \% \text{ EtOH} \cdot T_{\text{ext}} +$   
 290  $0.0010 \cdot \% \text{ EtOH}^2$

291 Citric acid =  $2871 + 818 \cdot n_{\text{ext}} - 9.4 \cdot \% \text{ EtOH} + 14 \cdot T_{\text{ext}} + 2.3 \cdot n_{\text{ext}} \cdot \% \text{ EtOH} -$   
 292  $5.5 \cdot n_{\text{ext}} \cdot t_{\text{ext}} - 2.3 \cdot n_{\text{ext}} \cdot T_{\text{ext}} - 0.067 \cdot \% \text{ EtOH} \cdot t_{\text{ext}} - 0.19 \cdot t_{\text{ext}} \cdot T_{\text{ext}} - 0.34 \cdot$   
 293  $t_{\text{ext}}^2$

294 L-malic acid =  $309 - 17 \cdot n_{\text{ext}} - 0.81 \cdot \% \text{ EtOH} + 2.3 \cdot t_{\text{ext}} + 0.50 \cdot T_{\text{ext}} + 0.29 \cdot n_{\text{ext}}$   
 295  $\cdot \% \text{ EtOH} - 0.0070 \cdot t_{\text{ext}} \cdot T_{\text{ext}} - 0.025 \cdot t_{\text{ext}}^2$

296 Quinic acid =  $4.6 - 0.0061 \cdot \% \text{ EtOH} - 0.073 \cdot t_{\text{ext}} + 0.028 \cdot T_{\text{ext}} + 0.0066 \cdot n_{\text{ext}} \cdot$   
 297  $\% \text{ EtOH} + 0.00014 \cdot \% \text{ EtOH} \cdot T_{\text{ext}} + 0.00016 \cdot t_{\text{ext}} \cdot T_{\text{ext}} + 0.0013 \cdot t_{\text{ext}}^2$

298 Succinic acid =  $369 - 1.9 \cdot \% \text{ EtOH} + 1.1 \cdot t_{\text{ext}} - 3.9 \cdot T_{\text{ext}} + 0.39 \cdot n_{\text{ext}} \cdot t_{\text{ext}} -$   
 299  $0.012 \cdot \% \text{ EtOH} \cdot t_{\text{ext}} + 0.015 \cdot \% \text{ EtOH} \cdot T_{\text{ext}} + 0.018 \cdot T_{\text{ext}}^2$

300 To determine the optimal levels of the studied factors on organic acids extraction,  
 301 three-dimensional surface graphs were constructed. Some of the graphs, selected from  
 302 among those obtained using the experimental model, are shown for papaya (Fig. 1) and for  
 303 pineapple (Fig. 2). The relation between peak area for each run and the peak area for the  
 304 run with maximal response was used as the normalised response value for each organic acid

305 as a way to measure the extraction efficiency. In these graphics, the variable with the  
306 greatest effect on the extraction, composition of the extractant, is shown along with number  
307 of extractions (Fig. 1a and Fig. 2a). The extraction efficiency increased when the  
308 percentage of ethanol in the extractant decreased. Thus, the optimum percentage of ethanol  
309 in the extractant was 0% (extraction with water). This conclusion is similar for all of the  
310 organic acids detected in the fruits except for the extraction of oxalic acid from papaya  
311 (Fig. 1a) and for quinic acid from pineapple (Fig. 2a) where the extraction was optimum  
312 when the extractant was 50% ethanol (in water) and 100% ethanol, respectively. The  
313 number of extractions affected the extraction of organic acids from papaya more than from  
314 pineapple. The extraction efficiency improved generally by increasing the number of  
315 extractions. This effect was also noticed in Fig. 1b and Fig. 2b which represent extraction  
316 temperature versus number of extractions. This fact suggested that 3 was the optimal  
317 number of extractions. Extraction temperature versus extraction time is represented in Fig.  
318 1c and Fig. 2c. The temperature of the extraction mainly affected pineapple. It can be  
319 observed that at a fixed extraction time the optimum extraction efficiency was reached at  
320 high extraction temperatures for acids such as oxalic and quinic; however, the highest  
321 extraction of citric and succinic acids was obtained with low extraction temperatures. In  
322 papaya, the highest extraction was obtained with high extraction temperatures for quinic  
323 acid (Fig. 1b and Fig. 1c) and with low extraction temperatures for succinic acid (Fig. 1c).  
324 Regarding extraction time, it was clear that in the cases in which it was a significant factor  
325 the highest extraction efficiency was reached at high extraction times, so, the optimal  
326 extraction time was found to be 60 min.

### 327 3.3. Verification of the model: analysis of organic acids in papaya and pineapple

328 In order to determine all the different acids in the same extract a pragmatic approach  
329 must be used when selecting the extraction conditions. Using water as extractant, 3  
330 extractions, a temperature of 65 °C and a time of 60 min, the highest extraction was  
331 obtained for the whole acids. In those conditions, the extraction for most of the acids  
332 (oxalic, citric, tartaric, L-malic and fumaric acids in papaya or pineapple) was higher than  
333 85% (compared to extractions obtained using the optimal conditions for the extraction of  
334 each acid); the extraction of succinic acid was 75% from papaya and 85% from pineapple.  
335 However, the lower extraction of quinic acid (60% from both types of fruit) was due to the  
336 important influence, on the extraction of this acid, of the extractant composition (optimal  
337 extraction with a percentage of ethanol in the extractant of 30-60% for papaya and of 100%  
338 for pineapple) and of the temperature (optimal extraction at 100 °C). The differences on the  
339 extraction of quinic acid could be related to the chemical structure of this acid since it is the  
340 only acid that has an aromatic ring with one carboxylic and four hydroxyl groups.  
341 Therefore, the optimal extraction of quinic acids was obtained with 60% of ethanol (in  
342 water), at 100 °C for 60 min and with 3 repetitions of the extraction procedure. In case that  
343 quinic acid was determined in the extract obtained to determine all the organic acids (water  
344 as extractant, 3 extractions, 65 °C, 60 min), a more accurate content for quinic acid could be  
345 calculated using a correction factor ( $f = 1.67$ ). This correction was estimated from the  
346 equation derived from the experimental design used to optimise quinic acid extraction and  
347 it is defined as the ratio between mg quinic acid/100 g obtained in optimal conditions for  
348 extracting quinic acid and mg quinic acid/100 g obtained in selected conditions for  
349 extracting all the organic acids.

350 To determine the accuracy of the extraction method, five independent extractions  
351 were carried out using the newly established experimental conditions. The experimental  
352 values for organic acids, except for quinic acid, were quite close to the predicted values  
353 which proved that the model was valid. Citric acid was the main organic acid in ripe papaya  
354 ( $335 \pm 32$  mg/100 g FW) and in ripe pineapple ( $891 \pm 15$  mg/100 g FW) followed by L-  
355 malic acid [ $209 \pm 12$  (papaya) and  $356 \pm 8$  (pineapple) mg/100 g FW, respectively]. The  
356 amounts of these acids found in this study were similar to those found by Cano et al. (1994)  
357 for papaya cv. “Sunrise” and by Bartolomé et al. (1996) for pineapple cv. “Red Spanish”.  
358 However, lower amounts of citric and L-malic acids were established in papaya (Harvey et  
359 al., 1971; González-Aguilar et al., 2003) and in pineapple cv. “Smooth Cayenne” (Cano et  
360 al., 1994; Brat et al., 2004; Saradhulhat et al., 2007). Oxalic, tartaric, quinic, succinic and  
361 fumaric acid contents in papaya were:  $10 \pm 1$ ,  $13 \pm 2$ ,  $52 \pm 5$ ,  $52 \pm 3$  and  $1.1 \pm 0.1$  mg/100 g  
362 FW, respectively. The corrected value for quinic acid was  $87 \pm 4$  mg/100 g FW. Cano et al.  
363 (1994) and González-Aguilar et al. (2003) reported lower quantities of quinic acid.  
364 Moreover, Cano et al. (1994) could not detect the presence of tartaric acid in papaya cv.  
365 “Sunrise”. In pineapple, oxalic, quinic and succinic acids were quantified as:  $8.5 \pm 0.8$ ,  $4.2$   
366  $\pm 0.1$  (corrected value,  $7.0 \pm 0.6$ ) and  $4.5 \pm 0.2$  mg/100 g FW, respectively. Bartolomé et al.  
367 (1996) described four times less amount of oxalic acid in the same pineapple cultivar  
368 analysed in this work and quinic and succinic acids could not be quantified. These  
369 discrepancies on organic acid contents described are probably related with the different  
370 methods used to extract these compounds from the fruit matrices.



371 **4. Conclusions**

372           Organic acids found in papaya and pineapple can be efficiently extracted using an  
373 adequate selection of the experimental conditions optimised in this study. The most  
374 important factor among the optimised conditions is the extractant composition, although the  
375 number of extractions and the extraction temperature contribute notably to the extraction  
376 efficiency from papaya and pineapple, respectively. Optimum values of the variables that  
377 influence the extraction of organic acids from these fruits are: 3 extractions, water as  
378 extractant, a 60 min extraction time and a 65 °C extraction temperature. The results of this  
379 work confirm the necessity to optimise the solvent extraction as a critical step in the  
380 analytical determination of organic acids in tropical fruits such as papaya and pineapple.

381

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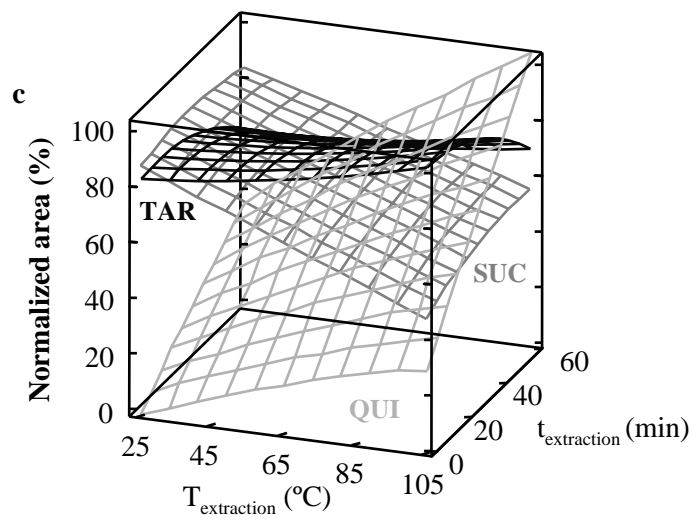
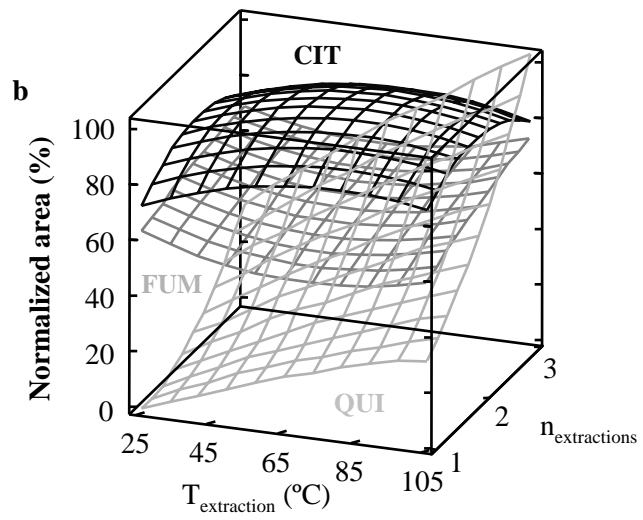
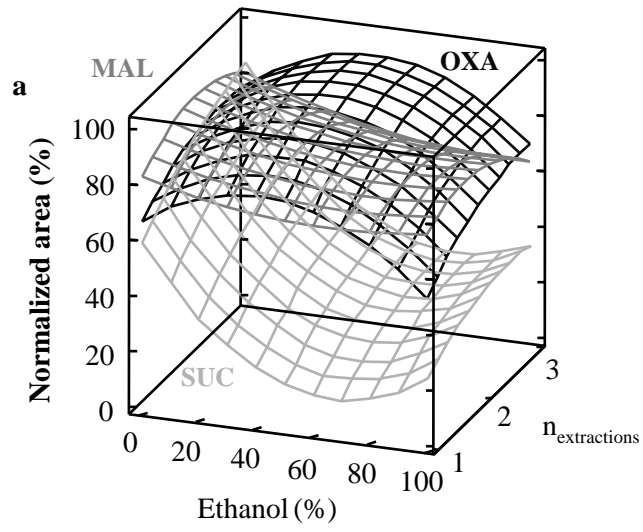
454 **Figure captions**

455

456 **Fig. 1.** Estimated response surfaces in the design experiment obtained by plotting (a) the  
457 ethanol concentration in the extractant mixture and number of extractions for oxalic  
458 (OXA), L-malic (MAL) and succinic (SUC) acids fixing temperature at 65 °C and time at  
459 60 min; (b) extraction temperature and number of extractions for citric (CIT), quinic (QUI)  
460 and fumaric (FUM) acids fixing ethanol percentage at 0% and time at 60 min; and (c)  
461 extraction temperature and extraction time for tartaric (TAR), quinic (QUI) and succinic  
462 (SUC) acids fixing ethanol percentage at 0% and number of extractions at 3, from papaya.

463

464 **Fig. 2.** Estimated response surfaces in the design experiment obtained by plotting (a) the  
465 ethanol concentration in the extractant mixture and number of extractions for oxalic  
466 (OXA), citric (CIT) and quinic (QUI) acids fixing temperature at 65 °C and time at 60 min;  
467 (b) extraction temperature and number of extractions for citric (CIT), L-malic (MAL) and  
468 quinic (QUI) acids fixing ethanol percentage at 0% and time at 60 min; and (c) extraction  
469 temperature and extraction time for oxalic (OXA), L-malic (MAL) and succinic (SUC)  
470 acids fixing ethanol percentage at 0% and number of extractions at 3, from pineapple.



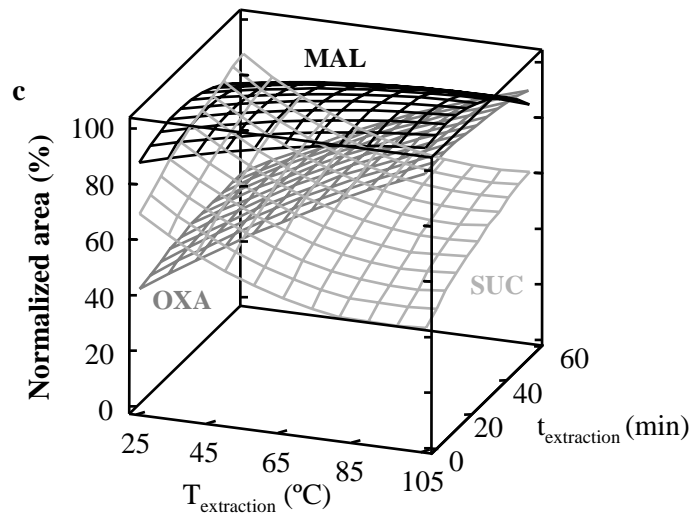
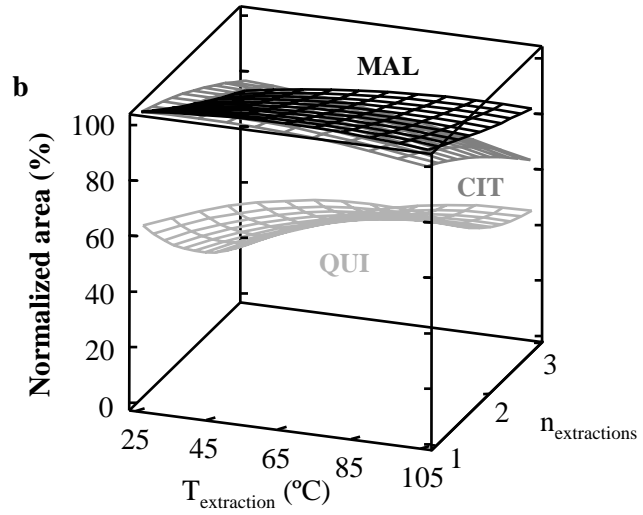
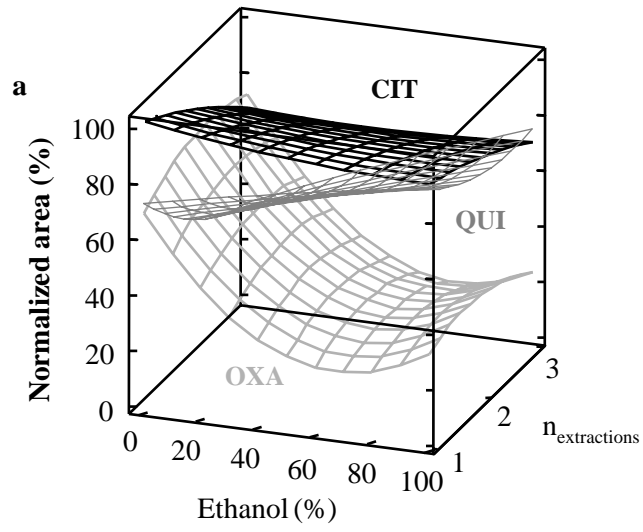


Table 1

Quality parameters for the chromatographic determination of organic acids

Organic acid	Slope	Intercept	R <sup>2</sup>	Linear range (mg/l)	Detection limit (mg/l)	RSD (%)
Oxalic	16,145	0.64	0.998	0.5-150	0.002	3.4
Citric	275	0.21	0.997	2.5-2,000	0.20	5.8
Tartaric	274	-0.40	0.997	2.5-2,000	0.20	7.3
Pyruvic	4,426	0.02	0.994	2.5-150	0.01	3.8
L-malic	1,372	0.03	0.999	2.5-1,250	0.05	4.0
Quinic	845	-0.12	0.994	2.5-2,000	0.05	5.2
Succinic	99	0.20	0.997	2.5-2,000	0.50	6.1
Fumaric	21,298	0.01	0.997	0.5-150	0.002	2.5



Table 2

Design matrix for the optimization of the extraction of papaya organic acids, experimental values<sup>a</sup> (EV, mg/100 g FW) obtained for each identified organic acid and predicted values (PV) for the central composite design ( $2^4 + \text{star}$ )

Experiment	$n_{\text{ext}}$	% EtOH	$t_{\text{ext}}$	$T_{\text{ext}}$	Oxalic acid		Citric acid		Tartaric acid		L-malic acid		Quinic acid		Succinic acid		Fumaric acid	
					EV	PV	EV	PV	EV	PV	EV	PV	EV	PV	EV	PV	EV	PV
1	1	0	60	100	9.5 <sup>0.023</sup>	8.5	248 <sup>0.079</sup>	259	11 <sup>0.043</sup>	11	168 <sup>0.025</sup>	162	21 <sup>0.094</sup>	24	29 <sup>0.12</sup>	29	0.70 <sup>0.088</sup>	0.76
2	3	50	32.5	62.5	11 <sup>0.032</sup>	11	308 <sup>0.029</sup>	295	12 <sup>0.23</sup>	12	178 <sup>0.064</sup>	173	46 <sup>0.076</sup>	42	27 <sup>0.15</sup>	24	0.98 <sup>0.065</sup>	0.98
3	3	0	60	100	11 <sup>0.048</sup>	12	332 <sup>0.025</sup>	332	14 <sup>0.071</sup>	14	202 <sup>0.026</sup>	202	86 <sup>0.068</sup>	82	49 <sup>0.15</sup>	49	1.1 <sup>0.056</sup>	1.1
4	2	50	32.5	62.5	11 <sup>0.035</sup>	11	319 <sup>0.041</sup>	299	11 <sup>0.096</sup>	11	182 <sup>0.038</sup>	182	29 <sup>0.12</sup>	25	25 <sup>0.18</sup>	25	0.90 <sup>0.073</sup>	0.87
5	2	0	32.5	62.5	9.3 <sup>0.064</sup>	9.2	250 <sup>0.0094</sup>	338	13 <sup>0.020</sup>	13	214 <sup>0.025</sup>	214	11 <sup>0.11</sup>	13	63 <sup>0.010</sup>	57	1.0 <sup>0.032</sup>	1.1
6	3	0	5	100	7.2 <sup>0.090</sup>	7.0	322 <sup>0.079</sup>	335	13 <sup>0.073</sup>	14	177 <sup>0.040</sup>	180	23 <sup>0.054</sup>	27	29 <sup>0.15</sup>	35	1.4 <sup>0.013</sup>	1.3
7	3	0	5	25	7.2 <sup>0.055</sup>	7.4	361 <sup>0.057</sup>	358	13 <sup>0.036</sup>	13	202 <sup>0.057</sup>	202	n.d.	-1.0	74 <sup>0.0092</sup>	68	1.3 <sup>0.028</sup>	1.3
8	3	100	60	100	11 <sup>0.029</sup>	10	209 <sup>0.038</sup>	209	8.8 <sup>0.11</sup>	9.1	144 <sup>0.021</sup>	146	27 <sup>0.066</sup>	30	34 <sup>0.11</sup>	30	0.75 <sup>0.11</sup>	0.74
9	3	100	60	25	8.1 <sup>0.12</sup>	8.0	176 <sup>0.056</sup>	184	11 <sup>0.16</sup>	11	147 <sup>0.068</sup>	145	n.d.	0.67	20 <sup>0.28</sup>	21	0.91 <sup>0.075</sup>	0.91
10	2	50	5	62.5	11 <sup>0.019</sup>	11	294 <sup>0.032</sup>	311	11 <sup>0.021</sup>	11	189 <sup>0.038</sup>	182	14 <sup>0.14</sup>	17	23 <sup>0.055</sup>	23	0.86 <sup>0.054</sup>	0.87
11	3	100	5	25	5.0 <sup>0.056</sup>	5.4	211 <sup>0.044</sup>	208	14 <sup>0.023</sup>	14	145 <sup>0.12</sup>	151	n.d.	-1.8	20 <sup>0.21</sup>	21	0.80 <sup>0.051</sup>	0.83
12	1	0	5	100	6.5 <sup>0.15</sup>	7.2	286 <sup>0.046</sup>	268	12 <sup>0.11</sup>	12	156 <sup>0.021</sup>	158	n.d.	-2.3	34 <sup>0.19</sup>	32	1.0 <sup>0.071</sup>	1.0
13	1	0	5	25	7.6 <sup>0.058</sup>	7.3	286 <sup>0.073</sup>	295	14 <sup>0.047</sup>	13	163 <sup>0.069</sup>	162	n.d.	-1.8	58 <sup>0.13</sup>	64	1.0 <sup>0.054</sup>	1.0
14	1	100	60	100	5.7 <sup>0.11</sup>	6.1	213 <sup>0.11</sup>	205	7.8 <sup>0.087</sup>	7.4	132 <sup>0.14</sup>	131	n.d.	-0.62	18 <sup>0.20</sup>	22	0.45 <sup>0.070</sup>	0.47
15	1	50	32.5	62.5	9.1 <sup>0.0046</sup>	9.1	238 <sup>0.045</sup>	261	11 <sup>0.082</sup>	11	142 <sup>0.022</sup>	146	20 <sup>0.037</sup>	26	14 <sup>0.14</sup>	18	0.69 <sup>0.020</sup>	0.71
16	3	100	5	100	5.7 <sup>0.084</sup>	5.5	235 <sup>0.096</sup>	227	13 <sup>0.080</sup>	13	137 <sup>0.010</sup>	134	n.d.	0.7	22 <sup>0.022</sup>	22	0.81 <sup>0.090</sup>	0.80
17	1	100	5	25	4.3 <sup>0.087</sup>	4.4	223 <sup>0.18</sup>	213	16 <sup>0.029</sup>	16	137 <sup>0.042</sup>	137	21 <sup>0.087</sup>	24	30 <sup>0.21</sup>	29	0.78 <sup>0.12</sup>	0.76
18	2	50	60	62.5	12 <sup>0.077</sup>	13	300 <sup>0.0026</sup>	294	9.0 <sup>0.020</sup>	8.9	176 <sup>0.060</sup>	182	33 <sup>0.12</sup>	32	21 <sup>0.17</sup>	22	0.74 <sup>0.011</sup>	0.74
19	2	50	32.5	100	11 <sup>0.019</sup>	11	280 <sup>0.059</sup>	288	10 <sup>0.046</sup>	10	162 <sup>0.036</sup>	166	32 <sup>0.072</sup>	31	18 <sup>0.23</sup>	19	0.89 <sup>0.017</sup>	0.91
20	1	100	60	25	3.7 <sup>0.11</sup>	3.3	188 <sup>0.035</sup>	183	12 <sup>0.028</sup>	12	115 <sup>0.071</sup>	113	n.d.	-2.0	17 <sup>0.099</sup>	12	0.73 <sup>0.11</sup>	0.72
21	3	0	60	25	10 <sup>0.071</sup>	10	344 <sup>0.011</sup>	349	14 <sup>0.027</sup>	14	206 <sup>0.051</sup>	206	23 <sup>0.067</sup>	26	70 <sup>0.015</sup>	75	1.2 <sup>0.052</sup>	1.2
22	2	50	32.5	62.5	11 <sup>0.012</sup>	11	313 <sup>0.020</sup>	299	10 <sup>0.095</sup>	11	179 <sup>0.016</sup>	182	27 <sup>0.14</sup>	25	27 <sup>0.23</sup>	25	0.89 <sup>0.080</sup>	0.87
23	1	100	5	100	4.9 <sup>0.045</sup>	4.8	226 <sup>0.088</sup>	229	13 <sup>0.029</sup>	13	137 <sup>0.019</sup>	137	n.d.	-2.3	34 <sup>0.044</sup>	31	0.66 <sup>0.091</sup>	0.66
24	2	50	32.5	25	9.8 <sup>0.039</sup>	9.6	287 <sup>0.012</sup>	289	12 <sup>0.058</sup>	12	173 <sup>0.062</sup>	168	14 <sup>0.084</sup>	16	30 <sup>0.12</sup>	31	1.0 <sup>0.077</sup>	1.0
25	2	100	32.5	62.5	6.8 <sup>0.088</sup>	6.9	212 <sup>0.083</sup>	236	11 <sup>0.088</sup>	12	173 <sup>0.054</sup>	173	n.d.	0.1	23 <sup>0.069</sup>	30	0.72 <sup>0.039</sup>	0.73
26	1	0	60	25	5.6 <sup>0.044</sup>	6.4	286 <sup>0.12</sup>	279	13 <sup>0.098</sup>	13	147 <sup>0.085</sup>	150	n.d.	-2.3	56 <sup>0.0030</sup>	54	0.72 <sup>0.065</sup>	0.73
$R^2$					0.977		0.953		0.984		0.982		0.979		0.954		0.993	
Standard error of the estimate					4.9		4.8		2.3		2.4		5.0		7.6		1.9	
Durbin-Watson statistic					1.5 ( $p = 0.101$ )		2.3 ( $p = 0.215$ )		1.9 ( $p = 0.375$ )		1.8 ( $p = 0.270$ )		1.9 ( $p = 0.422$ )		2.3 ( $p = 0.192$ )		1.8 ( $p = 0.343$ )	

$n_{\text{ext}}$ , number of extractions; % EtOH, ethanol:water ratio in the extractant;  $t_{\text{ext}}$ , extraction time (min);  $T_{\text{ext}}$ , extraction temperature (°C)

<sup>a</sup>, experimental values are the mean <sup>CV</sup> (coefficient of variation) of  $n = 3$  determinations

Table 3

Design matrix for the optimization of the extraction of pineapple organic acids, experimental values<sup>a</sup> (EV, mg/100 g FW) obtained for each identified organic acid and predicted values (PV) for the central composite design ( $2^4 + \text{star}$ )

Experiment	$n_{\text{ext}}$	% EtOH	$t_{\text{ext}}$	$T_{\text{ext}}$	Oxalic acid		Citric acid		L-malic acid		Quinic acid		Succinic acid	
					EV	PV	EV	PV	EV	PV	EV	PV	EV	PV
1	1	0	60	100	8.5 <sup>0.11</sup>	8.5	871 <sup>0.030</sup>	873	322 <sup>0.076</sup>	327	4.6 <sup>0.19</sup>	4.6	3.3 <sup>0.14</sup>	3.2
2	3	50	32.5	62.5	2.9 <sup>0.18</sup>	3.2	939 <sup>0.034</sup>	944	353 <sup>0.039</sup>	359	3.8 <sup>0.096</sup>	3.7	3.1 <sup>0.13</sup>	3.0
3	3	0	60	100	10 <sup>0.066</sup>	9.7	821 <sup>0.019</sup>	811	355 <sup>0.018</sup>	350	4.2 <sup>0.13</sup>	4.0	3.9 <sup>0.041</sup>	3.9
4	2	50	32.5	62.5	2.8 <sup>0.23</sup>	3.3	927 <sup>0.035</sup>	925	341 <sup>0.012</sup>	336	3.1 <sup>0.24</sup>	3.3	2.2 <sup>0.28</sup>	2.7
5	2	0	32.5	62.5	7.9 <sup>0.11</sup>	7.6	974 <sup>0.024</sup>	987	355 <sup>0.047</sup>	357	2.5 <sup>0.28</sup>	2.6	3.4 <sup>0.17</sup>	3.5
6	3	0	5	100	9.0 <sup>0.12</sup>	8.9	920 <sup>0.074</sup>	904	339 <sup>0.035</sup>	336	3.0 <sup>0.24</sup>	3.1	2.5 <sup>0.15</sup>	2.4
7	3	0	5	25	4.1 <sup>0.12</sup>	4.4	927 <sup>0.048</sup>	936	314 <sup>0.026</sup>	317	2.6 <sup>0.32</sup>	2.7	4.2 <sup>0.040</sup>	4.2
8	3	100	60	100	2.3 <sup>0.11</sup>	2.5	701 <sup>0.011</sup>	719	324 <sup>0.016</sup>	327	6.6 <sup>0.12</sup>	6.5	3.6 <sup>0.068</sup>	3.5
9	3	100	60	25	3.3 <sup>0.038</sup>	3.2	933 <sup>0.019</sup>	933	353 <sup>0.033</sup>	355	4.1 <sup>0.16</sup>	4.4	3.0 <sup>0.079</sup>	3.1
10	2	50	5	62.5	2.8 <sup>0.12</sup>	2.8	823 <sup>0.021</sup>	868	297 <sup>0.051</sup>	309	3.9 <sup>0.24</sup>	3.9	2.2 <sup>0.025</sup>	2.3
11	3	100	5	25	3.5 <sup>0.22</sup>	3.3	951 <sup>0.029</sup>	943	337 <sup>0.055</sup>	330	4.5 <sup>0.13</sup>	4.2	2.9 <sup>0.013</sup>	2.8
12	1	0	5	100	6.6 <sup>0.018</sup>	6.9	841 <sup>0.052</sup>	844	324 <sup>0.053</sup>	322	3.4 <sup>0.15</sup>	3.3	2.5 <sup>0.11</sup>	2.5
13	1	0	5	25	2.6 <sup>0.15</sup>	2.2	830 <sup>0.031</sup>	806	322 <sup>0.052</sup>	318	3.5 <sup>0.16</sup>	3.4	4.9 <sup>0.097</sup>	4.8
14	1	100	60	100	2.7 <sup>0.24</sup>	2.6	694 <sup>0.052</sup>	689	247 <sup>0.038</sup>	246	5.7 <sup>0.13</sup>	5.8	2.3 <sup>0.054</sup>	2.4
15	1	50	32.5	62.5	2.5 <sup>0.28</sup>	2.1	860 <sup>0.0026</sup>	864	324 <sup>0.0028</sup>	319	3.5 <sup>0.13</sup>	3.6	2.7 <sup>0.11</sup>	2.8
16	3	100	5	100	3.8 <sup>0.11</sup>	3.5	894 <sup>0.053</sup>	886	332 <sup>0.010</sup>	331	5.5 <sup>0.078</sup>	5.6	3.2 <sup>0.027</sup>	3.3
17	1	100	5	25	1.8 <sup>0.12</sup>	2.4	709 <sup>0.027</sup>	722	268 <sup>0.014</sup>	274	3.1 <sup>0.12</sup>	3.5	2.9 <sup>0.13</sup>	3.1
18	2	50	60	62.5	3.6 <sup>0.096</sup>	3.6	914 <sup>0.038</sup>	878	335 <sup>0.027</sup>	325	4.7 <sup>0.20</sup>	4.7	2.8 <sup>0.072</sup>	2.8
19	2	50	32.5	100	3.3 <sup>0.025</sup>	4.0	830 <sup>0.029</sup>	861	316 <sup>0.060</sup>	324	3.1 <sup>0.23</sup>	3.4	2.6 <sup>0.068</sup>	2.7
20	1	100	60	25	3.3 <sup>0.018</sup>	3.2	826 <sup>0.045</sup>	835	288 <sup>0.051</sup>	290	4.6 <sup>0.16</sup>	4.2	2.5 <sup>0.034</sup>	2.5
21	3	0	60	25	6.1 <sup>0.052</sup>	6.2	990 <sup>0.034</sup>	999	358 <sup>0.029</sup>	359	3.0 <sup>0.22</sup>	2.9	5.8 <sup>0.030</sup>	5.8
22	2	50	32.5	62.5	3.6 <sup>0.067</sup>	3.3	952 <sup>0.051</sup>	925	335 <sup>0.025</sup>	336	3.5 <sup>0.17</sup>	3.3	3.1 <sup>0.16</sup>	2.7
23	1	100	5	100	3.2 <sup>0.11</sup>	2.8	749 <sup>0.086</sup>	734	261 <sup>0.0018</sup>	258	4.7 <sup>0.15</sup>	4.5	3.2 <sup>0.15</sup>	3.1
24	2	50	32.5	25	2.8 <sup>0.067</sup>	2.0	970 <sup>0.031</sup>	949	342 <sup>0.032</sup>	336	2.6 <sup>0.33</sup>	2.4	3.9 <sup>0.053</sup>	3.7
25	2	100	32.5	62.5	3.8 <sup>0.26</sup>	4.1	902 <sup>0.0018</sup>	866	324 <sup>0.041</sup>	323	3.9 <sup>0.22</sup>	3.9	2.6 <sup>0.19</sup>	2.4
26	1	0	60	25	4.4 <sup>0.094</sup>	4.8	980 <sup>0.042</sup>	992	350 <sup>0.0052</sup>	352	3.9 <sup>0.21</sup>	4.1	5.5 <sup>0.047</sup>	5.5
$R^2$					0.976		0.959		0.966		0.960		0.969	
Standard error of the estimate					5.3		2.7		2.3		4.6		4.4	
Durbin-Watson statistic					2.3 ( $p = 0.189$ )		2.3 ( $p = 0.180$ )		2.3 ( $p = 0.202$ )		1.8 ( $p = 0.280$ )		1.5 ( $p = 0.067$ )	

$n_{\text{ext}}$ , number of extractions; % EtOH, ethanol:water ratio in the extractant;  $t_{\text{ext}}$ , extraction time (min);  $T_{\text{ext}}$ , extraction temperature (°C)

<sup>a</sup>, experimental values are the mean<sup>CV</sup> (coefficient of variation) of  $n = 3$  determinations

Table 4

Estimation of the effects and interactions in the central composite design

Extraction factor		Papaya acids							Pineapple acids				
		Oxalic	Citric	Tartaric	L-malic	Quinic	Succinic	Fumaric	Oxalic	Citric	L-malic	Quinic	Succinic
$n_{\text{ext}}$	F ratio	62	17	17	121	62	5.0	472	18	40	104	0.01	4.2
	$p$ value <sup>a</sup>	<b>0.000</b>	<b>0.002</b>	<b>0.002</b>	<b>0.000</b>	<b>0.000</b>	<b>0.046</b>	<b>0.000</b>	<b>0.002</b>	<b>0.000</b>	<b>0.000</b>	0.920	0.065
% EtOH	F ratio	64	152	44	287	41	106	743	196	51	75	86	84
	$p$ value <sup>a</sup>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>
$t_{\text{ext}}$	F ratio	48	4.2	141	0.09	52	0.14	119	9.0	0.38	16	29	14
	$p$ value <sup>a</sup>	<b>0.000</b>	0.066	<b>0.000</b>	0.773	<b>0.000</b>	0.715	<b>0.000</b>	<b>0.012</b>	0.551	<b>0.002</b>	<b>0.000</b>	<b>0.003</b>
$T_{\text{ext}}$	F ratio	17	0.00	117	1.2	53	20	69	62	50	11	49	65
	$p$ value <sup>a</sup>	<b>0.002</b>	1.000	<b>0.000</b>	0.302	<b>0.000</b>	<b>0.001</b>	<b>0.000</b>	<b>0.000</b>	<b>0.000</b>	<b>0.007</b>	<b>0.000</b>	<b>0.000</b>
$n_{\text{ext}} \cdot \% \text{ EtOH}$	F ratio	2.7	16	16	24	37	4.5	62	6.4	11	49	19	2.2
	$p$ value <sup>a</sup>	0.129	<b>0.002</b>	<b>0.002</b>	<b>0.001</b>	<b>0.000</b>	0.058	<b>0.000</b>	<b>0.029</b>	<b>0.006</b>	<b>0.000</b>	<b>0.001</b>	0.164
$n_{\text{ext}} \cdot t_{\text{ext}}$	F ratio	35	0.13	15	12	42	8.9	22	2.5	20	0.76	2.2	11
	$p$ value <sup>a</sup>	<b>0.000</b>	0.725	<b>0.003</b>	<b>0.006</b>	<b>0.000</b>	<b>0.013</b>	<b>0.001</b>	0.145	<b>0.001</b>	0.401	0.170	<b>0.007</b>
$n_{\text{ext}} \cdot T_{\text{ext}}$	F ratio	0.26	0.02	31	10	43	0.07	11	0.11	6.8	4.8	2.5	3.4
	$p$ value <sup>a</sup>	0.618	0.880	<b>0.000</b>	<b>0.008</b>	<b>0.000</b>	0.796	<b>0.007</b>	0.746	<b>0.024</b>	0.051	0.143	0.094
% EtOH $\cdot t_{\text{ext}}$	F ratio	0.01	0.77	117	3.9	34	1.4	39	12	7.8	4.8	0.00	28
	$p$ value <sup>a</sup>	0.920	0.399	<b>0.000</b>	0.073	<b>0.000</b>	0.259	<b>0.000</b>	<b>0.005</b>	<b>0.017</b>	0.051	0.958	<b>0.000</b>
% EtOH $\cdot T_{\text{ext}}$	F ratio	0.67	5.9	18	0.88	36	38	9.4	63	0.87	4.8	11	82
	$p$ value <sup>a</sup>	0.429	<b>0.034</b>	<b>0.001</b>	0.368	<b>0.000</b>	<b>0.000</b>	<b>0.011</b>	<b>0.000</b>	0.371	0.051	<b>0.009</b>	<b>0.000</b>
$t_{\text{ext}} \cdot T_{\text{ext}}$	F ratio	15	0.13	15	9.2	41	1.8	36	3.4	33	12	5.0	0.12
	$p$ value <sup>a</sup>	<b>0.003</b>	0.725	<b>0.003</b>	<b>0.012</b>	<b>0.000</b>	0.213	<b>0.000</b>	0.091	<b>0.000</b>	<b>0.005</b>	<b>0.047</b>	0.737
$n_{\text{ext}}^2$	F ratio	5.6	4.0	9.5	48	10	1.2	69	3.8	1.2	0.28	3.8	1.4
	$p$ value <sup>a</sup>	<b>0.037</b>	0.071	<b>0.010</b>	<b>0.000</b>	<b>0.009</b>	0.303	0.203	0.078	0.298	0.605	0.078	0.266
% EtOH <sup>2</sup>	F ratio	63	1.4	53	14	51	28	1.5	56	1.0	0.78	0.10	3.0
	$p$ value <sup>a</sup>	<b>0.000</b>	0.266	<b>0.000</b>	<b>0.003</b>	<b>0.000</b>	<b>0.000</b>	0.253	<b>0.000</b>	0.340	0.396	0.756	0.113
$t_{\text{ext}}^2$	F ratio	4.9	0.10	13	0.01	0.05	0.43	15	0.33	10	13	28	1.1
	$p$ value <sup>a</sup>	<b>0.048</b>	0.758	<b>0.004</b>	0.928	0.833	0.525	<b>0.002</b>	0.577	<b>0.009</b>	<b>0.004</b>	<b>0.000</b>	0.325
$T_{\text{ext}}^2$	F ratio	5.6	1.0	1.8	21	0.49	0.00	35	0.77	1.2	1.5	4.3	10
	$p$ value <sup>a</sup>	<b>0.037</b>	0.336	0.208	<b>0.001</b>	0.500	0.984	<b>0.000</b>	0.398	0.298	0.250	0.064	<b>0.009</b>

$n_{\text{ext}}$ , number of extractions; % EtOH, ethanol:water ratio in the extractant;  $t_{\text{ext}}$ , extraction time (min);  $T_{\text{ext}}$ , extraction temperature (°C)

<sup>a</sup>, bold numbers denote significant effects or interactions of the extraction method at a 95% confidence level