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Analytical Methods

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

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ABSTRACT

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

1. Introduction

Organic acids are widely distributed in fruits and originate from biochemical processes or from the activity of some microorganisms such as yeasts and bacteria. These carboxylic acids determine pH and total acidity of fruits, inhibit the action of enzymes and are chelating agents of metals and therefore impede chemical precipitation and oxidation. Moreover, the non-volatile organic acids influence fruit sensorial properties (flavour, colour and aroma). Therefore, the determination of organic acids allows different fruit cultivars (Usenik, Fabcic, & Stampar, 2008) and the effect of pre-harvest factors (Keutgen & Pawelzik, 2007) on the organoleptic quality of the fruit to be evaluated. It also allows the development (Saradhuldhat & Paull, 2007) or ripening (Sturm, Koron, & Stampar, 2003) of the fruit to be monitored, as well as provides a way to check and control the fermentation processes (Avenoza, Busto, Canal, & Peregrina, 2006). Moreover, their analysis is a powerful tool to characterise the authenticity of fruit products (Saavedra, Rupérez, & Barbas, 2001) or to control post-harvest (Beirao-Da-Costa et al., 2008) or technological processes (Silva, Andrade, Mendes, Seabra, & Ferreira, 2002) and storage (Marsh et al., 2004) based on their relative stability.

A variety of analytical methods for determining organic acids in fruits and fruit juices have been reported to date. The individual determination of organic acids in these food matrices is usually carried out using a spectrophotometric detector (Luque-Pérez, Ríos, & Valcárcel, 1998) but the resolution of complex mixtures requires the use of chemometric techniques or near-infrared spectroscopy (Chen, Zhang, & Matsunaga, 2006). The non-specificity of classical electrochemical methods applied to determine organic acids has been surpassed by their combination with enzymatic biosensors (Kim, 2006). However, because the organic acids are present in mixtures, the preferred choice for organic acids determination is the use of separation techniques: capillary electrophoresis (Mato, Suárez-Luque, & Huidobro, 2007; Saavedra et al., 2001), gas chromatography (GC) and liquid chromatography (LC). In spite of its high sensitivity and selectivity, GC has been less used for the determination of organic acids than LC, because these compounds need to be derivatised (Chan, Chenchin, & Vonnahme, 1973; González-Aguilar, Buta, & Wang, 2003). Most of the liquid chromatographic methods to determine organic acids have been carried out by ion-exclusion (Bartolomé, Rupérez, & Fúster, 1995; Bartolomé, Rupérez, & Fúster, 1996; Cano, Torija, Marín, & Cámara, 1994; Saradhuldhat and Paull, 2007).

To ensure that analysis by LC is effective, it is very important to optimise the sample extraction when analysing organic acids in complex samples such as fruits, because of their diverse matrices. Moreover, fruits contain large amounts of potentially interfering compounds. For these reasons considerable caution should be exercised in the employment of methods that have been developed for the analysis of specific plant tissue types. To provide clean

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extracts, fruits require a pre-treatment that includes organic solvent extraction and cleanup processes by solid-phase extraction. A critical step in the analytical determination of organic acids in tropical fruits such as papaya and pineapple, which has not been studied in detail, is the solvent extraction of these compounds as a function of these vegetable matrices. Several solvents have been used to extract organic acids from fruits: water (Sturm et al., 2003; Usenik et al., 2008), methanol (Bartolomé et al., 1995, 1996; Cano et al., 1994; Silva et al., 2002) and mixtures ethanol:water (Holcroft & Kader, 1999; Pérez, Olías, Espada, Olías, & Sanz, 1997; Saradhuldhat and Paull, 2007). Other variables that influence the organic acids extraction are time and temperature. So, it has been used extraction times between 1 and 30 min (Bartolomé et al., 1995; Cano et al., 1994; Holcroft & Kader, 1999; Pérez et al., 1997; Usenik et al., 2008) and extraction temperatures between room temperature and 65 °C (Bartolomé et al., 1995; Cano et al., 1994; Silva et al., 2002; Usenik et al., 2008).

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

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

2. Materials and methods

2.1. Plant material

Papaya (*Carica papaya* L., cv. "Baixinho do Santa Amalia") was harvested from fields located in Tejina in northwest Tenerife (Canary Islands, Spain) and pineapple (*Ananas comosus* L., cv. "Red Spanish") from Frontera in El Hierro (Canary Islands). Papaya was harvested at physiological maturity stage (mature-green) and allowed to ripen (full-ripeness or consumption stage) at 18 °C and 80–90% relative humidity; however, pineapple (which is a non-climacteric fruit) was collected at full-ripeness.

The assay was performed using nine homogeneous units of each fruit in a similar ripening stage, characterised by peel and pulp colour, firmness, total soluble solids (TSS), pH and titratable acidity. Lightness, hue angle and chromaticity of papaya peel, at full-ripeness, were 63 ± 2, 80 ± 1 and 60 ± 2, respectively. These colour attributes in pineapple peel (51 ± 2, 72 ± 3 and 34 ± 1, respectively) were lower than in papaya. The colours of papaya and pineapple pulp were characterised by a lightness of 60 ± 2 and 75 ± 5, a hue angle of 70 ± 2 and 109 ± 2 and a chromaticity of 46 ± 2 and 16 ± 2, respectively. Pulp firmness, measured as penetration force, was 4.3 ± 0.2 N and 15 ± 2 N for papaya and pineapple, respectively. TSS were similar for both fruits: 13 ± 1 °Brix for papaya and 15 ± 1 °Brix for pineapple. However, acid content was higher for pineapple (pH 3.5 ± 0.1; and titratable acidity 1175 ± 83 mg citric acid/100 g) than for papaya (pH 5.6 ± 0.1; and titratable acidity 90 ± 3 mg citric acid/100 g).

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

2.2. Solvent extraction method

Four grams of accurately weighed of frozen pulverised fruit samples were mixed with 8 ml of extractant [concentration of ethanol (Panreac, Madrid, Spain) and water varied depending on the particular experiment; ranging between 0 and 100%]. The mixture was homogenised with a Politron PT 6000 (Kinematica AG, Lucerne, Switzerland) high speed blender at 12,000 g for 1 min. Then organic acids present in the fruits were extracted (extraction time varied depending on the particular experiment; ranging between 5 and 60 min) in a water bath (extraction temperature varied depending on the particular experiment; ranging between 25 and 100 °C). Extracts were centrifuged at 5000 g for 30 min in a Jouan CR 312 centrifuge (Thermo Electron Corporation, Madrid, Spain). Depending on the experiment, this procedure was repeated (ranging between 1 and 3 times); the resulting supernatants were mixed together and a final volume of 25-ml was achieved. An aliquot of 4 ml of the extract was evaporated to dryness at 45 °C (approximately 20 h) in a Heto VR 1 evaporator (Allerod, Denmark). The residue was re-dissolved in 4 ml of water milli-Q and passed through a 300 mg Alltech (Laarne, Belgium) Sep-pack RP-C18 column to eliminate possible interferences. The eluate was collected, an aliquot of 1 ml was filtered through a 0.45 μm nylon membrane and subsequently used to analyse organic acids. After each determination, the adsorbent was cleaned with 6 ml of water to remove interfering compounds adsorbed; finally, the adsorbent was conditioned with 2 ml of ethanol.

2.3. Chromatographic determination of organic acids

The liquid chromatographic method used for the determination of organic acids (oxalic, citric, tartaric, pyruvic, L-malic, quinic, succinic and fumaric acids) consisted of an isocratic elution procedure with UV–Vis detection. The analyses were carried out on a Shimadzu modular chromatographic system (Kyoto, Japan) equipped with a LC-10AD pump, a SPD-10AV UV–Vis detector and controlled via Class LC-10 data acquisition software (also from Shimadzu). The injection valve was a Rheodyne 7725i (Cotati, USA) with an injection loop of 20 μl. Organic acid separation was carried out on a Shodex (Showa Kenko, Tokyo, Japan) RSpack KC-811 column (5 μm particle size, 250 × 4.6 mm i.d.), using an isocratic 0.1% orthophosphoric acid (Panreac) mobile phase at a flow-rate of 0.8 ml/min. Detection wavelength for the UV–Vis detector was set at 210 nm.

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

2.4. Experimental design

Statgraphics Plus version 4.1 (Statistical Graphics, Rockville, USA) was employed to generate design, regression analysis and to obtain the response surface plots. A central composite design