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Use of ion chromatography for monitoring microbial spoilage in the fruit juice industry

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Abstract

Fruit juices and purees are defined as fermentable, but unfermented, products obtained by mechanical processing of fresh fruits. The presence of undesired metabolites derived from microbial growth can arise from the use of unsuitable fruit or from defects in the production line or subsequent contamination. This involves a loss in the overall quality that cannot be resolved by thermal treatment following the start of fermentation. With these considerations, together with microbiological control, the analysis of different metabolites, which can be considered as microbial growth markers, such as alcohols (i.e. ethanol, etc.), acids (i.e. acetic, fumaric, lactic, etc.) is fundamental in order to achieve a better evaluation of product quality. Enzymatic determination and other single-component analytical techniques are often used for the determination of these metabolites. When the microbial spoilage is not well known, this results in a long and cumbersome procedure. A versatile technique that is capable of determining many metabolites in one analysis could be helpful in improving routine quality control. For this purpose, an ion chromatographic technique, such as ion exclusion, for separation, and diode array spectrophotometry and conductivity, for detection, were evaluated. Both different industrial samples and inoculated samples were analyzed.

Keywords: Fruit juice; Food analysis; Organic acids

1. Introduction

Microbiological changes in acidic foods (pH< 4.5), such as fruit juices and purees, generally do not involve toxicological hazard (with the exception of mycotoxin formation in the presence of some types of mould), but they can cause a degradation in quality, with notable commercial damages.

Among the different alteration pathways, degradation processes of carbohydrates (with the production of metabolites such as lactic acid and the precursors, acetic acid, ethanol and carbon dioxide [1–4]), lactic acid bacteria, yeasts and moulds were considered. The content of these compounds is normally used as a product quality marker. In fact, the presence of noticeable amounts of any of these metabolites is considered to be an index of inadequate raw materials or partial alteration during the production steps, despite any treatments for microbial stabilization. The AIJN (Association of the Industry of Juices and Nectars from Fruits and Vegetables of the European Economic Community) proposed the following limiting concentrations for metabolite content in fruit juices: 0.5 g/kg of lactic acid (as D- and/or L-isomers), 0.4 g/kg of acetic acid (as volatile acidity) and 3 g/kg of ethanol [5].

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Recent studies focused attention on the formation of fumaric acid in fruit juices spoiled by moulds (noticeably by *Rhizopus stolonifer*) [6]. The presence of fumaric acid can also arise from the addition of synthetic malic acid [7], and it could be considered as an index of adulteration, when confirmed by the analysis of the D-malic acid that is not present in malic acid from natural sources [7]. The actual AIJN guideline is to consider 5 mg/kg as the limiting concentration for fumaric acid content in apple juices [5], whilst the natural content of fumaric acid in different fruit juices and purees was not reported in the literature.

Metabolite determinations were normally carried out using enzymatic kits (ethanol, acetic acid, D- and L-lactic acid) or by colorimetric analyses (diacetyl and acetoin) [8,9]. HPLC determination of organic acids in fruit juices is very common [10–14], using different separation techniques (reversed-phase, ion exclusion, ion chromatography) and detectors (refractive index, UV absorption, conductimetry), but specific applications of these chromatographic techniques in microbial spoilage control were not found. The above-mentioned traditional enzymatic analysis requires single determinations for each metabolite, resulting in time-consuming procedures and high costs of analysis.

One purpose of this study is to improve the speed of routine quality control using versatile instrumentation that can be employed for both ion-exclusion chromatography and other determinations, such as sugars, amino acids, polyphenols, anions, cations, vitamins, etc. [17].

The new ion-exclusion chromatography materials [15] have led to the production of columns with different selectivities and higher efficiency in comparison with the traditional ones, which results in a reduction in interferences in very complex matrices, such as fruit and vegetable juices.

This paper evaluates the application of ion-exclusion chromatography (IEC) coupled with both conductivity and UV detection for the simultaneous determination of the acidic metabolites, acetic, lactic and fumaric acid.

Pear and peach nectars, before and after inoculation with lactic acid bacteria, yeasts and moulds, were analyzed, in order to determine the effect of microbial growth on metabolite content.

Several clarified apple juices that were produced industrially were analyzed in order to monitor the current commercial products and to verify the validity of the proposed limits and, in particular, the fumaric acid content.

2. Experimental

2.1. Instrumentation

The chromatographic system consisted of a Model 305 pump, a Model 805 pump pulsation electronic damper, a Model 231 automated sample injector, equipped with a 25-μl loop (Gilson Medical Electronics, Villiers-le-Bel, France), a Model 431 conductivity detector and a Model 990 diode-array spectrophotometric detector (Waters, Milford, MA, USA).

Acquisition and integration of chromatograms were performed with a 486-compatible personal computer linked to a Gilson GSIOC 506 C system interface.

An IonPac ICE AS06 ion-exclusion column, based on poly(styrene–acrylate–divinylbenzene) copolymer functionalized with both sulphonic and carboxylic groups, was used, together with an anionic micromembrane suppressor AMMS ICE (Dionex, Sunnyvale, CA, USA).

The analytical conditions are summarized in Table 1.

2.2. Reagents and standard solutions

Heptafluorobutyric acid (PFBA) and tetrabutylammonium hydroxide (TBAOH) were of ion chroma-

Table 1
Ion chromatographic conditions

Column	IonPac ICE-AS6
Eluent	PFBA 0.5 m*M*
Eluent flow-rate	1 ml/min
Suppressor	AMMS-ICE
Regenerant	TBAOH 5 m*M*
Regenerant flow-rate	5 ml/min
Detection	Suppressed conductivity, spectrophotometry (207 nm)

tography grade (Novachimica, Milan, Italy), sodium fumarate, L(+)-monolithium lactate and acetic acid were of RPE grade (Carlo Erba, Rodano, Italy). An analytical water purification system (SMEG, Parma, Italy) was used to produce deionized water (DI water) of ultrapure quality (<0.1 μS).

Working standard solutions were prepared daily by diluting 1000 mg/l standard solutions of each organic anion (fumarate 1–10 mg/l; lactate and acetate 5–50 mg/l).

2.3. Sample inoculum

Samples of pear and peach nectars (30 Tetra-Brik packages, each having the same production code), were inoculated with different microbial strains and incubated until evident growth before being analysed. Lactic acid bacteria, yeasts and moulds, as in Table 2, were singularly employed for the inoculum.

2.4. Lactic acid bacteria

A 0.1-ml volume of 24 h culture (MRS broth, Oxoid) was injected directly into the Tetra-Brik package using a 1-ml sterile syringe, in order to obtain a microbial concentration of about $1 \cdot 10^4$ CFU/ml of sample. After the inoculum, the cartons were sealed with silicon and then incubated at 30°C. Cartons inoculated with strains L40 and LN4 swelled in seven days. Microbial growth of the other strains was confirmed microscopically.

2.5. Yeasts

The strains were cultured in MEB (Malt Extract Broth, Oxoid) for two–three days at 28°C, with the exclusion of Y92 and Y26 osmofile strains, which were cultured in MYGP broth (malt extract 3 g, yeast extract 3 g, peptone 5 g, glucose 500 g, water to 1 l) for three–five days at 28°C. Sterile glass jars (125 ml) were aseptically filled with 100 ml of nectar and 0.1 ml volumes of single culture were added. The inoculated samples ($1 \cdot 10^3$–$1 \cdot 10^4$ CFU/ml of sample) were incubated for two–three days at 28°C. The microbial growth was confirmed by gas production and microscopic control.

2.6. Moulds

Each strain was cultured for four–six days at 25°C in MEA (Malt Extract Agar, Oxoid) at pH 4.6 (50%, m/v, citric acid solution as acidifier) to produce the spores for inoculum. Suspensions were prepared from these cultures ($1 \cdot 10^6$ spore/ml) in sterile distilled water (with 0.1% Tween 80 from Sigma). The 125 ml sterile glass jars (three jars for every test) were aseptically filled with 50 ml of nectar and 0.1 ml of single spore suspension were added. The inoculated samples were incubated at 28°C until visible micelial growth (seven–ten days for the RN and AN strains, 15–20 days for the PCh and TR strains). Altered samples were aseptically filtered on sterile glass wool.

Table 2
Microbial strains used for the inoculation of samples

Lactic acid bacteria		Yeasts		Moulds	
Code	Strain	Code	Strain	Code	Strain
LC1	*Lactococcus lactis*	Y78	*Metschnikowia pulcherrima*	RN	*Rhizopus stolonifer* (ex *R. nigricans*)
LN4	*Leuconostoc* sp.	Y72	*Candida parapsilosis*	AN	*Aspergillus niger*
P1	*Pediococcus acidilactici*	Y69	*Saccharomyces cerevisiae*	PCh	*Penicillium chrysogenum*
L40	*Lactobacillus fermentum*	Y66	*Saccharomyces cerevisiae*	TR	*Trichoderma harzianum*
L12	*Lactobacillus casei* subsp. *casei*	Y92	*Zygosaccharomyces bailii*		
		Y26	*Zygosaccharomyces rouxii*		
		Y36	*Kluyveromyces marxianus*		
		Y3	*Brettanomyces claussennii*		
		Y90	*Torulaspora delbrueckii*		

All strains were from the Stazione Sperimentale per l'Industria delle Conserve Alimentari (SSICA) collection.

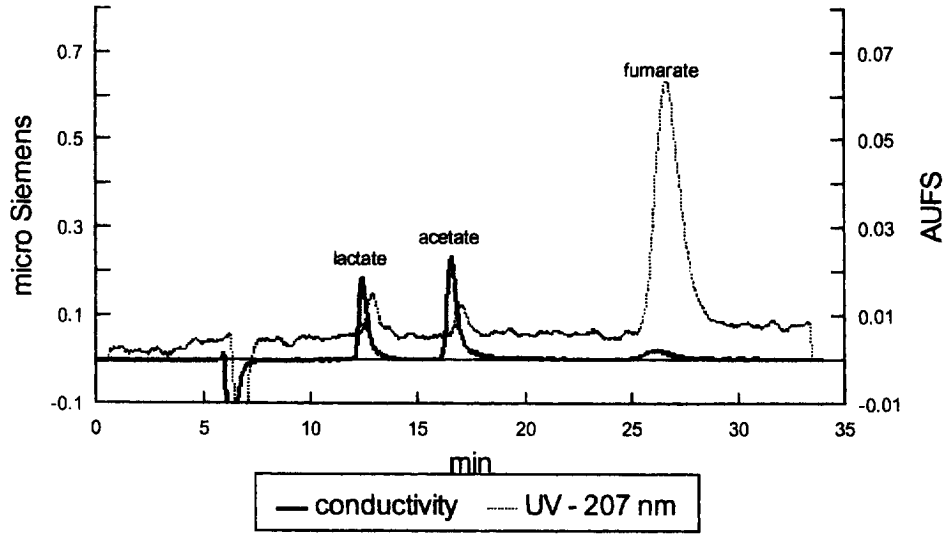

Fig. 1. Chromatograms of a standard solution of organic acids for both detectors. Lactic acid, 20 mg/l; acetic acid, 20 mg/l and fumaric acid, 4 mg/l. Chromatographic conditions as in Table 1.

2.7. Sample preparation

Analyses were carried out, before and after inoculation, on peach nectar, pear nectar and on 53 samples of clarified concentrated apple. The samples were simply diluted with DI water, to fit within the linear range of the analytes, filtered through paper and passed through a 0.45-μm filter before injection.

In the case of clarified apple concentrates, the results refer to juice diluted to 11.2°Brix.

2.8. Enzymatic analysis

Specific kits from Boehringer Mannheim (Germany) and a UV–visible spectrophotometer (Model

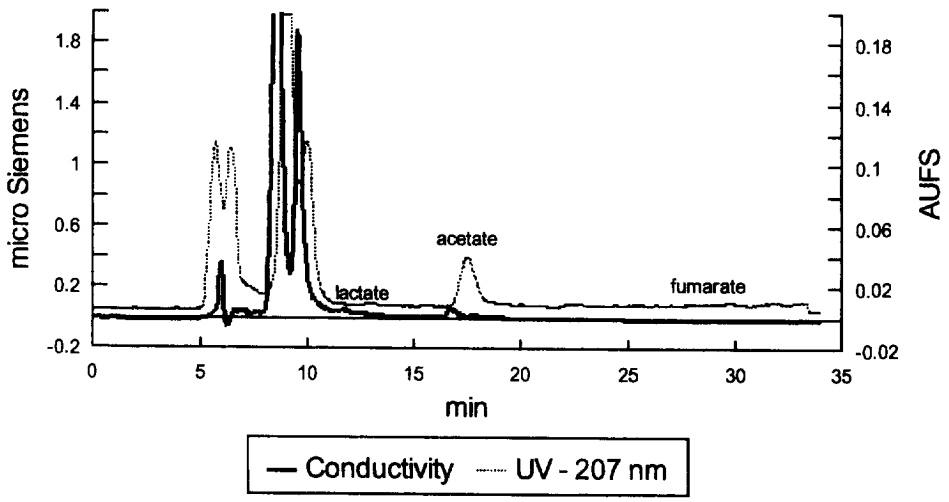

Fig. 2. Chromatograms of a pear nectar for both detectors (chromatographic conditions as in Table 1).

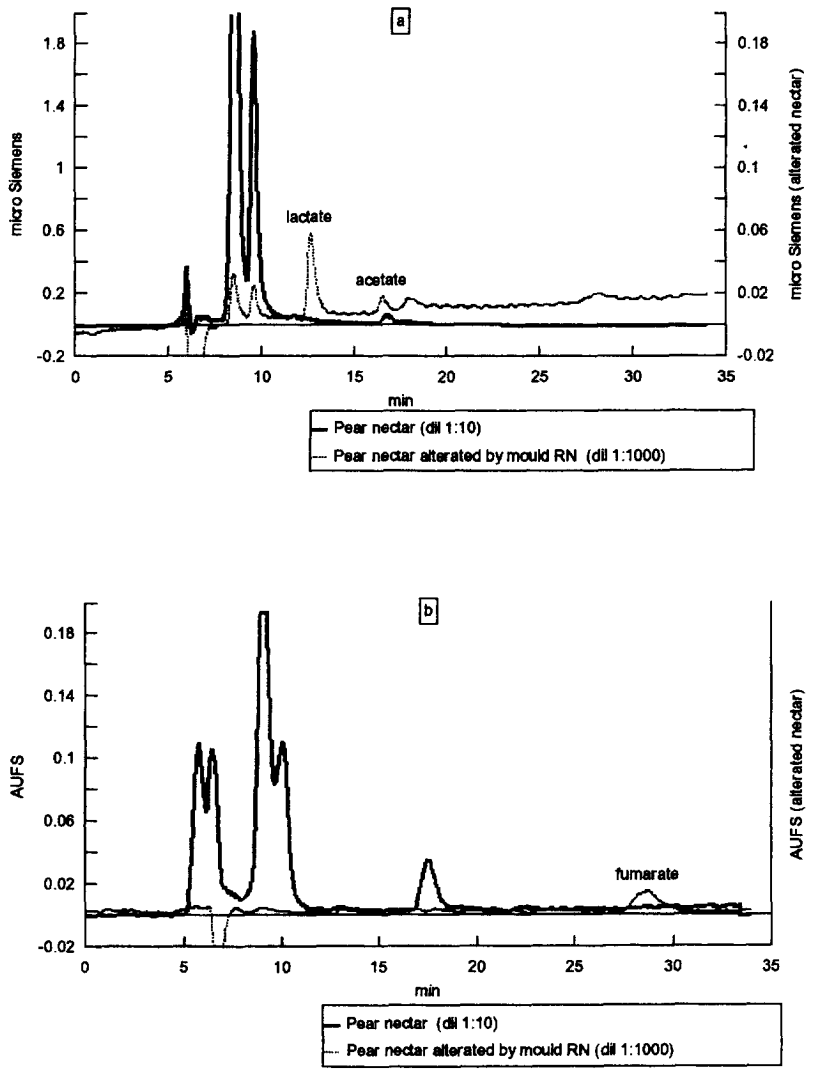

Fig. 3. Chromatograms of pear nectar before and after alteration by the mould *R. stolonifer* (chromatographic conditions as in Table 1). (a) Conductimetric detection and (b) spectrophotometric detection.

UV-160 Shimadzu, Milan, Italy) were used for enzymatic analyses of acetate and D- and L-lactate.

3. Results and discussion

3.1. Ion-exclusion chromatography

The use of two different detection techniques is required because of the relatively low concentration of the components that must be determined, i.e., UV set at 207 nm for fumaric acid and conductimetry for both acetic and lactic acids. In this way the detection limits were optimized and the interferences due to the complexity of the matrix were greatly reduced.

The ion-exclusion chromatography method allows for the determination of total lactic acid content, but cannot be used to determine the content of D- and L-isomers, as can the enzymatic method, however, this is only a minor problem because it has been demonstrated in previous studies [9] that microbial growth can cause the formation of one or both

isomers, thus, specific determination is not significant for quality control.

For the determination of acetate, lactate and fumarate, conductivity and spectrophotometric detectors were used in series (Fig. 1). The best sensitivity for fumarate was achieved with UV detection at 207 nm. Lactate and acetate were also detected at 207 nm, but better sensitivities were achieved with conductivity detection.

Furthermore, it is evident from Fig. 2 (pear nectar) that UV detection at 207 nm overestimates the content of acetic acid, in comparison with conductivity.

Diode array spectral analysis suggests the presence of a UV-absorbing substance coeluting with acetic acid, however, acetic acid data obtained by conductivity detection were in good agreement with enzymatic analyses, $r=0.948$, therefore, the UV interference had no influence on the conductimetric determination of acetic acid. Lactic acid ion-exclusion chromatographic data showed good correlation with the enzymatic method also ($r=0.998$).

The spectral purity of fumaric acid was tested using the diode array detector. Interferences were not found.

3.2. Reproducibility

The reproducibility of the peak area was calculated by performing five replicate analyses of a 10 mg/l standard.

The relative standard deviations (R.S.D.) for peak area, calculated using a standard solution of fumaric (2 mg/l), acetic (10 mg/l) and lactic acid (10 mg/l), were 4.5, 1.8 and 1.5%, respectively ($n=5$).

3.3. Detection limit

The detection limits calculated according to IUPAC suggestions [16] were 1 mg/l for lactic and acetic acid and 0.5 mg/l for fumaric acid.

3.4. Inoculated samples

Different microorganism inoculation tests demonstrate the ability of ion-exclusion chromatography to determine the metabolites of interest in a single run. Fig. 3a–b shows the chromatograms of a pear nectar before and after alteration by the mould *R. stolonifer*; the presence of a 100-times higher amount of fumaric acid and some variation in acetic

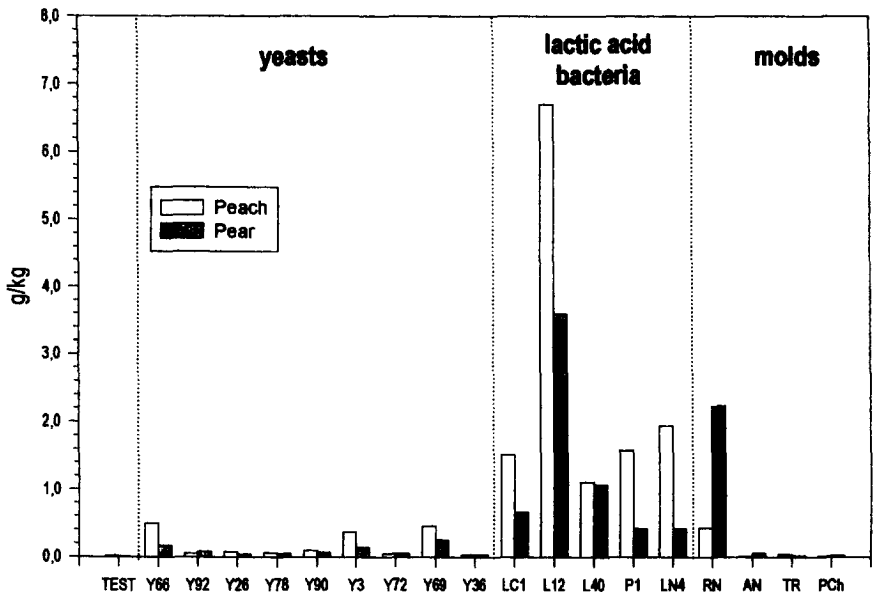

Fig. 4. Effect of spoilage by different strains on the lactic acid content of fruit nectar.

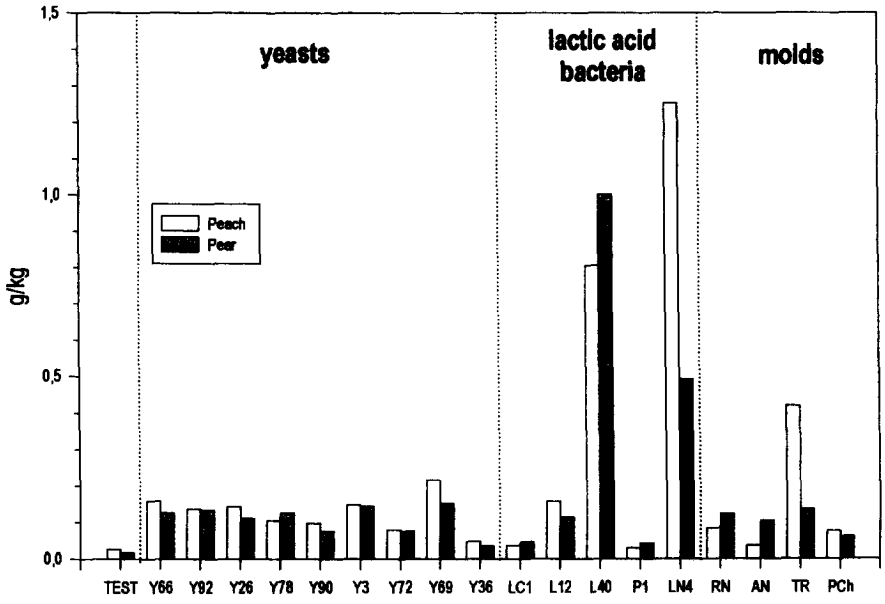

Fig. 5. Effect of spoilage by different strains on the acetic acid content of fruit nectar.

acid content in the spoiled product are evident. This behaviour is similar in pear nectar inoculated with the yeast *M. pulcherrima*, where there is an increase in acetic and fumaric acid concentration after product alteration. Conversely in pear nectar inoculated with the lactic acid bacteria *Leuconostoc* sp., an increase

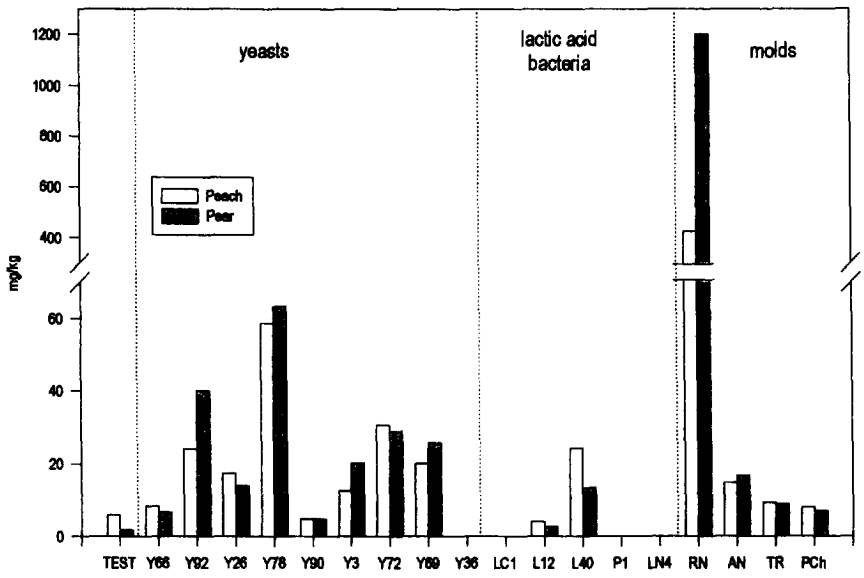

Fig. 6. Effect of spoilage by different strains on the fumaric acid content of fruit nectar.

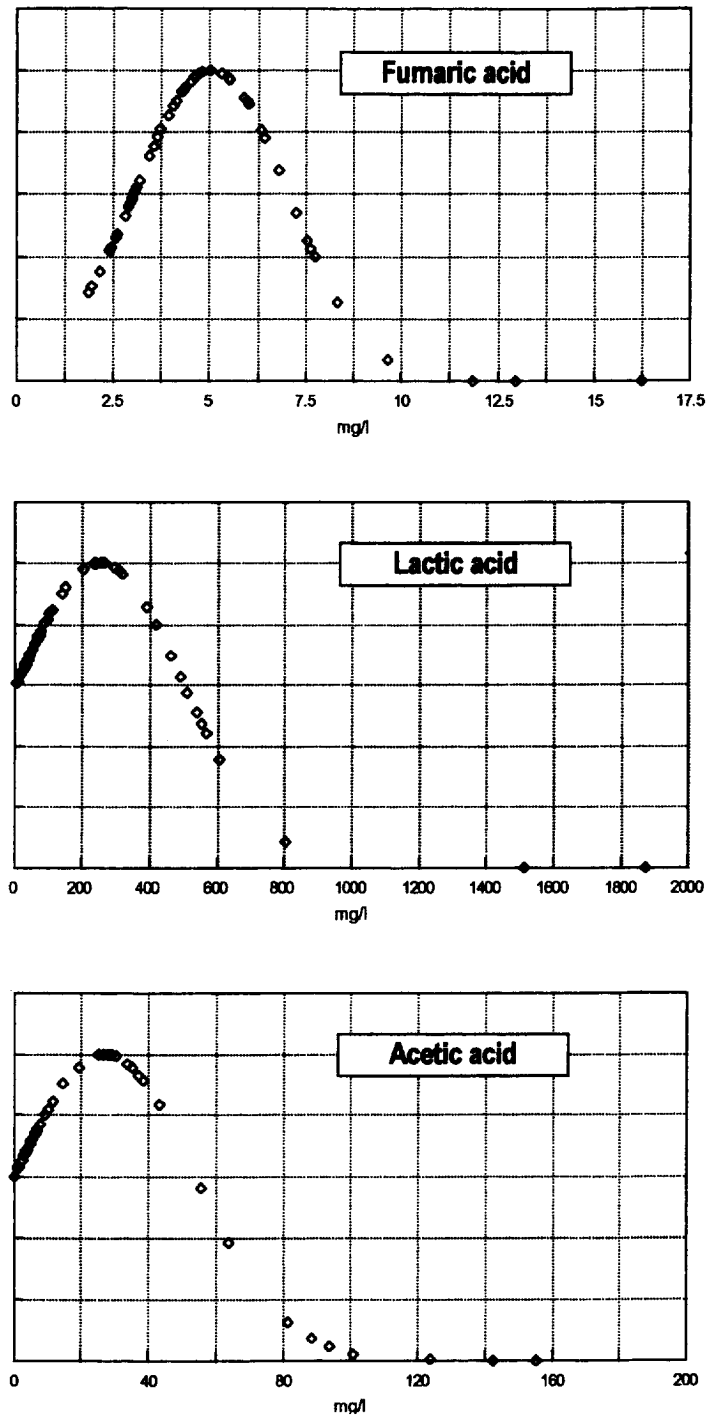

Fig. 7. (Top) Fumaric acid data for commercial samples of apple juices. (Middle) Lactic acid data for commercial samples of apple juices. (Bottom) Acetic acid data for commercial samples of apple juices.

of acetic and lactic acid concentrations is evident, while there is no increase in fumaric acid.

The analyses of inoculated samples confirmed that the formation of lactic acid was caused mainly by lactic acid bacteria (Fig. 4), while both yeasts and moulds, with the exception of the RN strain, did not produce lactic acid amounts that were higher than the proposed limits.

All of the yeasts produced low amounts of acetic acid (Fig. 5), always less than 0.4 g/kg. The highest amounts of acetic acid were produced by L40 and LN4 strains of lactic acid bacteria and only peach nectar altered by *T. harzianum* mould had acetic acid concentrations that were slightly higher than the limit.

The alteration induced by *R. stolonifer* confirmed bibliographic data [6] and produced a large amount of fumaric acid (400–500 mg/kg in peach nectar and 1200–1300 mg/kg in pear nectar), as shown in Fig. 6. Other moulds produced fumaric acid only to a minor extent (10–20 mg/kg).

For the lactic acid bacteria, fumaric acid (20–30 mg/kg) was found only in the sample altered by the L40 strain. Most yeasts, with the exception of Y66, Y90 and Y36 strains, produced concentrations of fumaric acid that were higher than 15 mg/kg and were up to 60–70 mg/kg for the Y78 strain-inoculated sample (Fig. 6).

Considering that ethanol, the main metabolite of yeast alterations, can easily be eliminated with an appropriate technological process due to its high volatility, the presence of high concentrations of the non-volatile fumaric acid can be used as an important indication of the quality of the product.

3.5. Commercial samples

By using the same method, a study of several industrial samples was performed. In order to evaluate the existing situation on the market, several samples of industrial apple juice were analyzed and the distribution curves of acetic, lactic and fumaric acid data are shown in Fig. 7.

Acetic acid data showed that the concentration of this metabolite is always lower than 200 mg/l; this fact can be explained by the relatively high volatility of acetic acid and by the fact that the production of samples involves a concentration step by evaporation. In the case of lactic acid, only a few samples have concentrations that are clearly higher than 500 mg/l. Fumaric acid was characterized by lower dispersion of the data (R.S.D.=56% instead of 137 and 140% for lactic and acetic acid, respectively), and values ranged from 1.5 to 10 mg/l. Only three samples contained fumaric acid values that were between 10 and 20 mg/l.

4. Conclusions

The proposed method allows the simultaneous determination of some important acidic metabolites used for quality control in complex matrices such as vegetable and fruit juices, nectars and purees.

Inoculation tests demonstrate that microbial spoilage of peach and pear nectar results in the formation of appreciable quantities of one or more metabolites of interest.

The significance of the proposed limit for fumaric acid was evaluated considering that microorganisms other than *R. stolonifer* are able to produce this metabolite also. According to our results, based on data obtained from apple juice analysis, fumaric acid determination is valuable in defining the quality of the product, and the limit of 5 mg/kg proposed by AIJN appears to be too restrictive.

Reported results arise from a preliminary screening where complete alteration of the product occurs. Studies are in progress to evaluate how the influence of bacterial contamination could effect the fumaric acid formation rate.

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