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Simultaneous determination of soluble sugars and organic acids as their trimethylsilyl derivatives in apricot fruits by gas–liquid chromatography

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Abstract

The aim of this study was to determine a reliable procedure for the quantification of organic acids, alcohol soluble sugars and sugar alcohols in fruit flesh by means of a rapid GLC method, without resorting to methoximation of sugars and employing apricot as a model. The use of two internal standards, an accurate derivatization and a proper calibration of the GLC conditions allowed an accurate quantitative analysis of the compounds detected in the unknown samples. This simple procedure improves the speed of preparation of the trimethylsilyl derivatives and is highly reproducible. Variability was found between years for each of the five cultivars studied and for each compound in terms of absolute values, whereas the percentage incidence of the single sugars as a total was more stable over the two years of observation.

Keywords: Fruits; Derivatization, GC; Carbohydrates; Organic acids; Sugar alcohols

1. Introduction

Qualitative and quantitative composition of organic acids and soluble sugars has been often regarded as indicator of fruit quality traits [1–3]. In addition, these compounds are important to evaluate fruit maturity, ripeness and storage conditions and could be used as chemical markers in distinguishing different cultivars.

Since different techniques (refractometer, titratable acidity, reducing sugars, paper chromatography, gas chromatography, HPLC) are employed to detect these compounds, comparison of the results with previously published data is often very difficult. Acid and sugar content in flesh of apricot fruits has already been studied, but with different analytical techniques and with sometimes divergent results [3–7]. Recent papers [8–11] proved gas–liquid chromatography (GLC) to be a suitable technique for this purpose due to the applicability of the silylation technique to all compounds containing active hydrogen functions, simplicity, short analysis time and low cost. Nevertheless, the presence of compounds characterized by different functional groups and in extremely different concentration ranges requires an accurate choice of silylation parameters. Fruit sam-

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pling and calibration of the stock solutions containing the compounds to be studied also have to be properly carried out.

The aim of the present study was the determination of a reliable procedure for the quantification of organic acids, alcohol soluble sugars and sugar alcohols in fruit flesh by means of a rapid GLC method, without resorting to the methoximation of sugars and employing apricot as a model.

2. Experimental

2.1. Materials, reagents and samples

Chemicals and reagents were of analytical-reagent grade. Silylation and oximation reagents were obtained from Sigma (St. Louis, MO, USA), model sugars and acids were from Fluka (Buchs, Switzerland), and two internal standards used were from Aldrich (Buchs, Switzerland). Apricot fruits were collected in 1992 and 1994 from four cultivars ("Amabile Vecchioni", "Cricot", "Mono" and "Reale di Imola") and one selection ("NJ A 1") grown at the experimental fields of the University of Bologna, Italy. Data from 1993 were not available due to a late frost that prevented a sufficient yield. These apricots are widely used in the breeding programme at the University of Bologna because of their high quality and horticultural traits.

2.2. Preparation of the samples for GLC

Fifteen fruits harvested at eating maturity for each cultivar were collected. Penetrometer analysis of each of these 15 fruits determined the four fruits closest to the average firmness for the GLC analysis (data not shown). Each fruit was peeled and a mixture of 5 g of flesh and 1 ml of β-phenyl-glucopyranoside (2.5 g/100 ml) as an internal standard, was ground in 10 ml of 50% ethanol for 5 min in a Sanderson blender. Samples were then centrifuged at 1000 *g* for 5 min. The supernatant was withdrawn and diluted to a volume of 50 ml with 50% ethanol. One ml of this stock solution was dried by air stream after adding 0.5 ml of methyl arachidate (100 mg/100 ml) as an additional standard.

2.3. Preparation of the standard solutions

Various amounts of organic acids (succinic, malic, citric, tartaric, quinic and ascorbic acids), alcohol soluble sugars (xylose, fructose, glucose, sucrose, maltose, trehalose and raffinose) and sugar alcohols (sorbitol, mannitol and inositol) were dissolved in 50% ethanol together with imidazole buffer 0.1 *M*, pH 7, in order to avoid acid hydrolysis of sucrose in fructose and glucose (Fig. 1). In fact, it is known that sucrose is hydrolyzed to glucose and fructose by dilute acids and by invertase, a yeast enzyme. Thus, imidazole buffer creates a neutral pH avoiding the formation of an acid environment, which sometimes occurs in apricot flesh. For each compound 13 different concentrations with seven repetitions were tested on the basis of the range taken from the literature [4,5,7,12]. Two stock solutions (A and B) containing the same total amount of the following standard compounds were prepared (details in Table 1): solution A: ascorbic acid, citric acid, fructose, malic acid, maltose, quinic acid, raffinose, succinic acid, tartaric acid, trehalose and xylose; the amount of each compound was gradually decreased from run No. 1 to No. 13; solution B: glucose, inositol, mannitol, sorbitol and sucrose; the amount of each compound was gradually increased from run No. 1 to No. 13.

For each of the 13 different calibration runs 1.7 ml of the two stock solutions were dried by air stream.

2.4. Preparation of the trimethylsilyl (TMS) and TMS-methoxime derivatives

The dehydrated residues of stock solutions and flesh extracts were treated with 400 μl of pyridine, 200 μl of hexamethyldisilazane (HMDS) and 100 μl of trimethylchlorosilane (TMCS) (modifying the method described by Sweeley et al. [13]) and heated at 60°C for 2 h. The 4:2:1 ratio and imidazole addition allow a good derivatization despite the presence of traces of water in the samples [13,14]. The cooled samples were stored at 4°C and 0.3-μl amounts were injected for each analysis. The same procedure was used for preparing the methoximated samples, dissolving methylhydroxylamine hydrochloride into the pyridine (2 g/100 ml), heating the

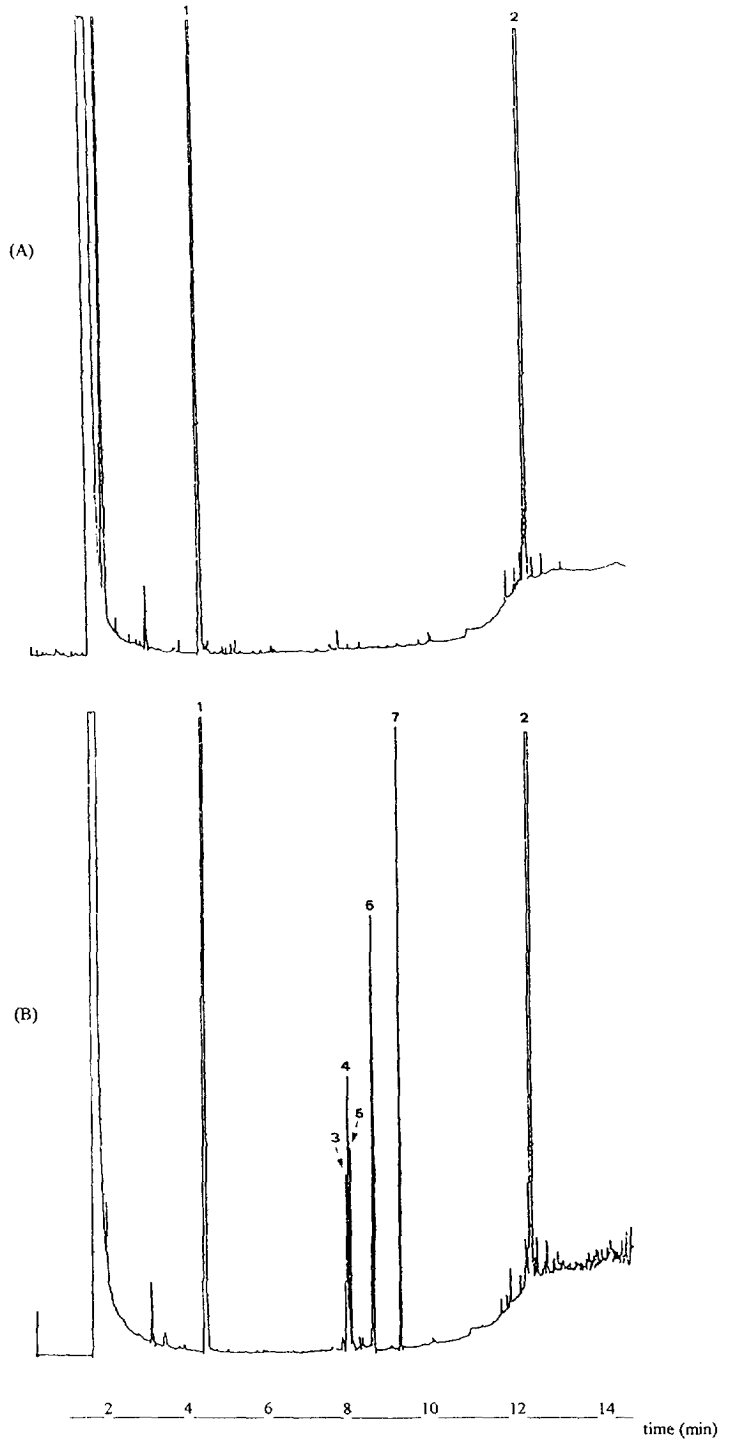

Fig. 1. GLC identification of sucrose with (A) and without (B) imidazole in the presence of malic acid. Peaks in order of number: 1=malic acid; 2=sucrose; 3=fructose$_1$; 4=fructose$_2$; 5=fructose$_3$; 6=glucose$_1$; 7=glucose$_2$.

Table 1
Composition of the two standard solutions used for the calibration runs

	Run												
	1	2	3	4	5	6	7	8	9	10	11	12	13
Solution A (μl)	1675	1600	1450	1300	1150	1000	850	700	550	400	250	100	25
Solution B (μl)	25	100	250	400	550	700	850	1000	1150	1300	1450	1600	1675
A+B	1700	1700	1700	1700	1700	1700	1700	1700	1700	1700	1700	1700	1700
Solution A (mg)													
Ascorbic acid	1.34	1.28	1.16	1.04	0.92	0.80	0.68	0.56	0.44	0.32	0.20	0.08	0.02
Citric acid	1.34	1.28	1.16	1.04	0.92	0.80	0.68	0.56	0.44	0.32	0.20	0.08	0.02
Fructose	2.68	2.56	2.32	2.08	1.84	1.60	1.36	1.12	0.88	0.64	0.40	0.16	0.04
Malic acid	1.34	1.28	1.16	1.04	0.92	0.80	0.68	0.56	0.44	0.32	0.20	0.08	0.02
Maltose	0.67	0.64	0.58	0.52	0.46	0.40	0.34	0.28	0.22	0.16	0.10	0.04	0.01
Quinic acid	1.34	1.28	1.16	1.04	0.92	0.80	0.68	0.56	0.44	0.32	0.20	0.08	0.02
Raffinose	0.67	0.64	0.58	0.52	0.46	0.40	0.34	0.28	0.22	0.16	0.10	0.04	0.01
Succinic acid	0.67	0.64	0.58	0.52	0.46	0.40	0.34	0.28	0.22	0.16	0.10	0.04	0.01
Tartaric acid	0.67	0.64	0.58	0.52	0.46	0.40	0.34	0.28	0.22	0.16	0.10	0.04	0.01
Trehalose	0.67	0.64	0.58	0.52	0.46	0.40	0.34	0.28	0.22	0.16	0.10	0.04	0.01
Xylose	0.67	0.64	0.58	0.52	0.46	0.40	0.34	0.28	0.22	0.16	0.10	0.04	0.01
	12.06	11.52	10.44	9.36	8.28	7.20	6.12	5.04	3.96	2.88	1.80	0.72	0.18
Solution B (mg)													
Glucose	0.04	0.16	0.40	0.64	0.88	1.12	1.36	1.60	1.84	2.08	2.32	2.56	2.78
Inositol	0.01	0.04	0.10	0.16	0.22	0.28	0.34	0.40	0.46	0.52	0.58	0.64	0.67
Mannitol	0.01	0.04	0.10	0.16	0.22	0.28	0.34	0.40	0.46	0.52	0.58	0.64	0.67
Sorbitol	0.04	0.16	0.40	0.64	0.88	1.12	1.36	1.60	1.84	2.08	2.32	2.56	2.78
Sucrose	0.08	0.32	0.80	1.28	1.76	2.24	2.72	3.20	3.68	4.16	4.64	5.12	5.36
	0.18	0.72	1.80	2.88	3.96	5.04	6.12	7.20	8.28	9.36	10.44	11.52	12.06
Solution A+B:	12.24	12.24	12.24	12.24	12.24	12.24	12.24	12.24	12.24	12.24	12.24	12.24	12.24

sample at 60°C for 60 min and then silylating with HMDS and TMCS as described above.

2.5. Separation of TMS and the TMS-methoxime derivatives

The gas chromatograph used was a Chrompack CP 9000 (Chrompack, Middelburg, Netherlands) equipped with a flame ionization detector and a capillary fused-silica column (25 m×0.25 mm I.D.), coated with CP-Sil-5CB, DF 0.12 (Chrompack). Injector and detector temperatures were 280°C and 320°C, respectively. The following temperature programme was set: 120°C for 1 min, followed from 120 to 152°C at 8°C/min, from 152 to 176°C at 12°C/min, from 176 to 198°C at 16°C/min, from 198 to 238°C at 20°C/min, from 238–300°C at 24°C/min, and finally 300°C for 5 min. Using this programme, 20 min were required to elute the trimethylsilyl derivatives of the above cited compounds. This particular programme makes it possible to obtain well resolved peaks with comparable width (1–1.6 s). Only mannitol and sorbitol appear to be partially separated, but this is due to their very close retention times. Flow-rates of He, H_2, air and N_2 (used as a make-up gas) were 2, 30, 250 and 30 ml/min, respectively, with a split ratio of 80:1. Individual sugars and acids were identified by comparison of their retention times with those of TMS derivatives of the authentic compounds (Fig. 2 and

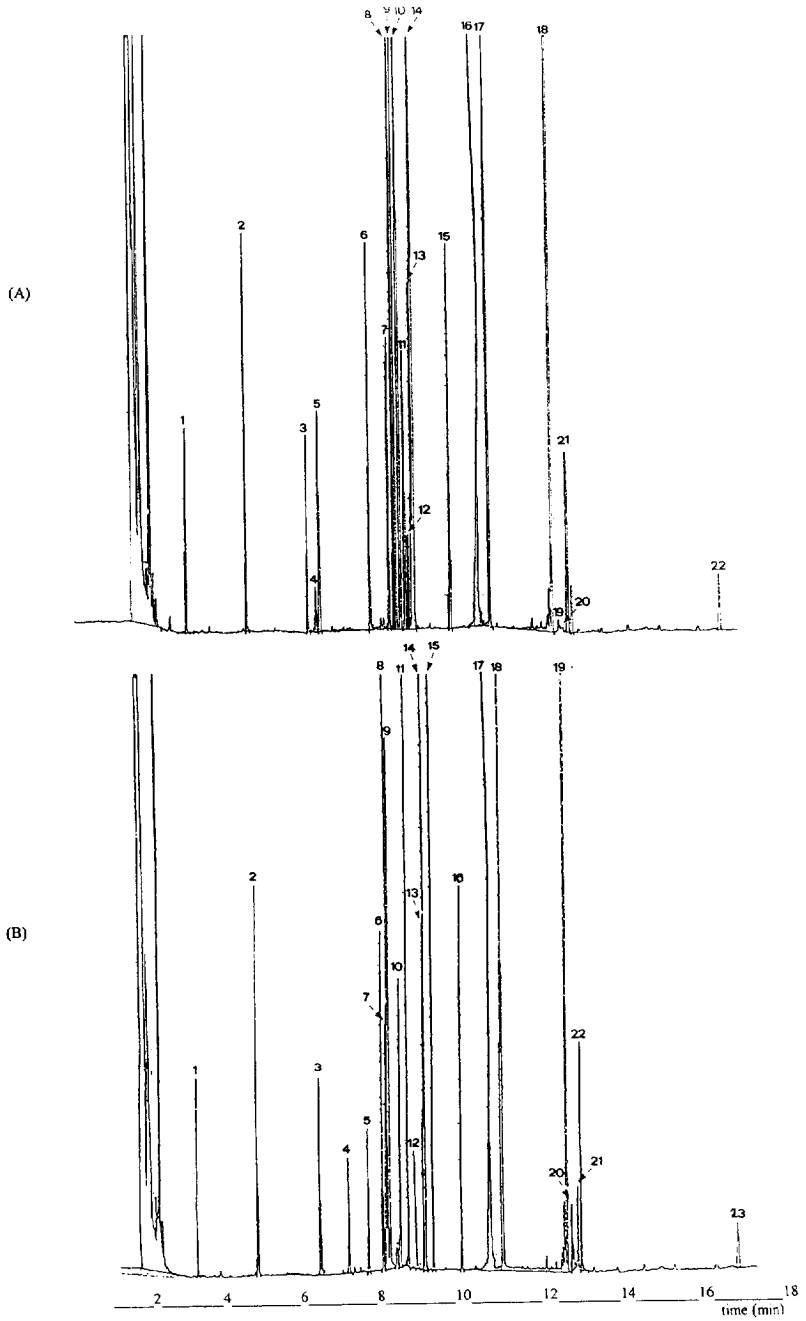

Fig. 2. GLC identification of a stock solution with (A) and without (B) methoximation of sugars. Peaks in order of retention times for chromatogram A: 1=succinic acid; 2=malic acid; 3=tartaric acid; 4=xylose$_1$; 5=xylose$_2$; 6=citric acid; 7=quinic acid; 8=fructose$_1$; 9=fructose$_2$; 10=glucose$_1$; 11=glucose$_2$; 12=ascorbic acid; 13=mannitol; 14=sorbitol; 15=inositol; 16=methyl arachidate; 17=β-phenylglucopyranoside; 18=sucrose; 19=maltose$_1$; 20=maltose$_2$; 21=trehalose; 22=raffinose. Peaks in order of retention times for chromatogram B: 1=succinic acid; 2=malic acid; 3=tartaric acid; 4=xylose$_1$; 5=xylose$_2$; 6=citric acid; 7=fructose$_1$; 8=fructose$_2$; 9=fructose$_3$; 10=quinic acid; 11=glucose$_1$; 12=ascorbic acid; 13=mannitol; 14=sorbitol; 15=glucose$_2$; 16=inositol; 17=methyl arachidate; 18=β-phenylglucopyranoside; 19=sucrose; 20=maltose$_1$; 21=maltose$_2$; 22=trehalose; 23=raffinose. The amounts of each compound in both solutions refer to run No. 8 reported in Table 1.

Fig. 3). The quantification of each compound was performed using the internal standard calculation method.

2.6. Statistical analysis

Data observed after the calibration and calculated as the ratio between each compound area and the internal standard area, were analyzed statistically by means of a linear regression model using the Statistix program. Data from fruit samples were submitted to ANOVA (analysis of variance) and Student–Newman–Keuls test for comparison of means within and between years, using a GLM (general linear model) procedure by SAS (SAS Institute Inc., Cary, NC, USA) program ($P=0.05$). Both absolute values and relative composition of acids and sugars were used for statistical analysis.

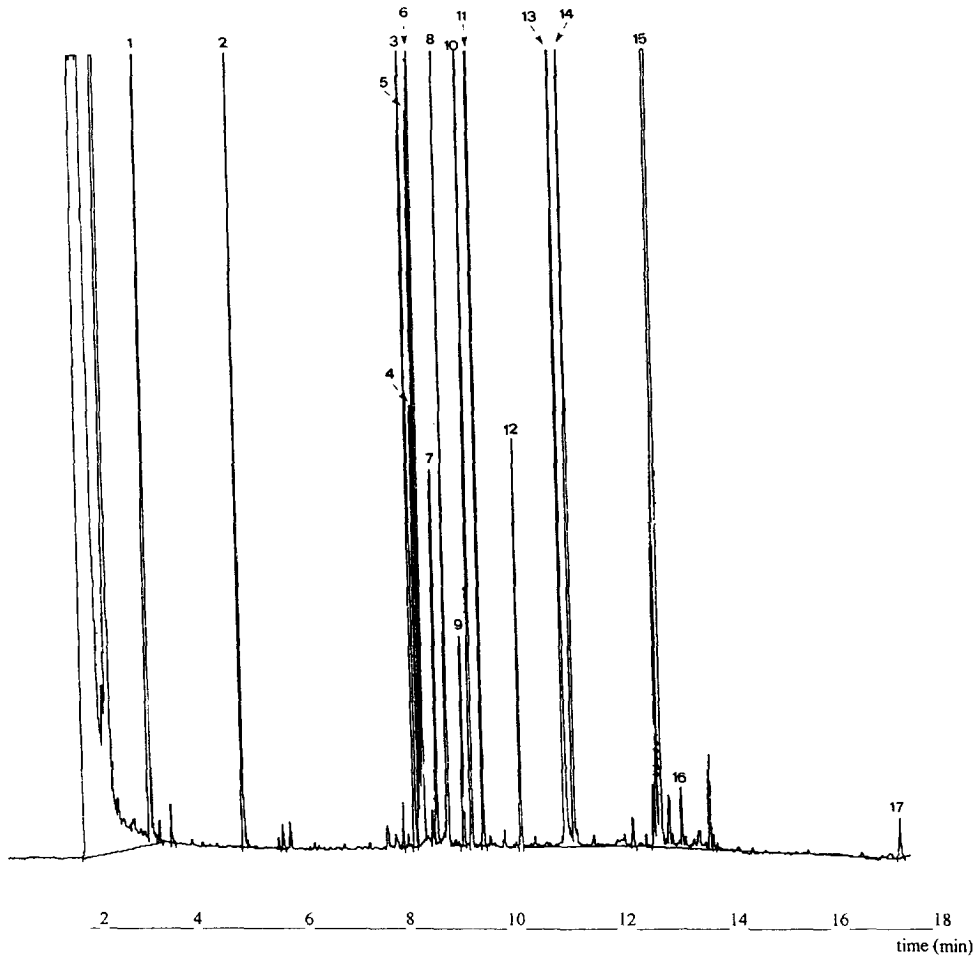

Fig. 3. GLC identification of acids and sugars in apricot flesh, cv. Cricot. Peaks in order of retention times (amount, g/100 g fresh mass, in parentheses): 1=succinic acid (0.013); 2=malic acid (0.762); 3=citric acid (0.642); 4=fructose$_1$ (0.123); 5=fructose$_2$ (0.270); 6=fructose$_3$ (0.415); 7=quinic acid (0.075); 8=glucose$_1$ (0.481); 9=ascorbic acid (0.092); 10=sorbitol (0.258); 11=glucose$_2$ (0.630); 12=inositol (0.068); 13=methyl arachidate (I.S.); 14=β-phenylglucopyranoside (I.S.); 15=sucrose (5.045); 16=trehalose (0.020); 17=raffinose (0.032).

3. Results and discussion

3.1. GLC procedure

Chromatograms of non-methoximated standard compounds did not particularly differ from the methoximated ones (Fig. 2), even if changes in retention times can be seen for reducing sugars analyzed by the two different methods. Multiple peaks found for reducing sugars, which correspond to the various isomeric forms present at equilibrium, did not compromise the reading of chromatograms and were well separated and recognizable even in the non-methoximated samples. Anyway, the number of isomeric forms did not dramatically increase using the trimethylsilylation procedure, and this can be explained since only reducing sugars can react with methylhydroxylamine hydrochloride. Also, in our analyses only three isomers for fructose and two isomers for the other reducing sugars (xylose, glucose, maltose) were considered, because the other expected isomers were represented by insignificant peaks. Moreover, as previously reported in other papers where methoximation of sugars was carried out [15], linear regression between expected and observed data from our non-methoximated samples showed a very high r coefficient for almost all of the compounds studied, and a low standard error of estimate (Table 2 and Fig. 4). Also, the seven repetitions for each concentration tested gave a high reproducibility (data not shown). Only ascorbic acid showed a little lower r coefficient (0.919). This is probably due to the use of air in the drying step of stock solutions and apricot extracts, and also to the susceptibility of ascorbic acid to light, which makes the GLC analysis unreliable for this compound.

The use of two internal standards is advised as the first is useful for determining sample losses during the extraction and the second is to control the silylation procedures. Following the right extraction procedures [16] and the derivatization and GLC conditions from the present work, GLC was found to be a reliable means for the detection of acids and sugars extracted from fruit flesh, with no need to resort to the methoximation of sugars.

Table 2
Linear regresion coefficient (r) and standard error of estimate (S.E.) for the compounds syudied by GLC

	r	S.E.
Fructose$_1$	0.9734	0.067
Fructose$_2$	0.9930	0.044
Fructose$_3$	0.9421	0.070
Maltose$_1$	0.9973	0.006
Maltose$_2$	0.9963	0.009
Glucose$_1$	0.9686	0.051
Glucose$_2$	0.9978	0.031
Xylose$_1$	0.9978	0.005
Xylose$_2$	0.9986	0.006
Ascorbic acid	0.9194	0.147
Quinic acid	0.9974	0.031
Citric acid	0.9989	0.020
Inositol	0.9979	0.013
Mannitol	0.9975	0.014
Raffinose	0.9974	0.015
Sorbitol	0.9976	0.059
Succinic acid	0.9990	0.009
Sucrose	0.9961	0.155
Tartaric acid	0.9984	0.012
Trehalose	0.9976	0.013

3.2. Fruit composition

As far as the composition of apricot flesh is concerned, a large variability among cultivars was found, as confirmed by the statistical analysis (Table 3). In both years malic acid was the prevalent acid in all the cultivars studied, and sucrose was the most abundant sugar, followed by glucose and fructose. Succinic and quinic acids, xylose, maltose, raffinose, trehalose, mannitol and inositol were present in traces occasionally, while tartaric acid was absent (data not shown). All the apricots tested showed the presence of ascorbic acid, but the quantification of this compound was unreliable for the reasons cited above. Citric acid and sorbitol were relevant compounds also. Data confirms the possibility to use these compounds as chemical markers. For instance, "Amabile Vecchioni" showed a very high amount of malic acid, whereas citric acid was almost absent compared to the other varieties studied. Regarding sugars and sugar alcohols, cv. Mono had the highest amount of fructose and glucose, and the lowest content of sucrose, whereas "Reale" had the highest sorbitol content of all the apricots studied.

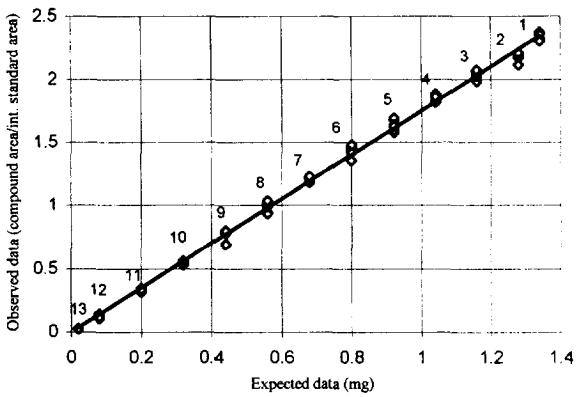

Fig. 4. Regression obtained for citric acid after 13 calibration runs: each run had 7 replications. Observed data $=-1.12\cdot10^{-5}+1.75\cdot$ expected data. Pearson's correlation: $r=0.9989$.

Variability was found also between years for each cultivar and for each compound in terms of absolute values (data not shown), whereas the percentage incidence of the single sugars on the total was more stable over the two years (Table 4).

4. Conclusions

According to the literature two separate, but simultaneous derivatization procedures were required for GLC analysis of organic acids and sugars [14,17]. More recently methoximation and silylation of sugars have been reduced to a single step [10], but the prior conversion of sugars to their oximes is still advisable. Our results showed the methoximation of sugars to be unnecessary in apricots. In fact, data obtained from calibration, derivatization and GLC conditions of the present study allowed us to obtain an accurate quantitative analysis of the compounds detected in the unknown samples. This method permitted us to obtain extremely readable chromatograms even without the methoximation of sugars, and by means of a simpler and less expensive procedure.

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Table 3
Amounts of the most important organic acids, sugars and sugar alcohols detected in five apricots

Cultivar	Malic acid	Citric acid	Fructose	Glucose	Sucrose	Sorbitol	Total acids	Total sugars
A. Vecchioni	2.02 a	0.07 c	0.50 c	1.10 b	5.18 ab	0.06 b	2.13 a	6.90 b
Cricot	0.71 c	0.62 a	0.76 b	1.02 b	5.24 ab	0.16 b	1.32 b	7.22 b
Mono	0.73 c	0.66 a	1.24 a	1.89 a	4.37 b	0.28 b	1.39 b	7.81 ab
NJ A 1	0.87 c	0.29 b	0.77 b	1.35 b	5.37 ab	0.28 b	1.16 b	7.83 ab
Reale di Imola	1.24 b	0.28 b	0.72 b	1.02 b	6.52 a	0.73 a	1.52 b	9.05 a

Mg/100 mg fresh mass: two-year mean.
Means in the same column with the same letter are not significantly different ($P=0.05$).

Table 4
Incidence of each compound on total acids and sugars in two years of observations in five apricots (%)

Cultivar	Malic acid		Citric acid		Fructose		Glucose		Sucrose		Sorbitol	
	1992	1994	1992	1994	1992	1994	1992	1994	1992	1994	1992	1994
A. Vecchioni	93.3 b	97.0 a	6.7 a	3.0 b	6.6 b	7.8 a	15.5 a	16.6 a	76.1 a	73.8 a	0.9 a	0.7 b
Cricot	61.7 a	42.0 a	38.3 a	58.0 a	12.0 a	9.8 a	15.5 a	13.6 a	71.0 a	72.8 a	1.3 a	2.8 a
Mono	75.4 a	42.8 b	24.6 b	57.2 a	14.7 a	16.4 a	23.7 a	24.3 a	59.6 a	54.3 a	2.0 a	4.4 a
NJ A 1	72.4 a	77.7 a	27.6 a	22.3 a	9.1 a	11.0 a	14.8 a	19.7 a	72.8 a	63.7 a	2.8 b	4.6 a
Reale di Imola	76.5 b	88.3 a	23.5 a	11.7 b	7.6 a	8.3 a	11.3 a	11.3 a	77.2 a	67.6 b	3.4 b	12.2 a

Means between years for each cultivar with the same letter are not significantly different ($P=0.05$).

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