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# Determination of mono-, poly- and hydroxy-carboxylic acid profiles of beverages as their 2-nitrophenylhydrazides by reversed-phase ion-pair chromatography

Hiroshi Miwa*, Magobei Yamamoto

*Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1, Nanakuma, Jonan-ku, Fukuoka 814-80, Japan*

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**Abstract**

A simple, rapid and accurate method for the derivatization and quantitative reversed-phase ion-pair chromatographic analysis of mono-, poly- and hydroxy-carboxylic acids in wines, fruit juices, beer and Japanese "sake" is presented. Nine hydrazine derivatives were prepared in high yield by direct derivatization of the carboxylic acids with 2-nitrophenyl-hydrazine hydrochloride in sample matrix. Isocratic separation of the carboxylic acid hydrazides was performed within 18 min by a suitable combination of pH, the polarity of eluent and the size of the counter ion. The analytical results showed good recovery and reproducibility using 3-methylglutaric acid as an internal standard. Due to its superior selectivity and sensitivity, the present method can serve as a useful tool for routine analysis of carboxylic acids in foods and beverages.

## 1. Introduction

The nature and concentration of carboxylic acids, such as mono-, poly- and hydroxy-carboxylic acids, in foods and beverages are of wide interest with respect to quality control. Although several high-performance liquid chromatographic (HPLC) methods, e.g. ion-exchange and ion-exclusion chromatography, solvophobic chromatography, ion-pair chromatography and reversed-phase chromatography, have been extensively studied for the determination of carboxylic acids in various samples, their determination is still object of research. This is due to weak chromophoric properties of the carboxyl group, giving relatively poor sensitivity and selectivity the detection.

To achieve more sensitive and selective detection with HPLC, pre-column derivatization methods have been developed [1–10]. However, most of these methods did not consider quantitative aspects, were not always successful in the simultaneous separation of mono-, poly- and hydroxy-carboxylic acid derivatives and also need a fairly long analysis time and/or a rigorous sample clean-up procedure. It is therefore desirable to establish a more convenient HPLC method that is rapid and easy to use, involves minimum sample preparation and is suitable for routine analysis.

In a previous study, mono-, poly- and hydroxy-carboxylic acids were selectively separated as their 2-nitrophenylhydrazides with reversed-

* Corresponding author.

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phase ion-pair chromatography (RP-IPC) by the influence of pH, the polarity of the eluent and the size of the counter ion [11]. The present paper demonstrates an extension of the direct derivatization procedure of the above carboxylic acids in various beverages without any pre-treatment, and the quantitative analysis of citric, tartaric, malic, succinic, fumaric, glycolic, L-pyroglutamic, lactic and acetic acids using an improved RP-IPC method with 3-methyllgutaric acid as an internal standard.

## 2. Experimental

### 2.1. Materials and reagents

All carboxylic acid (ethanol solutions) were obtained from Yamamura Chemical Laboratories (Kyoto, Japan). Tetramethylammonium (TMA), tetraethylammonium (TEA) and tetra-*n*-propylammonium (TPA) were purchased as their bromides from Tokyo Kasei Kogyo (Tokyo, Japan). A 2-nitrophenylhydrazine hydrochloride (2-NPH·HCl) (Tokyo Kasei Kogyo) solution (0.02 *M*) was prepared by dissolving the reagent in 0.1 *M* hydrochloric acid–ethanol (1:1, v/v). A 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (1-EDC·HCl) (Sigma, St. Louis, MO, USA) solution (0.25 *M*) was prepared by dissolving the reagent in a solution of pyridine (3%, w/v) in ethanol. A potassium hydroxide (10%, w/v) solution was prepared by dissolving the reagent in methanol–water (1:1, v/v). All the reagent solutions were stable for at least three months when kept below 5°C, and were commercially available from Yamamura Chemical Labs. All other chemicals were reagent grade, unless otherwise mentioned.

For beverage materials, commercially available red wine, white wine, apple juice, orange juice, beer and Japanese "sake" were used.

### 2.2. HPLC analysis

Chromatographic analyses were carried out using a Shimadzu LC-6A liquid chromatograph (Shimadzu Seisakusho, Kyoto, Japan) equipped with an on-line degasser ERC-3310 (Erma, Tokyo, Japan) and a Shimadzu SPD-6AV variable-wavelength UV-Vis detector. The detector signals were recorded on a Rikadenki multi-pen recorder (Tokyo, Japan). The column temperature was kept constant at 35°C using a Shimadzu GTO-6A column oven. The column was a J'sphere ODS-M 80 main column (particle size 4 $\mu$m, 250 × 6 mm I.D.) with a BBC-5-$C_8$ guard column (particle size 5 $\mu$m, 10 × 5 mm I.D.), packed at Yamamura Chemical Labs.

All analyses were carried out isocratically using phosphate buffer–acetonitrile–methanol as the eluent at a flow-rate of 2 ml/min. The pH was adjusted to the desired value by mixing 0.005 *M* $KH_2PO_4$–acetonitrile–methanol with 0.005 *M* $Na_2PO_4$–acetonitrile–methanol and then dissolving counter ions at a concentration of 0.005 *M*. The counter ions studied were TMA, TEA and TPA as their bromides. The solvents were filtered through Nucleopore filter (pore size 0.2 $\mu$m) (Nomura Micro Science, Osaka, Japan).

### 2.3. Assay procedure

For samples, 50 $\mu$l of the wines, 25 $\mu$l of the fruit juices, 100 $\mu$l of beer and 50 $\mu$l of Japanese "sake" were exactly measured and each sample, with the exception of beer, was diluted with water to 100 $\mu$l. To the sample solutions, 200 $\mu$l of ethanol containing 400 nmol of 3-methylglutaric acid as the internal standard, 200 $\mu$l of 2-NPH·HCl solution and 200 $\mu$l of 1-EDC·HCl solution were added and the mixture was heated at 80°C for 5 min. After the addition of 200 $\mu$l of 10% (w/v) potassium hydroxide solution, the mixture was further heated at 80°C for 5 min and then cooled. Aliquots (5–10 $\mu$l) of the hydrazide mixture were injected onto the chromatographic system.

## 3. Results and discussion

### 3.1. Derivatization conditions

In HPLC analysis, some components in the beverage matrix sometimes interfered with the

refractive index or ultraviolet detection (at about 210 nm) of the carboxylic acids. Because the interfering substances do not absorb at 400 nm, this problem can be solved by using pre-column derivatization in conjunction with visible detection. During the direct derivatization of various carboxylic acids, such as mono-, poly- and hydroxy-carboxylic acids in beverages without lengthy and cumbersome sample workup, the pH of the reaction mixture may be slightly increased by basic substances occurring in the beverages, resulting in a decrease of the yields of the carboxylic acid hydrazides [12,13]. To overcome this problem, the reaction mixture was kept at constant pH by the addition of hydrochloric acid.

Another investigation was needed to decrease the derivatization period required. The derivatization rate gradually increased with increasing temperature, but the detector response decreased with reproducible yields. Using an optimum treatment of 5 min at 80°C, the carboxylic acids studied were rapidly converted to their hydrazides. In this derivatization process, di- and tri-carboxylic acids converted to their monohydrazine derivatives, i.e., acidic acid hydrazides, which were characterized as weak acidic compounds due to the residual carboxyl group [11,14].

### 3.2. Chromatographic conditions

RP-IPC, in which a hydrophobic stationary phase and an aqueous buffer containing a low concentration of counter ion are used, facilitates the separation of both ionized and non-ionized compounds under the same chromatographic conditions. We have already studied the separation of mono-, poly- and hydroxy-carboxylic acid hydrazides by RP-IPC [11]. Unfortunately, the internal standard 3-methylglutaric acid hydrazide, which was necessary in order to obtain good quantitative accuracy, had a similar retention time as fumaric and/or glycolic acid hydrazides in all instances.

In the present paper, we attempted the separation of ten carboxylic acid hydrazides, including 3-methylglutaric acid hydrazide, by using the new developed J'sphere ODS-M 80 column, optimizing the pH of the eluent and the size of the ion-pair reagent. A pH of 7 was chosen to convert the acidic acid hydrazides into their ionized forms. A small-size ion-pair reagent should be selected to not dominate the chromatographic behavior of the ion-pair reagent. The eluents containing quaternary alkylammonium compounds ranging from TMA to TPA were prepared by mixing known volumes of acetonitrile, methanol and aqueous phosphate buffers.

When TPA was used as the counter ion, large retention volumes were observed with a significant loss of resolution. TMA and TEA gave similar resolution, but TEA was selected as the optimum counter ion since it yielded a higher capacity factor with no loss in resolution.

The column temperature has a significant effect on the separation of various pairs of carboxylic acids and an increase from 30°C to 50°C lead to an inversion in the selectivity. By increasing the temperature separation of L-pyroglutamic and lactic and acetic acid hydrazides could be achieved, but this change also resulted in a decreased separation of tartaric and malic and succinic acid hydrazides. Vice versa, decreasing the temperature caused greater separation of these acidic acid hydrazides, but resulted in a loss of separation between lactic and acetic acid hydrazides.

Fig. 1 shows a typical chromatogram of the ten carboxylic acid hydrazides by RP-IPC analysis with phosphate buffer–acetonitrile–methanol (80:10:10, v/v) containing 0.005 *M* TEA as the isocratic eluent at 35°C and with detection in the visible region. In the chromatogram, two peaks appeared for both citric and malic acid hydrazides; these are attributes to stereochemical isomers of the derivatives.

### 3.3. Quantitative analysis

Calibration curves were constructed by derivatizing increasing amounts of carboxylic acids in the presence of 400 nmol of 3-methylglutaric acid as the internal standard and analysing as described above. The calibration test was replicated five times. From the chromatograms ob-

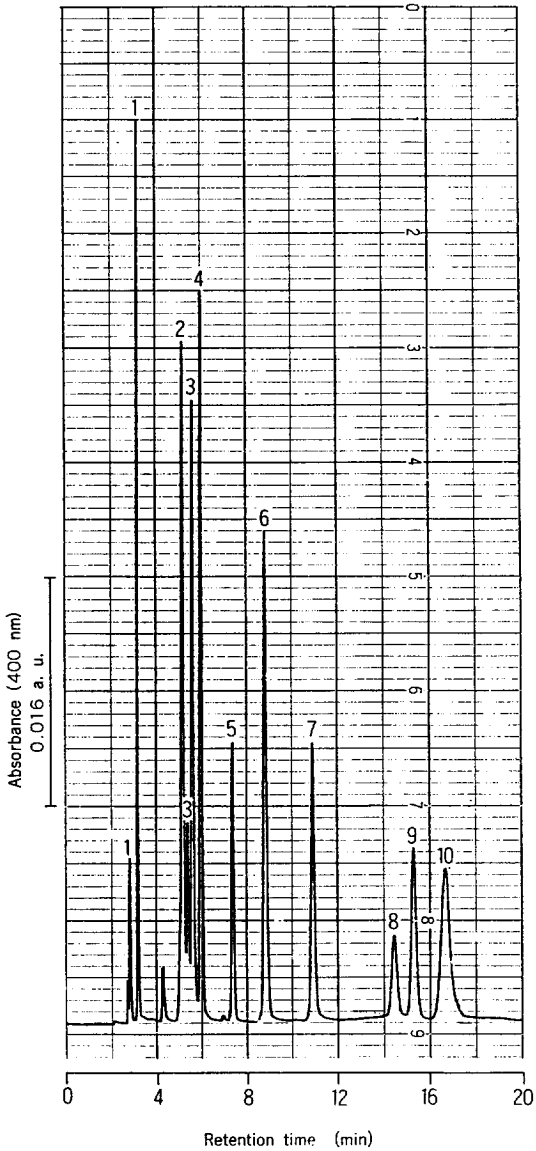


Fig. 1. Chromatogram of the 2-nitrophenylhydrazides of a standard mixture of mono-, poly- and hydroxy-carboxylic acids obtained with visible detection. Peaks: 1 = citric; 2 = tartaric; 3 = malic; 4 = succinic; 5 = fumalic; 6 = 3-methylglutaric (I.S.); 7 = glycolic; 8 = L-pyroglutamic; 9 = lactic; 10 = acetic acid hydrazide. Each peak corresponds to 1 nmol.

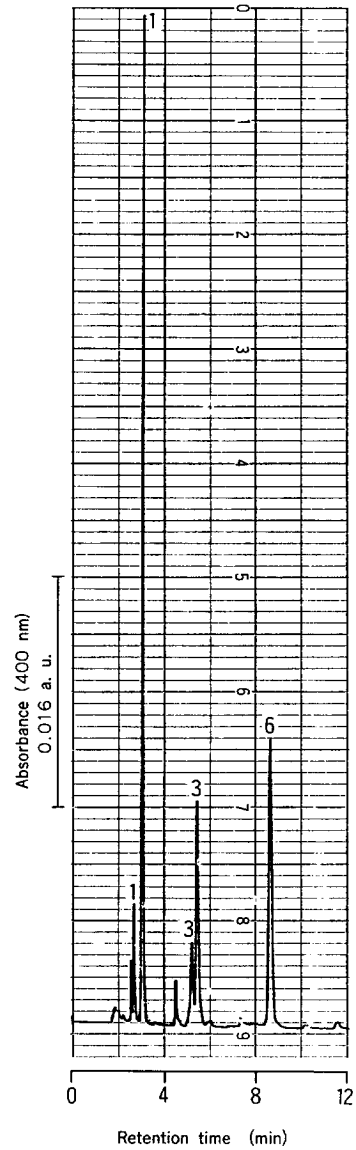


Fig. 2. Carboxylic acid profile of orange juice. For peak identification see Fig. 1.

tained, the relationships between the peak-height ratios of the acid hydrazides to that of the internal standard and the concentrations of the acids were calculated by the least-squares method. Table 1 lists the parameters and correlation coefficients of the calibration plots. Each plot was linear for 2 pmol–5 nmol per injection, and passed through the origin. The limits of detection, based on a signal-to-noise ratio of 2, were 1–4 pmol per injection.

Table 1
Parameters and correlation coefficients ($r$) of calibration plots for carboxylic acids

| Carboxylic acid | $a$ (mean ± S.D.) | $b$ (mean ± S.D.) | $r$ |
|---|---|---|---|
| Citric | 2.178 ± 0.065 | −0.118 ± 0.004 | 0.9991 |
| Tartaric | 1.393 ± 0.021 | −0.021 ± 0.001 | 0.9995 |
| Malic | 1.682 ± 0.044 | 0.042 ± 0.002 | 0.9993 |
| Succinic | 1.486 ± 0.016 | −0.014 ± 0.0003 | 1.0000 |
| Fumaric | 0.570 ± 0.019 | −0.007 ± 0.0001 | 0.9992 |
| Glycolic | 0.561 ± 0.004 | 0.010 ± 0.0003 | 1.0000 |
| L-Pyroglutaric | 0.168 ± 0.005 | 0.002 ± 0.0000 | 0.9998 |
| Lactic | 0.355 ± 0.005 | 0.008 ± 0.0004 | 0.9996 |
| Acetic | 0.308 ± 0.007 | −0.012 ± 0.001 | 1.0000 |

Calibration plots are expressed as regression lines of the form $y = ax + b$, where $y$ is the peak height ratio and $x$, the amount of acid in nmol. The calibration test was replicated five times. Linear range: 2 pmol–5 nmol.

Table 2
Analytical recovery of carboxylic acids added to red wine

| Carboxylic acid | Added: 50 nmol | | Added: 200 nmol | |
|---|---|---|---|---|
| | Recovery (%) | C.V. (%) | Recovery (%) | C.V. (%) |
| Citric | 97.5 ± 2.9 | 3.0 | 99.1 ± 2.5 | 2.5 |
| Tartaric | 100.8 ± 2.4 | 2.4 | 98.8 ± 1.5 | 1.5 |
| Malic | 98.7 ± 1.7 | 1.7 | 102.5 ± 3.7 | 3.6 |
| Succinic | 101.5 ± 0.9 | 0.9 | 100.6 ± 1.1 | 1.1 |
| Fumaric | 103.9 ± 3.2 | 3.1 | 99.5 ± 2.8 | 2.8 |
| Glycolic | 99.2 ± 1.7 | 1.7 | 102.8 ± 0.8 | 0.8 |
| L-Pyroglutaric | 100.4 ± 2.6 | 2.6 | 101.6 ± 3.3 | 3.2 |
| Lactic | 99.6 ± 1.3 | 1.3 | 98.7 ± 2.0 | 2.0 |
| Acetic | 97.8 ± 2.2 | 2.2 | 99.3 ± 1.4 | 1.4 |

Data are expressed as the mean ± S.D. ($n = 9$).

Table 3
Precision of the present method for determination of carboxylic acids in red wine

| Carboxylic acid | Intra-assay ($n = 6$) | | Inter-assay ($n = 6$) | |
|---|---|---|---|---|
| | Mean ± S.D. (g/l) | C.V. (%) | Mean ± S.D. (g/l) | C.V. (%) |
| Citric | 0.21 ± 0.004 | 1.9 | 0.19 ± 0.004 | 2.1 |
| Tartaric | 1.93 ± 0.03 | 1.6 | 1.96 ± 0.01 | 0.5 |
| Malic | 0.28 ± 0.002 | 0.7 | 0.27 ± 0.003 | 1.1 |
| Succinic | 0.67 ± 0.01 | 1.5 | 0.66 ± 0.02 | 3.0 |
| Lactic | 2.90 ± 0.08 | 2.8 | 2.94 ± 0.11 | 3.7 |
| Acetic | 0.58 ± 0.01 | 1.7 | 0.56 ± 0.01 | 1.8 |

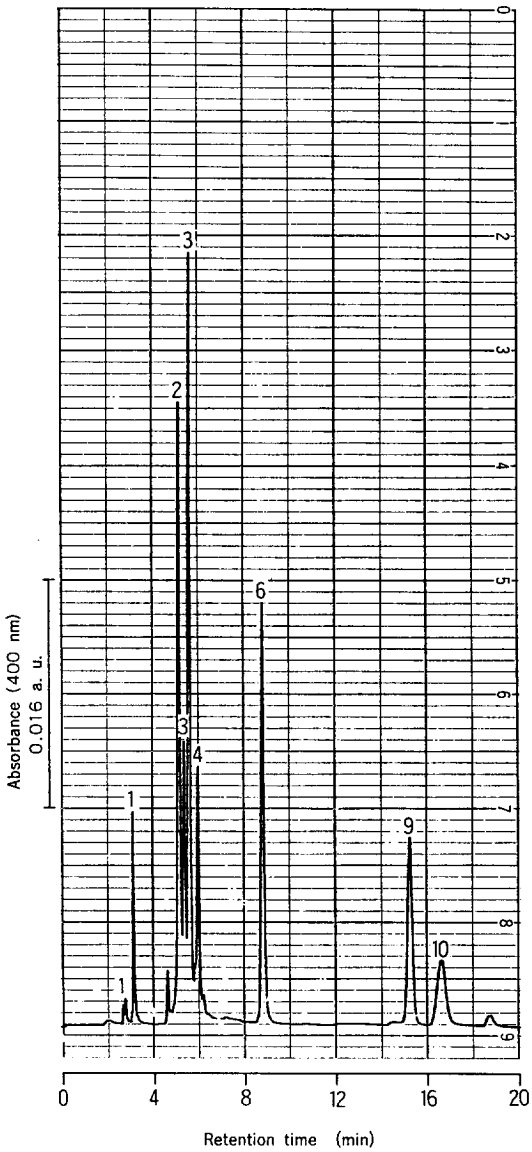


Fig. 3. Carboxylic acid profile of white wine. For peak identification see Fig. 1.

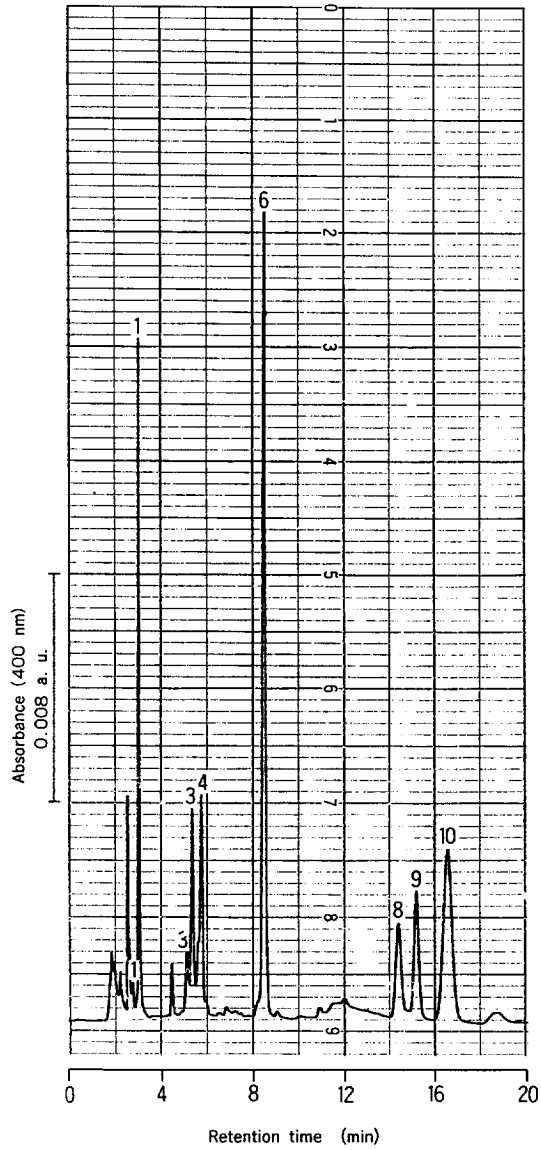


Fig. 4. Carboxylic acid profile of beer. For peak identification see Fig. 1.

### 3.4. Recovery and precision

Known amounts (50 and 200 nmol) of mixtures of the carboxylic acids were added to pooled red wine (50 $\mu$l) to examine the precision of carboxylic acid measurements across a broad range of carboxylic acid concentrations and to test the efficiency of carboxylic acid recovery in the assay procedure. Each aliquot was analysed by nine separate measurements of the carboxylic acid contents. The recoveries are corrected for the initial presence of carboxylic acids in red wine. Table 2 shows the recoveries of the carboxylic acids; the range of 97.5–103.9% is sufficient for practical applications.

The intra-assay precision was evaluated by

Table 4
Determination of major carboxylic acids in beverages by the present method

| Beverage | Carboxylic acid (g/l) | | | | | | |
|---|---|---|---|---|---|---|---|
| | Citric | Tartaric | Malic | Succinic | L-Pyroglutaric | Lactic | Acetic |
| Apple juice | 0.52 ± 0.01 | N.D. | 2.37 ± 0.04 | N.D. | N.D. | N.D. | N.D. |
| Orange juice | 5.35 ± 0.14 | N.D. | 1.34 ± 0.03 | N.D. | N.D. | N.D. | N.D. |
| White wine | 0.41 ± 0.01 | 1.28 ± 0.01 | 1.59 ± 0.02 | 0.39 ± 0.01 | N.D. | 0.91 ± 0.03 | 0.24 ± 0.01 |
| Red wine | 0.20 ± 0.01 | 1.95 ± 0.02 | 0.29 ± 0.004 | 0.66 ± 0.02 | N.D. | 2.99 ± 0.05 | 0.58 ± 0.01 |
| Beer | 0.16 ± 0.01 | N.D. | 0.05 ± 0.001 | 0.04 ± 0.001 | 0.18 ± 0.004 | 0.08 ± 0.001 | 0.08 ± 0.002 |
| Japanese "sake" | 0.14 ± 0.004 | N.D. | 0.07 ± 0.002 | 0.17 ± 0.003 | 0.27 ± 0.01 | 1.19 ± 0.03 | 0.03 ± 0.001 |

Data are expressed as the mean ± S.D. ($n = 3$). N.D.: not detectable.

assaying six times the same red wine. The inter-assay precision was determined by analysing spiked red wine on different days over one week ($n = 6$). Table 3 shows that the present method has a satisfactory precision, the intra- and inter-assay coefficients of variation being ≦2.8% and ≦3.7%, respectively. These results indicate that the present method can be used for quantitative analyses of carboxylic acids in various beverages.

### 3.5. Applicability

The present method was tested for the identification and determination of carboxylic acids in some beverages. The carboxylic acid profiles of typical orange juice, white wine and beer are shown in Figs. 2–4. The chromatograms monitored by visible absorbance showed very clean visible backgrounds, and thus the carboxylic acids in the samples were easily identified by comparison of the retention times of their hydrazides with those of standards.

The amount of each carboxylic acid was calculated from the calibration curve. The detection limits of the real samples, which were determined by diluting the real samples, were in the range of 0.5–2 μmol/l. The results of the determination of the major carboxylic acids in the beverages tested are listed in Table 4. According to the literature [5,10], apple and orange juices contain citric (0.25–8.47 g/l) and malic (0.20–7.20 g/l) acids. In wine, citric (0.10–3.80 g/l), tartaric (0.80–2.50 g/l), malic (0.06–3.73 g/l), succinic (0.10–0.76 g/l), lactic (0.10–4.50 g/l) and acetic (0.20–0.80 g/l) acids were found [5,9,10,15–17]. The values observed by us are compatible with those literature values.

## 4. Conclusions

The main carboxylic acids – e.g. citric, tartaric, malic, succinic, fumaric, glycolic, L-pyroglutamic, lactic and acetic acids – in beverages can be directly converted into their hydrazine derivatives which absorb visible radiation. The advantage of using visible detection is that the chromatograms are simpler and more selective. The RP-IPC analyses described here permit the isocratic separation of the above carboxylic acids with good accuracy, precision and sensitivity owing to the minimum sample preparation required. Despite its simplicity and speed the present method enables a remarkably long column life time and therefore the method is particularly suitable for routine determinations of carboxylic acids in foods and beverages.

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