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Detection of Exogenous Citric Acid in Fruit Juices by Stable Isotope Ratio Analysis

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A new method has been developed for measuring the D/H ratio of the nonexchangeable sites of citric acid by isotope ratio mass spectrometry (IRMS). Pure citric acid is transformed into its calcium salt and subsequently analyzed by pyrolysis-IRMS. The citric acid isolated from authentic fruit juices (citrus, pineapple, and red fruits) systematically shows higher D/H values than its nonfruit counterpart produced by fermentation of various sugar sources. The discrimination obtained with this simplified method is similar to that obtained previously by applying site specific isotopic fractionation−nuclear magnetic resonance (SNIF-NMR) to an ester derivative of citric acid. The combination of carbon 13 and deuterium measurements of extracted citric acid is proposed as a routine method for an optimum detection of exogenous citric acid in all kinds of fruit juices.

KEYWORDS: Citric acid; fruit juice; authentication; carbon 13; deuterium

INTRODUCTION

Citric acid is one of the most widely used food additives, especially in beverages (*1*). However, in Europe, under EC Directive 2001/112/EC relating to fruit juices and certain similar products, its use is strictly limited and must be mentioned in the ingredients list (*2*). Therefore, to be able to enforce this legislation, there is a need for an analytical tool capable of detecting and quantifying citric acid addition.

The traditional method for assessing whether citric acid has been added uses the ratio between citric acid and isocitric acid concentrations, but because the natural range for this ratio in fruits is very wide (*3*), fairly large additions of citric acid can go undetected. The industrial production of citric acid is based on the fermentation of cheap carbohydrate sources from plants with C3 and C4 metabolisms. As a result, the $^{13}C/^{12}C$ ratio of commercial citric acid covers the whole range of values associated with both C4 and C3 metabolisms. The isotope ratio mass spectrometry (IRMS) determination of the $^{13}C/^{12}C$ ratio has therefore been proposed as a means of detecting the addition of citric acid from C4 plant sources (cane or maize) in C3 fruits such as citrus (*4*). More recently, it has been shown that the simultaneous use of the $^{13}C/^{12}C$ ratios of citric acid and other fruit juice components such as sugars or other organic acids also determined by IRMS leads to a lower detection limit (*5*−*8*). However, the addition of citric acid produced from a plant with the same primary metabolism type as the fruit, for example, citric acid from beet molasses in citrus fruits, where both are C3 plants, has remained undetectable.

The approach based on stable isotope analysis was further complemented by the site specific isotopic fractionation−nuclear magnetic resonance (SNIF-NMR) analysis of the nonexchangeable sites of citric acid (*9*), which was shown to detect all possible sources of commercial citric acid in lemon juice, with a detection limit approximately equal to 20%. Nevertheless, this pioneering method is rather cumbersome, due to the high amount of pure citric acid required (about 2.5 g) for the analysis and the preparation of an ester derivative in order to be able to carry out the SNIF-NMR analysis of the nonexchangeable sites in suitable conditions.

To simplify this procedure, but still with the idea of using hydrogen isotopes as additional authenticity criteria, a new method based on the transformation of citric acid to its calcium salt followed by a pyrolysis-IRMS measurement has been developed. This new procedure, its validation, the results obtained on fruit-extracted and commercially available citric acid, and finally its practical application to the authenticity control of fruit juices are described in this paper. This work was performed in the context of an EU-funded project.

MATERIALS AND METHODS

Samples Description. This work is based on the study of 20 commercial citric acids and 79 citric acids extracted from fruit juices (citrus, red fruits, and pineapple). Most of the fruit juices and commercial citric acid samples included in this study were taken in fruit juice factories around the world by SGF-IRMA inspectors (SGF-IRMA is an International Raw Material Assurance system used as a self-control tool by the fruit juice industry worldwide). The samples are therefore fully representative of the products in use in today's industry. Some further commercial samples were purchased from chemical suppliers, and finally, some fruit samples from major producing countries were squeezed in the laboratory to obtain additional authentic references.

Sample Preparation. Citric acid was isolated from fruit juices using the ion exchange and preparative high-performance liquid chromatog-

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Table 1. Study of the Repeatability of D/H Results at Various Stages of the Analysis

	no. of samples	repetitions per sample	repeatability standard deviation, S_r (ppm)	repeatability limit, r (ppm)
Py-IRMS measurement	10	3	0.2	0.5
overall method (from the juice)	5	2	0.2	0.5

raphy (HPLC) steps described previously (*8*), resulting in a crystallized product similar to the commercial acid. All samples of extracted or purchased citric acid were transformed into tricalcium citrate using the following procedure. To 250 mg of pure citric acid, 1.5 mL of a 50% $CaCl_2$ solution was added. After homogenization, the pH of the mixture was adjusted to 8−9 by adding 25% $NH_4(OH)$. The solution was heated at 98 °C for 10 min in an oil bath and then kept at 4 °C overnight (in a fridge). The precipitate was spread on a glass frit and rinsed with at least 100 mL of water, until the pH dropped back to 7. The salt was then put in a drying oven at 130 °C overnight in order to remove residual water. Then the dry salt was kept in a desiccator containing some P_2O_5 and placed under vacuum until the measurement.

IRMS Determinations. Hydrogen isotope ratios were determined using a EUROPyrOH 3100 Elemental Analyzer (Eurovector, Milan, Italy) coupled to an Isoprime Isotope Ratio Mass Spectrometer (GVI, Manchester, United Kingdom). Calcium citrate acid was introduced into the elemental analyzer using silver capsules. The analytical conditions were similar to those described by Kelly et al. (*10*), except for the following point: The chromium oven was operated at 1075 °C. The D/H values were calculated with reference to V.SMOW and corrected for the H3+ contribution by a mathematical algorithm included in the IRMS manufacturer's proprietary software (MassLynx 3.6i).

Carbon isotope ratios were determined using a NA1500C-N Elemental Analyzer (Carlo Erba, Milan, Italy) coupled to a Finnigan Mat DeltaS mass spectrometer (Thermo electron corporation, Bremen, Germany). Crystallized citric acid was introduced into the elemental analyzer using tin capsules.

Calculations. The $^{13}C/^{12}C$ results are expressed on the δ ‰ scale with respect to the international standard V.PDB according to the relation:

$$\delta^{13}C\ (‰) = (R_{product}/R_{standard} - 1) \times 1000$$

where $R = {}^{13}C/{}^{12}C$.

SNIF-NMR Determinations. The derivatization of citric acid to triethyl citrate (TEC) and SNIF-NMR analysis of this compound was performed using the analytical conditions previously described by Gonzalez et al. (*9*). The corresponding results are presented as the D/H_{TEC}, previously defined (*9*).

RESULTS AND DISCUSSION

Method Validation. Thanks to the recent development of pyrolysis systems for IRMS, the on-line measurement of D/H ratios in organic compounds has recently become available as a routine method, offering the combined advantages of low sample sizes and high throughput. After studying the linearity of our instrument, the optimum mass range for calcium citrate was found to be 0.6−0.8 mg per capsule. The precision of the measurement for calcium citrate was determined by repeating the analysis three times on 10 different calcium salt samples (under repeatability conditions). The precision of the overall procedure including the extraction of citric acid was tested by repeating the whole process twice on five different orange juices (blind duplicates). The results are shown in **Table 1**. The repeatability standard deviation of the measurement and of the overall procedure are of the same magnitude, showing that the main source of uncertainty is the measurement itself. The overall repeatability of the process (maximum acceptable difference between two D/H analyses on the same juice) is 0.5 ppm.

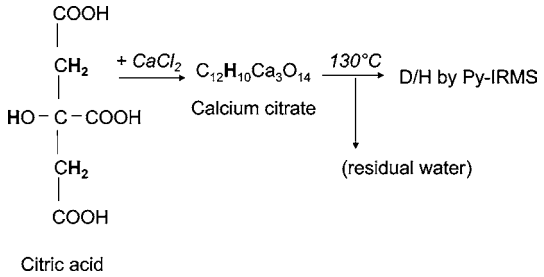

Figure 1. D/H analysis of citric acid: Principle of the IRMS method used in this work. In bold: hydrogen sites measured in the calcium salt.

Table 2. Comparison between the Results Obtained by SNIF-NMR (*9*) and by the IRMS Method Described in This Article on the Same Three Commercial Acids

sample	$D/H_{citrate\ (ppm)\ by\ IRMS}$ (this project)	$D/H_{TEC\ (ppm)\ by\ SNIF-NMR}$ (*9*)	difference (ppm)
A	149.0	146.5	2.5
B	147.8	146.9	0.9
C	147.4	146.3	1.1

One disadvantage of IRMS vs NMR is that for compounds such as citric acid, the exchangeable hydrogen sites bound to oxygen atoms are included in the overall D/H result. To limit this contribution, citric acid was transformed into its calcium salt, which eliminates the three protons of the carboxylic functions (**Figure 1**).

The final drying step of the procedure described above also eliminates the residual water trapped in the calcium salt. This was checked by gravimetric moisture determinations of the products. We then compared the results obtained by the IRMS procedure described in this paper and previous SNIF-NMR measurements (*9*) on three commercial acids. The results presented in **Table 2** show small differences considering the totally different techniques used and the fact that IRMS values were not corrected for the D/H value of the residual OH group in calcium citrate, which is exchangeable with the medium and cannot be measured accurately. Because the purpose of our work was to be able to find a similar discrimination between fruit sources and nonfruit sources as that shown by SNIF-NMR, this limited difference between the two techniques encouraged us to apply the new procedure to citric acid extracted from fruit juices.

Isotopic Pattern of Citric Acid from Various Origins. The carbon 13 deviations and D/H ratios obtained for citric acid of various origins are represented in **Figure 2** and summarized in **Table 3**. The mean $\delta^{13}C$ values reported in **Table 3** are fairly close to the mean values obtained previously on other data sets (*6*−*9*). As expected, the carbon isotope ratio separates C3 plants (citrus, soft fruits) from CAM plants (pineapple), but some partial overlap between commercial and fruit-extracted samples remains when considering this parameter on its own. On the other hand, a clear-cut discrimination is evident from the *y*-axis in **Figure 2**: All of the fruit-extracted citric acids show significantly higher D/H ratios than their nonfruit counterparts (as confirmed by p values being systematically lower than 0.01 in t-tests between each fruit and commercial sources). This can be interpreted by considering the filiations of carbon and hydrogen atoms along the glycolysis pathway and in the Krebs

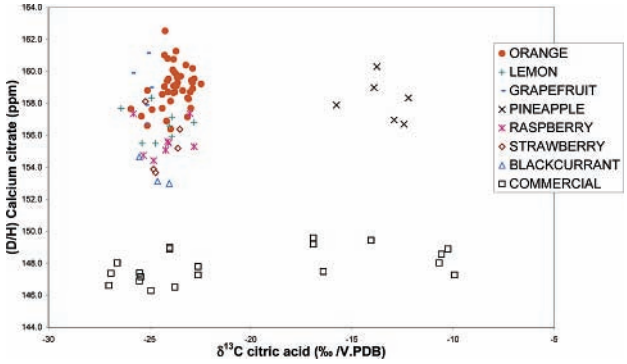

Figure 2. Bidimensional plot of the carbon and hydrogen isotopic ratios of different sources of citric acid.

Table 3. Mean and Standard Deviation of the Carbon and Hydrogen Isotopic Ratios of Different Sources of Citric Acid Analyzed by the IRMS Procedure Described in the Materials and Methods

origin of citric acid	no. of samples		$\delta^{13}C$ (‰)	D/H (ppm)
orange	42	mean	−23.8	159.0
		SD	0.7	1.3
lemon	10	mean	−24.8	156.7
		SD	1.1	1.0
grapefruit	5	mean	−25.3	158.9
		SD	0.3	1.7
pineapple	6	mean	−13.5	158.2
		SD	1.3	1.3
raspberry	8	mean	−24.3	155.7
		SD	1.0	1.1
strawberry	5	mean	−24.4	155.5
		SD	0.8	1.8
black currant	3	mean	−24.7	153.6
		SD	0.6	0.9
commercial C3	12	mean	−25.0	147.5
		SD	1.6	0.9
commercial other (C4 and C4+C3)	8	mean	−13.2	148.6
		SD	3.2	0.9

cycle. It appears that citric acid derives from all glucose carbon atoms and that two-thirds of the nonexchangeable hydrogen of the two methylene sites come from water of the medium. The ^{13}C deviations therefore reflect the primary metabolism of the sugars used as a carbon source, either by the fruit-bearing plant or by the microorganisms used in industrial processes. On the other hand, the well-known deuterium enrichment in plant water vs groundwater results in a significant enrichment of the deuterium content of citric acid in all fruits as compared to commercial samples.

The results of **Table 3** indicate that higher values are observed for citric acid from citrus and pineapple, while red fruits (raspberry, strawberry, and black currant) tend to have slightly lower values (albeit higher than industrial sources). This can be linked to the differences observed in the isotopic content of water from the same fruits: Because of rather limited evapotranspiration rates, red fruits tend to bear lower isotopic values than citrus and pineapple (*11*). On the other hand, no strong correlation has been observed between D/H and the D/H of the fruit water (nor with the concentration of citric acid), so that it seems impossible to predict the D/H value from other parameters in the juice.

Detection of Adulterations. The ability of the developed method to detect an addition of citric acid was further confirmed by spiking an orange juice with known amounts of citric acid (representing 10, 15, 30, and 50% of the total citric acid content). The spiked juices were analyzed for D/H of calcium citrate acid

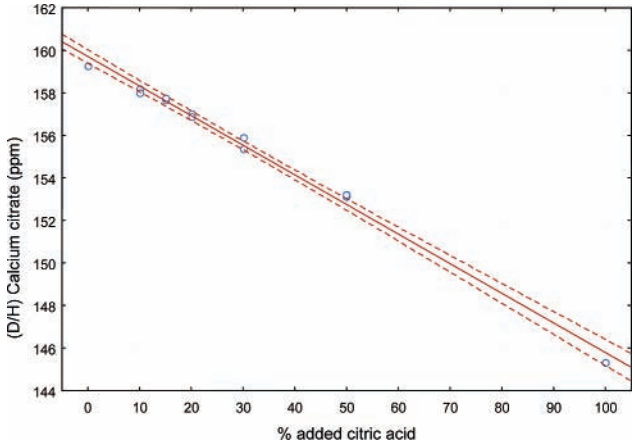

Figure 3. Spiking experiments performed by adding known amounts of nonfruit citric acid to an authentic orange juice. The continuous and dotted lines correspond respectively to the calculated regression line and corresponding 95% confidence range.

using the procedure described above. The results of these experiments are presented in **Figure 3**, showing a linear decrease of the D/H ratio when increasing amounts of nonfruit citric acid are present in the juice (correlation coefficient $r = -0.9965$). Conversely, the proportion of exogenous citric acid in an unknown sample can be estimated by linear interpolation using the average D/H values for a given fruit on one hand and for commercial citric acid on the other hand.

The detection limit in a given type of fruit juice will depend on the natural distribution of D/H ratios in this type of juice. In the case of orange, taking into account the mean values of **Table 3**, this limit typically lies around 20% (of the total citric acid) for C3 plants sources in C3 fruits. In the case of citric acid from a plant having a different metabolic type, the detection threshold will be lower (typically around 10−15%) thanks to the complementary information brought by the multicomponent ^{13}C profile of the sample (*8*).

The combined use of global $^{13}C/^{12}C$ and positional $^{2}H/^{1}H$ ratios allows a satisfactory discrimination between citric acid from fruits on one hand and all commercial sources of this acid on the other hand, which represents the most powerful way to detect undeclared citric acid addition in fruit juice. The fruits included in the study (citrus, red fruits, and pineapple) were chosen among the common fruits having the highest concentrations of citric acid and, therefore, the highest potential risk of adulteration. Provided that a suitable database is available, the same approach will be applicable to any kind of fruit juice containing citric acid.

Most importantly, the procedure developed during this project can be considered as a potential routine method in control laboratories equipped with suitable IRMS machines. The next step of the running project will consist in the validation of the interlaboratory reproducibility of the method, to demonstrate its ability to become a standard procedure of fruit juice authenticity control.

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