

Authentication of Lemon Juices and Concentrates by a Combined Multi-isotope Approach Using SNIF-NMR and IRMS

Javier Gonzalez,[†] Eric Jamin,[†] Gérald Remaud,^{*,†} Yves-Loïc Martin,[‡] Gilles G. Martin,[†] and Maryvonne L. Martin[§]

Eurofins Scientific, Site de la Géraudière, B.P. 42301, 44323 Nantes Cedex 3, France, LAIEM, Université de Nantes, CNRS, 2 rue de la Houssinière, B.P. 92208, F-44322 Nantes Cedex 3, France, and ENI Laboratories Inc., 2394 Route 130, Dayton, New Jersey 08810

A methodology for measuring the natural deuterium distribution in citric acid by the SNIF-NMR method is presented. Triethyl citrate (TEC) is retained as a molecular probe for ²H NMR. The method provides efficient criteria for characterizing the authenticity of lemon juices: it is a powerful approach to detect the addition of exogenous citric acid manufactured from beet sugar (or other C3 plants). The whole analytical chain includes the separation and purification of citric acid from lemon juices or concentrates via preparative HPLC, followed by the synthesis of TEC. The present procedure may also integrate a determination of the ¹³C contents of sugars, citric acid, and L-malic acid. The correlation between $\delta^{13}\text{C}$ values of sugars and of both acids is very specific and reinforces the discriminating power of the SNIF-NMR method.

Keywords: Citric acid; triethyl citrate; SNIF-NMR; malic acid; sugars; lemon juices; lemon concentrates; $\delta^{13}\text{C}$ profile; preparative HPLC; adulteration; authentication

INTRODUCTION

Among the existing range of analytical tools applied to the characterization of foods and beverages, isotope analyses have been proved to provide powerful authentication criteria. The SNIF-NMR method (a trademark of Eurofins Scientific, Nantes, France) uses nuclear magnetic resonance spectroscopy to measure ²H/¹H (D/H) ratios at specific sites of a given molecule (Martin and Martin, 1981). In the case of wines and fruit juices, in particular, the method can be used to detect undeclared addition of sugar as beet sucrose or modified sugar syrups to the juices or concentrates (Martin et al., 1991, 1996a,b). The isotope ratio mass spectrometry technique (IRMS), which measures overall molecular isotope ratios of different elements, provides additional authenticity indicators. The ¹³C content is especially efficient to distinguish organic products originating from C3 and C4 plants (O'Leary, 1988). Thus, addition of sweeteners can be detected in fruit juices (Bricout, 1982) and in honey (White and Winters, 1989), for instance, and the carbon isotope ratios can be used for characterizing vinegars (Remaud et al., 1992). Similarly, IRMS determination of the D/H and ¹⁸O/¹⁶O ratios of water of juices not made from concentrate provides useful criteria for detecting possible addition of water (Cohen and Saguy, 1984). In the case of fruit concentrates these parameters have also been used for characterizing adulteration by sugar syrup (Doner et al., 1992; Yuni-anta et al., 1995; Rossmann and Trimborn, 1996).

Isotopic methods can be applied to ingredients other than sugars present in fruit juices, that is, organic acids,

vitamins, polyols, etc. Such molecules not only are potentially good probes for detecting addition of the same chemical species but may also provide useful internal isotopic references. In this respect several internal referencing strategies using the IRMS technique have already been proposed (Gensler and Schmidt, 1994; Rossmann et al., 1995). A similar approach can be efficient in the SNIF-NMR methodology, and we shall consider the aptitude of citric acid to provide a hydrogen isotopic reference for lemon juices and concentrates.

As the cost of lemon juice is mostly based on the quantity of citric acid and sugar present in the juice, the main adulterations of this product consist in adding citric acid and/or sugar from a less costly origin. Compositional analyses, based on the definition of admissible ranges of variation for several metabolites naturally present in lemon juice, have been proposed to detect such frauds (Faethe et al., 1990; Association of German Fruit Juice Industries, 1993). However, the ranges of variation are usually large enough to allow substantial addition of the main components without having to adjust the relative quantities of the minor components (for instance, citric acid versus isocitric acid). Until now, only the adulteration by citric acid derived from C4 plants (cane or corn) was detectable using isotopic analyses based on $\delta^{13}\text{C}$ determinations (Doner, 1985). Recently, we have introduced the SNIF-IRMS method (specific natural isotopic profile measured by isotope ratio mass spectroscopy) for routine analysis on fruit juices. This strategy involves the measurement of the ¹³C contents of pure fractions of sugars and of citric and malic acids. Such ¹³C profiles of fruit juice metabolites have been determined for apple (Jamin et al., 1997a), pineapple (Jamin et al., 1997b), and orange and mandarin (Jamin et al., 1998). Correlations between $\delta^{13}\text{C}$ of sugars, citric acid, and malic acid were found to be specific for each fruit. This technique

* Author to whom correspondence should be addressed (fax 33 2 51 83 21 11; e-mail GeraldRemaud@Eurofins.com).

[†] Eurofins Scientific.

[‡] ENI Laboratories, Inc.

[§] LAIEM, Université de Nantes.

improves the detection threshold for the addition of ingredients from C4 plants (or C3 plants in the case of pineapple). We present herein the SNIF-IRMS results obtained for lemon. The limitation encountered in the detection of molecules originating from C3 plants prompted us to explore the application of the SNIF-NMR methodology to the characterization of citric acid. In the present work we especially address the problem of addition of citric acid manufactured from C3 plants such as beet, potato, wheat, and rice. The proposed methodology involves an optimized procedure for extraction and purification of citric acid from fruit juices and the choice of a derivative appropriate to quantitative ^2H -NMR determinations. The combined determinations of site-specific isotope ratios of citric acid by SNIF-NMR and of the ^{13}C fingerprint of sugars and acids are the basis for a routinely applicable authentication method of lemon juices and concentrates.

MATERIALS AND METHODS

Origin of the Samples. This work is based on the study of >100 different samples of citric acid from different origins. The commercial sources of citric acid are sugar derivatives: cane and corn (C4 plants); beet, potato, wheat, rice, sweet potato, and tapioca (manioc) (C3 plants). Some commercially available L-malic acid samples were also analyzed. Pure lemon juices were obtained from fresh lemons squeezed in the laboratory. The fruits were mainly from Argentina, Italy, and Spain, but samples from Brazil, Greece, Israel, Mexico, Turkey, Uganda, and Venezuela were also investigated. Authentic concentrates from Argentina were compared, and some commercially available lemon juices or lemon concentrates were analyzed to test the method.

Chemicals and Supplies. A Lichroprep C-18 (40–63 μm) was purchased from Merck (Merck Clevenot, Nogent-sur-Marne, France). Cation-exchange resin AG 50Wx8 (200–400 mesh, H^+ form) and anion-exchange resin AG1x2 (200–400 mesh, Cl^- form) were obtained from Bio-Rad (Bio-Rad SA, Ivry-sur-Seine, France). Glass columns (3 cm i.d.) were filled respectively with 50 g of cation-exchange resin, 150 g of anion-exchange resin, and 40 g of C-18.

Chromatographic Equipment. The chromatographic system consisted of an L-6250 Merck pump, a 7725i Rheodyne injector, and an Iota2 RI detector from Precision Instruments. The type of column used for preparative chromatography was a Lobar Lichroprep RP-8 size 6 (440 \times 37 mm) with 40–63 μm packing (Merck Clevenot).

Sample Preparation. The overall ^{13}C contents of organic acids can be measured on their calcium salts. However, in the precipitation of the latter, citric acid is contaminated with malic acid. Moreover, two important requirements must be fulfilled: (i) great care should be taken when the matrix contains high concentrations of sugars (Brause, 1995) and (ii) high grade calcium oxide should be used to ensure that no carbon dioxide has been trapped during its industrial production. Consequently, we have preferred to resort to separation and purification of citric acid by preparative HPLC.

The whole procedure for isolation of pure L-malic acid, citric acid, and sugars from lemon juices is described in Figure 1. Lemon juices were centrifuged (5000g, 10 min) and filtered. An appropriate amount of the filtrate (equivalent to ~ 4 g of citric acid) was introduced into the C-18 column previously conditioned with 100 mL of methanol and 100 mL of water. Nonpolar ingredients were retained, and the polar fraction was eluted with 200 mL of water. The complete eluate was concentrated, adjusted to pH 2.0, and submitted to the cation-exchange resin (previously conditioned at the same pH). Amino acids were retained, and elution was carried out with 400 mL of 10 mM HCl. The complete eluate was concentrated, adjusted to pH 7–8, and submitted to the anion-exchange resin (previously conditioned at the same pH). The sugars removed by washing with 1 L of water were ready for ^{13}C analysis, after

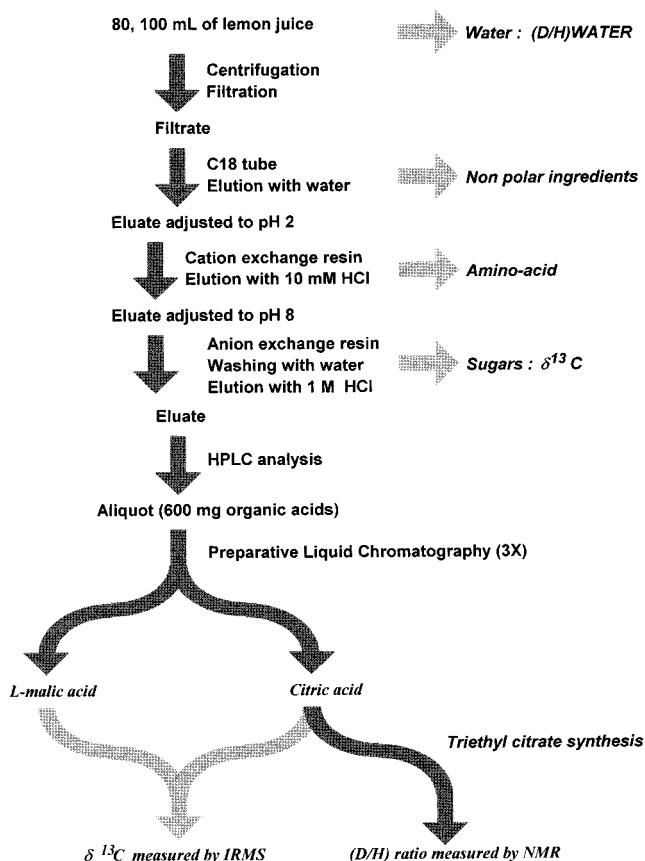


Figure 1. Flow chart of the preparation steps proposed for the determination of the isotopic fingerprint of lemon juices.

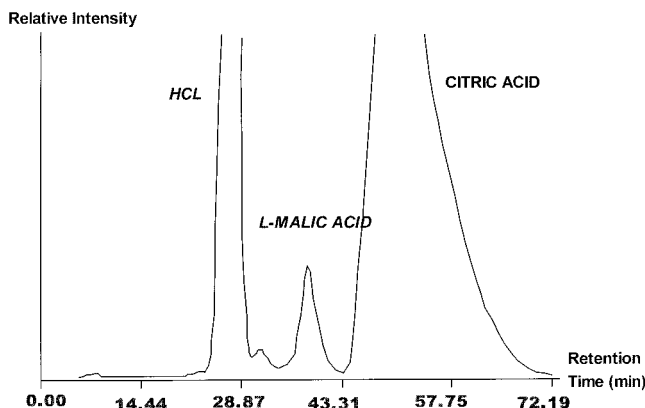


Figure 2. Chromatographic profile of lemon juice obtained in preparative HPLC (loading of about 600 mg of organic acids).

concentration. The organic acids were then eluted with 1.3 L of 1 M HCl. The eluate was concentrated to 100 mL, and its L-malic and citric acid contents were determined by enzymatic titration (using IFU methods 21 and 22; AIJN, 1993). The recovery yield for both L-malic and citric acid at this stage should be >95%. To avoid signal overlap with L-malic acid, the maximum amount of citric acid injected into the preparative column was 600 mg. Four injections per sample were therefore required to obtain ~ 2.5 g of pure citric acid. Elution was performed with 5.8 mM HCl (10 mL/min) at ambient temperature, and the fractions corresponding to the organic acids peaks were collected separately (Figure 2). Each fraction containing pure acid was concentrated under vacuum before ^{13}C determination by IRMS. Residual HCl was eliminated by air blowing. Citric acid powder could finally be obtained by recrystallization in acetone and addition of cold cyclohexane.

The esterification of citric acid to triethyl citrate (TEC) was performed in a Dean–Stark apparatus, according to a general

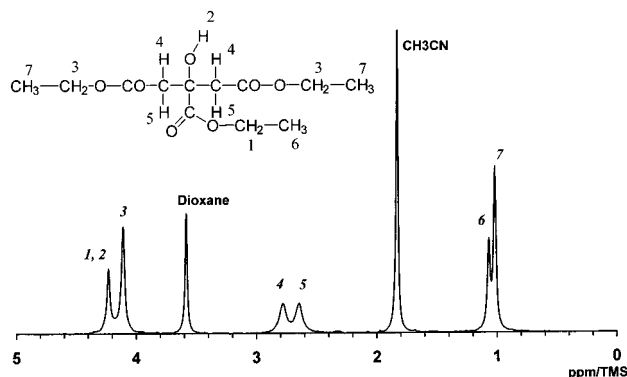


Figure 3. Deuterium NMR spectrum of TEC in a mixture of CH_3CN and dioxane. The dioxane signal is used as isotopic reference. The spectrum was run on a Bruker AM 500 instrument. Signals denoted 4 and 5 are associated with the two enantiomeric pairs of isotopomers $2_S + 4_R$ and $2_R + 4_S$ (see Figure 4).

procedure (March, 1992). Water formed during the reaction was eliminated. The azeotropic distillation of water was achieved using toluene. *p*-Toluenesulfonic acid was added in catalytic amounts. The reaction was complete after 6 h, and the organic solvents were then evaporated under vacuum. TEC was purified by means of silica gel chromatography. For ~2.5 g of TEC, 50 g of silica gel 60 A 70–200 μm (SDS, Peypin, France) was used. TEC was eluted by using a gradient of pentane/dichloromethane (from 100 to 0% of pentane). The composition of each fraction collected was controlled by gas chromatography. The fractions containing TEC were gathered and desiccated over Na_2SO_4 , and the organic solvents were evaporated under vacuum. TEC was obtained as a pure liquid (purity >99%), with a yield >98%.

Quantitative ^2H NMR on TEC. A Bruker AM500 spectrometer was used for the ^2H -NMR experiments. The spectra (76.77 MHz) were recorded at 308 K using a 10 mm o.d. deuterium probe, broad band decoupling, and ^{19}F locking (provided by C_6F_6). The quantitative measuring conditions were the following: 90° pulse (13 μs), repetition delay of $5T_{1\text{max}}$ (5 s), sweep width of 4800 Hz, time domain size 32K, and acquisition time of 3.4 s. The chemical shifts are expressed in parts per million (ppm) with respect to tetramethylsilane (TMS). A mixture of acetone- d_6 and dioxane was used as solvent to optimize chemical shift separation in the deuterium NMR spectrum of TEC. Dioxane also provides an internal isotopic reference (Martin and Martin, 1995) (Figure 3). This secondary reference was calibrated with respect to certified tetramethylurea (TMU) purchased from the European Union Institute for Reference Materials and Measurements (Geel, Belgium). The two signals corresponding to the methylenic hydrogens are separated enough to allow satisfactory integration using Eurospec-Liss software (Martin, 1994). The isotopic ratios, $(\text{D}/\text{H})_i$, expressed in ppm, are mean values over at least three spectra. With ~1.4 g of pure TEC, the spectrometer time was <8 h for recording three spectra with a signal-to-noise ratio >150 for the reference signal.

IRMS Measurements. The protocol for measurement of $\delta^{13}\text{C}$ of the organic materials has been adapted from that of Koziet et al. (1993). The carbon isotope ratios were determined using a Finnigan Mat DeltaE mass spectrometer fitted with a Carlo Erba NA1500C-N elemental analyzer. All $\delta^{13}\text{C}$ values are referred to the PDB standard on the δ ‰ scale.

RESULTS AND DISCUSSION

Isotopic Probe for the Study of Site-Specific Hydrogen Fractionation in Citric Acid. Citric acid itself is not a favorable probe for the SNIF-NMR method. It is sufficiently soluble in water only, and its deuterium NMR signals are broad. Sharper signals are

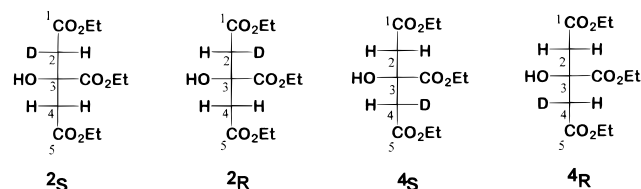


Figure 4. Monodeuteriated isotopomers of TEC. Isotopomers 2_S , 2_R , 4_S , and 4_R (stereochemical numbering) are distinguished on a genealogical basis since the two prochiral methylenic groups originate from different precursor atoms in the biosynthetic pathway. Et stands for the group CH_2CH_3 . Signals denoted 4 and 5 in Figure 3 are associated with the two enantiomeric pairs of isotopomers $2_S + 4_R$ and $2_R + 4_S$.

obtained above 70 $^\circ\text{C}$, but high-temperature conditions are not suitable for routine experiments. Moreover, pure crystals of citric acid, free of water, are not easily obtained. Therefore, we have searched for a chemical derivative of citric acid that fulfills the following conditions: (i) it has to be easy to prepare (simple purification procedure, good yield); (ii) it has to be produced in a repeatable manner without (or with strictly controlled) isotopic fractionation; (iii) its ^2H -NMR characteristics (sensitivity, integration of the peaks, internal reference) should be suitable for routine analyses.

To accelerate the low molecular dynamics encountered with citric acid, an increase in the number of CH groups with respect to the number of oxygen atoms has been considered. By becoming more hydrophobic, the corresponding derivatives are expected to exhibit sharper ^2H -NMR signals when diluted in organic solvents. Among the easily obtained ester derivatives, trimethyl citrate is not convenient because of its high viscosity and insufficient chemical shift separation between the methyl and methylene signals of the citric fragment. TEC, which is commercially available, offers the advantage of being very stable and relatively well adapted to quantitative ^2H NMR. Tetramethylurea (TMU), the usual and official reference for SNIF-NMR analyses, cannot be used in the case of TEC because of signal overlap. Therefore, a new reference had to be found. Dioxane exhibits a unique signal, well separated from those of TEC (Figure 3). Its deuterium content was calibrated with respect to TMU by means of ^2H NMR. The sample used in our experiments has a D/H ratio of 148 ppm. To further confirm this value, a ^2H -NMR analysis of benzaldehyde previously performed with the TMU standard (Remaud et al., 1997) has been repeated with dioxane from the same pool as a secondary reference. The NMR results obtained in the two series of experiments are in close agreement.

Unfortunately, due to symmetry degeneracy only two peaks, 4 and 5 (Figure 3), are observed for the four hydrogen positions 2_S , 2_R , 4_S , and 4_R on the methylenic carbons 2 and 4 of the citric skeleton (Figure 4). The isotopic ratios $(\text{D}/\text{H})_4$ and $(\text{D}/\text{H})_5$ therefore represent isotopic behaviors averaged over the $2_S + 4_R$ and $2_R + 4_S$ genealogies. Partial overlap of signals 4 and 5 is somewhat detrimental to the accuracy of the $(\text{D}/\text{H})_4$ and $(\text{D}/\text{H})_5$ determinations. More accurate values, $(\text{D}/\text{H})_{\text{TEC}}$, can be obtained by least-squares curve fitting of the whole 4,5 pattern, but it is at the price of further reduction in the specificity of the biochemical information. It should be noted that the chemical shift degeneracy of positions $2_S, 4_R$ and $2_R, 4_S$ can be resolved in ^1H NMR by interaction with chiral partners (Anet and Park, 1992). However, because of the smaller discrimi-

Table 1. Mean and Standard Deviation of the ^2H -NMR Parameters of TEC Prepared from Citric Acid Extracted from Authentic Lemon Juice Samples from Greece (a) and from Spain (b)^a

sample	trial no.	(D/H) ₄ (ppm)	(D/H) ₅ (ppm)	(D/H) _{TEC} (ppm)
(a) Greece	1	166.6	160.5	163.6
	2	167.5	157.6	162.6
	3	168.3	159.8	164.0
	mean	167.5	159.3	163.4
	SD	0.5	1.5	0.7
(b) Spain	1	159.9	157.8	158.9
	2	160.3	160.3	160.3
	3	161.0	157.6	159.3
	mean	160.4	158.6	159.5
	SD	0.6	1.5	0.7

^a The whole experimental procedure, described in Figure 1, was applied to samples a and b by two different operators.

nating potential of deuterium, this attractive strategy is not transposable to ^2H NMR.

Evaluation of the SNIF-NMR Method. To detect possible weakness of a given step of the procedure, we have evaluated separately the repeatability of ^2H -NMR quantitative analysis, of the synthesis of TEC, and finally of the extraction and purification of citric acid from lemon juices. Standard deviations of the ^2H -NMR determinations on TEC were estimated by recording, over 1 year, 42 different experiments, involving three spectra of the same commercial TEC sample. They reach 0.90, 0.95, and 0.60% for (D/H)₄, (D/H)₅, and (D/H)_{TEC}, respectively. Similar performances are obtained when the esterification (TEC synthesis) is performed 10 times on a sample of pure anhydrous citric acid, since the standard deviations are 1.00, 1.00, and 0.60% for (D/H)₄, (D/H)₅, and (D/H)_{TEC}, respectively. Finally, two lemon juice samples (from Greece and Spain) were subjected to three independent preparations according to the scheme described in Figure 1 by two different operators (Table 1). All of these results show that the present methodology is reliable.

Carbon Isotope Parameters. *Sugars and Malic and Citric Acids from Authentic Lemon Juices and Concentrates.* As discussed above, the ^{13}C content of a given compound is a good indicator of addition of the same molecular species originating from C4 plants. This discrimination is potentially improved by determining the correlation between the $\delta^{13}\text{C}$ values of the different metabolites. Therefore, we have investigated the carbon isotope ratios of sugars, citric acid, and malic acid of >70 authentic lemon samples, according to the procedure described in Figure 1. The IRMS methodology for $^{13}\text{C}/^{12}\text{C}$ measurement on different metabolites of a fruit juice has been already evaluated elsewhere (Jamin et al., 1997a,b, 1998). Mean values and standard deviations of the carbon isotopic parameters of sugars, $\delta^{13}\text{C}_s$,

citric acid, $\delta^{13}\text{C}_c$, and malic acid, $\delta^{13}\text{C}_m$, characterizing samples from most commercially relevant geographical areas, are collected in Table 2. Values of differences between these three molecular parameters have also been computed. The standard deviations of these differences are lower than those calculated for each individual component. This behavior confirms the existence of an isotopic correlation between organic acids and sugars and enables characteristic patterns of ^{13}C distribution between sugars, malic acid, and citric acid in lemon juices to be established. Similar behaviors, but with different patterns, have been observed for other fruits, such as apple (Jamin et al., 1997a), pineapple (Jamin et al., 1997b), and orange and mandarin (Jamin et al., 1998). When compared to these others fruits, lemon exhibits a remarkably high level of ^{13}C enrichment of the malic acid component. The minimum and maximum values of $\delta^{13}\text{C}$ illustrate the large natural dispersion of carbon isotope ratios in lemon juices. With a view to improve the authentication potential of the carbon isotope parameters, cutoff points can be proposed for the differences between the $\delta^{13}\text{C}$ values of sugars, s, malic acid, m, and citric acid, c. The minimum cutoff values retained are $\delta c_s - \delta c_m = -3.5\text{‰}$, $\delta c_s - \delta c_c = -0.2\text{‰}$, and $\delta c_m - \delta c_c = 1.1\text{‰}$. The corresponding maximum values are -0.2 , $+1.8$, and $+4.0\text{‰}$, respectively. The results on authentic samples presented in Table 2 being representative for a large number of production areas around the world, it can be supposed that all of the unadulterated commercial samples will fall within the $\delta^{13}\text{C}$ values intervals centered on the whole population mean plus or minus twice the standard deviation observed when the samples reported in this paper are analyzed. The cutoff points, given above, have been defined using this statistical approach. It should be emphasized that even the extreme experimental values of the differences between two parameters are comprised within the cutoff point intervals. The systematic differences between $\delta^{13}\text{C}_s$, $\delta^{13}\text{C}_m$, and $\delta^{13}\text{C}_c$ (Table 2) provide improved authenticity criteria with respect to individual parameters. It should also be noted that the standard deviations are smaller for concentrates than for isolated batches obtained by pressing 1 kg of fruits from the same production area. Such averaging effects of industrial processing have been discussed previously (Martin et al., 1996b; Jamin et al., 1997b, 1998).

Commercial Malic and Citric Acids. No citric acid from chemical synthesis is commercially available. Since this acid is currently produced from sugar derivatives, via biotechnological processes (Federici and Petruccioli, 1997), its $\delta^{13}\text{C}$ value reflects the ^{13}C content of the plant precursors. Thus, citric acids from C4 plants (corn or cane) exhibit high $\delta^{13}\text{C}$ values (from -9.8 to -13‰),

Table 2. Mean and Standard Deviation Values of Hydrogen and Carbon Isotope Parameters of Different Components Extracted from Lemon^a

	D/H ratios (ppm)				carbon isotope parameters $\delta^{13}\text{C}$ (‰)											
	triethyl citrate				sugar		malic acid		citric acid		differences					
	(D/H) ₄	(D/H) ₅	(D/H) _{TEC}	NS ^b	Cs	NS	CM	NS	CC	NS	Cs - Cm	NS	Cs - Cc	NS	Cm - Cc	NS
mean	162.2	158.8	160.6	70	-24.7	65	-22.9	84	-25.5	84	-1.8	55	0.8	55	2.5	82
SD	2.9	3.3	2.3		1.2		0.9		1.2		0.8		0.5		0.7	

^a Various production areas have been considered: lemon from Argentina, Spain, Italy, and Eastern Mediterranean countries (Israel, Greece, and Turkey); limes from South America (Mexico, Brazil, and Venezuela). The following parameters have been determined: hydrogen isotope ratios of TEC, (D/H)₄, (D/H)₅, (D/H)_{TEC}; carbon isotopic deviations, $\delta^{13}\text{C}$ for sugars (Cs), malic acid (Cm), citric acid (Cc); and differences between the $\delta^{13}\text{C}$ values of sugars and of malic acid (Cs - Cm), of sugars and of citric acid (Cs - Cc), of malic acid and of citric acid (Cm - Cc). ^b NS is the number of samples actually used for the calculation of means and standard deviations, SD.

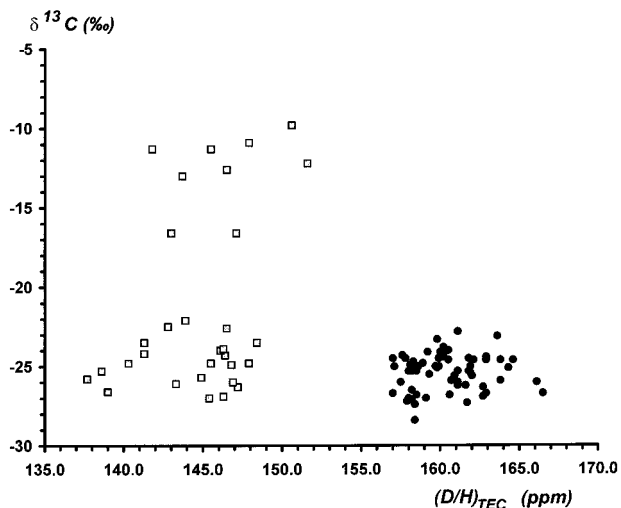


Figure 5. Representation of citric acids in the plane of the isotopic parameters $(D/H)_{TEC}$ of TEC and $\delta^{13}C$ of citric acid. Citric acids corresponding to industrial biosynthesis (squares) are clearly differentiated from authentic citric acids from lemons (circles).

whereas citric acids originating from C3 plants (mainly, beet, potato, wheat, rice, sweet potato, and manioc) are characterized by $\delta^{13}C$ values comprised between -23.5 and -26.6 ‰. Intermediate values are also found when mixtures of C3 and C4 raw materials are used in the production line. Fewer sources are commercially available for malic acid, which is usually produced by chemical reaction (Miles-Prouten, 1994). Consequently, relatively low $\delta^{13}C$ values are expected for commercial malic acids. In a previous study, no commercial sample with a $\delta^{13}C$ deviation > -23 ‰ was found (Jamin et al., 1997b).

Hydrogen Isotope Parameters. *Authentic Lemon Juices and Concentrates.* The mean values and standard deviations of $(D/H)_4$, $(D/H)_5$, and $(D/H)_{TEC}$ of TEC synthesized from citric acid extracted from authentic lemon juices and concentrates are gathered in Table 2. In the limit of repeatability of the 2H -NMR determinations performed on the individual sites 4 and 5 (Table 1), it may be concluded that the deuterium content of site 4 seems only slightly higher than that of site 5. The dispersion of $(D/H)_{TEC}$, which is obtained with a better precision, is rather large. No clear distinction in terms of geographical origin is provided by this parameter except in the case of lemon juices from Israel and Greece, which are characterized by significantly higher $(D/H)_{TEC}$ values than juices from other countries.

Commercial Citric Acids. The mean values of $(D/H)_{TEC}$ measured on TEC synthesized from commercial citric acids are 144.2 ± 3.4 ppm (19 samples) for C3 plant precursors and 146.8 ± 3.2 ppm (7 samples) for a C4 origin. In contrast with the situation exhibited by other molecules such as sugars (Martin et al., 1991), no significant difference is observed between the two main metabolisms, C3 and C4 (Figure 5). The deuterium content of citric acid biogenerated from sugar derivatives remains close to that of the tap or distilled water, which was used for the fermentation medium.

Detection and Quantification of Adulteration of Lemon Juices. A given lemon juice (from Greece) has been submitted to progressive spiking with commercial anhydrous citric acid (from beet), and the whole experimental procedure described in Figure 1 was carried out. As shown in Figure 6 the results are quite satisfactory.

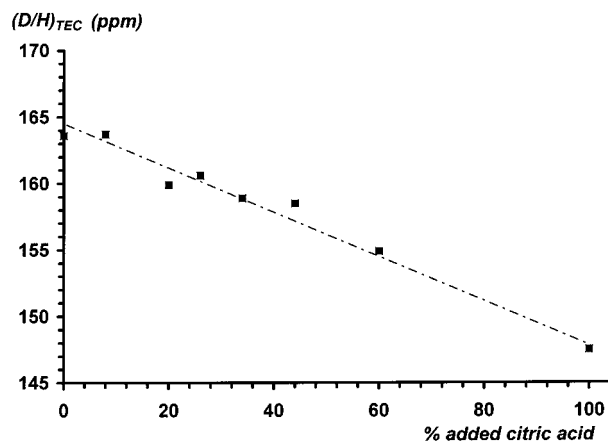


Figure 6. Spiking experiments performed by successive additions of commercial citric acid to an authentic lemon juice. The whole experimental procedure described in Figure 1 has been applied to all test points. The skew dotted line represents the average experimental behavior as a function of the amount of added commercial citric acid.

The dispersion of the values remains within the reproducibility of the experimental determination. On the sole basis of the 2H -NMR results (Figure 6) and using the reproducibility range (see above), the limit of detection of adulteration at a confidence level of 95% reaches $\sim 15\%$, when the origin of the lemon juice is known. In the case of unknown lemon juices or concentrates this limit is more difficult to appraise (15–30%, depending on the deuterium content before addition of citric acid). As already observed (Martin et al., 1996b; Jamin et al., 1997b, 1998), the dispersion of analytical values is decreased in the case of concentrates and the detection limit remains close to 15%. More generally, a multi-isotope strategy, that is, a combination of the SNIF-NMR and the SNIP-IRMS methods, improves significantly, in all cases, the detection limits of adulteration.

Conclusion. An efficient methodology has been developed to measure the $^{13}C/^{12}C$ ratios of sugars, citric acid, and malic acid extracted from lemon juices. Correlations have been observed between the $\delta^{13}C$ values of these components, whatever the origin of lemons. Consequently, with a view to improve the authentication potential of the carbon isotope parameters, cutoff points can be proposed for the differences between the $\delta^{13}C$ values of sugars, s, malic acid, m, and citric acid, c. The minimum cutoff values retained are $\delta c_s - \delta c_m = -3.5$ ‰, $\delta c_s - \delta c_c = -0.2$ ‰, and $\delta c_m - \delta c_c = 1.1$ ‰, and the corresponding maximum values are -0.2 , $+1.8$, and $+4.0$ ‰, respectively. Since methods based on the sole determination of ^{13}C can detect only the addition to lemon juices of molecules originating from C4 plants, complementary criteria are necessary to characterize citric acid produced from C3 plants such as beet. An analytical chain using TEC as a probe for determining site-specific hydrogen isotope ratios of citric acid has been checked. It is concluded that the SNIF-NMR method can be applied for detecting the addition to lemon juice of commercial citric acid derived from beet. Work is in progress to improve the sensitivity of detection, which is limited to ~ 15 – 30% (depending on the deuterium content before addition of citric acid) in the absence of knowledge on the geographic origin of the precursors. A combined use of the carbon and hydrogen isotope parameters is efficient for detecting the presence of exogenous citric acid from either C3 or

C4 origin. The analytical strategy developed here can be extended to other kinds of fruits.

ACKNOWLEDGMENT

We thank Drs. Hischenhuber and Prodoliet for their advice in this research project. We are grateful to Pr. N. Naulet for his contribution to the IRMS experiments and to M. E. Quintanilla, L. Adam, H. Valentin, A. Andrés Debon, I. Bengoechea, and G. Kerneur for their help in the implementation of the separation and synthesis technologies.

LITERATURE CITED

- AIJN. Individual reference guidelines. In *Code of Practice for Evaluation of Fruit and Vegetable Juices*; Association of the Industry of Juices and Nectars from fruits and vegetables of the European community: Brussels, Belgium, 1993.
- Anet, F. A. L.; Park, J. Proton chemical shift assignments in citrate and trimethyl citrate in chiral media. *J. Am. Chem. Soc.* **1992**, *114*, 411–416.
- Association of German Fruit Juice Industries. *RSK-Values—The Complete Manual 1987 and update 1993*; Verlag Flüssiges Obst: Schoenborn, Germany, 1993.
- Brause, A. R. The Application of the Matrix Method Approach to the Determination of fruit juice authenticity for a variety of juices. In *Methods To Detect Adulteration of Fruit Juice Beverages*; Nagy, S., Wade, R. L., Eds; AgScience: Auburndale, FL, 1995; Vol. 1, pp 1–27.
- Bricout, J. Possibilities of stable isotope analysis in the control of food products. *Stable Isotopes*; Elsevier: Amsterdam, Holland, 1982; pp 483–493.
- Cohen, E.; Saguy, I. Measurement of oxygen-18/oxygen-16 stable isotope ratio in citrus juices: a comparison of preparation methods. *J. Agric. Food Chem.* **1984**, *32*, 28–30.
- Doner, L. W. Carbon isotope ratios in natural and synthetic citric acid as indicators of lemon juice adulteration. *J. Agric. Food Chem.* **1985**, *33*, 770–772.
- Doner, L. W.; Brause, A. R.; Petrus, D. R. Delta ¹⁸O measurements in water for detection of sugar beet-derived syrups in frozen concentrated orange juice. Collaborative study. *J. Assoc. Off. Anal. Chem.* **1992**, *75*, 1107–1111.
- Faethe, W.; Fuchs, G.; Hofsommer, H. J.; Neuhäuser, K.; Wallrauch, S. Richtwerte und Schwankungsbreiten bestimmter Kennzahlen (RSK Werte) für Zitronensaft. *Fluess. Obst* **1990**, *57*, 357–359.
- Federici, F.; Petruccioli, M. Immobilization of filamentous fungi: a new frontier in the production of organic acids. *Ital. J. Food Sci.* **1997**, *9*, 171–182.
- Gensler, M.; Schmidt, H. L. Isolation of the main organic acids from fruit juices and nectars for carbon isotope ratio measurement. *Anal. Chim. Acta* **1994**, *299*, 231–237.
- Jamin, E.; Gonzalez, J.; Remaud, G.; Naulet, N.; Martin, G. G.; Weber, D.; Rossmann, A.; Schmidt, H.-L. Improved detection of sugar addition to apple juices and concentrates using internal standard ¹³C IRMS. *Anal. Chim. Acta* **1997a**, *347*, 359–368.
- Jamin, E.; Gonzalez, J.; Remaud, G.; Naulet, N.; Martin, G. G. Detection of exogenous sugars or organic acids addition in pineapple juices and concentrates by ¹³C IRMS analysis. *J. Agric. Food Chem.* **1997b**, *45*, 3961–3967.
- Jamin, E.; Gonzalez, J.; Bengoechea, I.; Kerneur, G.; Remaud, G.; Naulet, N.; Martin, G. G. ¹³C/¹²C ratios measured on sugars, malic and citric acids as authenticity probes of citrus juices and concentrates. *J. Assoc. Off. Anal. Chem.* **1998**, in press.
- Koziet, J.; Rossmann, A.; Martin, G. J.; Ashurst, P. R. Determination of carbon-13 content of sugars of fruit and vegetable juices/A European inter laboratory comparison. *Anal. Chim. Acta* **1993**, *271*, 31–38.
- March, J. *Advanced Organic Chemistry, Reactions, Mechanisms and Structure*, 4th ed.; Wiley: New York, 1992; pp 393–398.
- Martin, G. G.; Wood, R.; Martin, G. J. Detection of added beet sugar in concentrated and single strength fruit juices by Deuterium Nuclear Magnetic Resonance (SNIF-NMR Method): Collaborative Study. *J. Assoc. Off. Anal. Chem.* **1996a**, *79*, 917–928.
- Martin, G. G.; Hanote, V.; Lees, M.; Martin, Y. L. Interpretation of combined ²H–SNIF-NMR and ¹³C SIRA-MS analyses of fruit juices to detect added sugar. *J. Assoc. Off. Anal. Chem.* **1996b**, *79*, 62–72.
- Martin, G. J.; Martin, M. L. Deuterium labelling at the natural abundance level as studied by high field quantitative ²H NMR. *Tetrahedron Lett.* **1981**, *22* (36), 3525–3528.
- Martin, G. J.; Martin, M. L. Stable isotope analysis of food and beverages by nuclear magnetic resonance. *Annu. Rep. NMR Spectrosc.* **1995**, *31*, 81–104.
- Martin, G. J.; Danho, D.; Vallet, C. Natural isotope fractionation in the discrimination of sugar origins. *J. Sci. Food Agric.* **1991**, *56*, 419–434.
- Martin, Y. L. A Global Approach to Accurate and Automatic Quantitative Analysis of NMR Spectra by Complex Least-Squares Curve-Fitting. *J. Magn. Reson., Ser. A* **1994**, *111*, 1–10 (commercial information on the software used is available on request to the authors).
- Miles-Prouten A. P. The role and application of acidulants in fruit juice. *Fruit Process.* **1994**, *1/94*, 6–7.
- O'Leary, M. H. Carbon Isotope in Photosynthesis. *BioScience* **1988**, *38*, 328–336.
- Remaud, G.; Guillou, C.; Vallet, C.; Martin, G. J. A coupled NMR and MS isotopic method for the authentication of natural vinegars. *Fresenius' J. Anal. Chem.* **1992**, *342*, 457–461.
- Remaud, G.; Andres Debon, A.; Martin, Y.-L.; Martin, G. G.; Martin, G. J. Authentication of bitter almond oil and cinnamon oil: application of SNIF-NMR method to benzaldehyde. *J. Agric. Food Chem.* **1997**, *45*, 4042–4048.
- Rossmann, A.; Trimborn, P. Gehalte an stabilen sauerstoffisotopen in wasser von apfelsaftkonzentrat als kriterium für den nachweis einer zuckerung. *Z. Lebensm. Unters. Forsch.* **1996**, *203*, 277–282.
- Rossmann, A.; Rieth, W.; Schmidt, H. L. Stable isotope ratio determination and its combination with conventional analyses (RSK-Values) for fruit juice authenticity control. In *Methods To Detect Adulteration of Fruit Juice Beverages*; Nagy, S., Wade, R. L., Eds.; AgScience: Auburndale, FL, 1995; Vol. 1, pp 28–40.
- White, J. W.; Winters, K. Honey protein as internal standard for stable carbon isotope ratio detection of adulteration of honey. *J. Assoc. Off. Anal. Chem.* **1989**, *72*, 907–911.
- Yunianta; Zhang, B. L.; Lees, M.; Martin, G. J. Stable isotope fractionation in fruit juice concentrates: application to the authentication of grape and orange products. *J. Agric. Food Chem.* **1995**, *43*, 2411–2417.

Received for review December 15, 1997. Revised manuscript received March 19, 1998. Accepted March 23, 1998. Nestle Research Centre is acknowledged for financial support.

JF971066P