Food Chemistry 146 (2014) 36–40

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Analytical Methods

$^{13}C/^{12}C$ isotope ratios of organic acids, glucose and fructose determined by HPLC-*co*-IRMS for lemon juices authenticity

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ARTICLE INFO

Article history:
Received 25 June 2012
Received in revised form 29 August 2013
Accepted 4 September 2013
Available online 11 September 2013

Keywords:
HPLC-*co*-IRMS
Carbon 13 isotope ratio
$\delta^{13}C$
Organic acids
Glucose
Fructose
Lemon juices
Lemon concentrate
Authentication

ABSTRACT

High performance liquid chromatography linked to isotope ratio mass spectrometry via an interface allowing the chemical oxidation of organic matter (HPLC-*co*-IRMS) was used to simultaneously determine carbon 13 isotope ratio ($\delta^{13}C$) of organic acids, glucose and fructose in lime and lemon juices. Because of the significant difference between organic acids and sugars concentrations, the experimental protocol was optimised by applying a "current jump" to the IRMS device. The filament current is increased of 300 μA during elution in order to enhance IRMS sensitivity. Then, analysis were performed on 35 lemon and lime fruits from various geographical origins and squeezed in the laboratory. An overall average $\delta^{13}C$ values of $-25.40 \pm 1.62‰$, $-23.83 \pm 1.82‰$ and $-25.67 \pm 1.72‰$ is found for organic acids mixture mainly made up of citric acid, glucose and fructose, respectively. These authentic samples allowed the definition of a confidence domain to which have been confronted 30 commercial juices (24 "pure juices" and 6 coming from concentrate). Among these 30 samples, 10 present $\delta^{13}C$ values outside the defined range revealing an added "C4" type organic acids or sugars, addition not specified on the label that is not in agreement with EU regulation.

1. Introduction

Lemons and limes are commercialised as fresh fruits or as juices, oils can also be found on the market. Until now, geographical origin does not appear as a real commercial argument and few labels mentioned the lemon/lime juice origin. As a result, geographical origin studies on these matrix are scarce (Barnes, 1997; Pellerano, Mazza, Marigliano, & Marchevsky, 2008). The price of lemon and concentrate juice is determined on the basis of titratable acidity, i.e. citric acid concentration essentially, and sugar content. Thus, adulterations could be performed by the addition of exogenous sugar and/or citric acid. Moreover, European legislation elaborated regulations fixing the fruit juices trade including protected geographical indication (PGI). The addition of various acidifying compounds is authorised as well as the addition of sugars according to European reglementation (Directive, 1995; Directive, 2001). The use of such authorised compounds must be mentioned in the ingredients list of the product label and the simultaneous addition of sugar and acidifying product is prohibited (Directive, 2001). Moreover, sugar addition in fruit juices will be no more authorised from 28 October 2013 (Directive, 2012). As a result, most of the research works are focus on the characterisation of the authenticity of main components of lemon juice and several pathways were envisaged to detect such additions.

Concentration ratio between citric and isocitric acid has been used to detect added citric acid. Unfortunately, this concentration ratio range is large leading to undetected citric acid addition (AIJN). Citric acid, industrially produced, results from the fermentation of various sources like beet and cane molasses, corn syrups i.e. from plants with either "C3" or "C4" type metabolisms. One of the main differences between these plants carbohydrates is their carbon 13 isotope ratio ($\delta^{13}C$ or $^{13}C/^{12}C$): "C3" carbohydrates $\delta^{13}C$ values are in the range $-34 < \delta^{13}C < -24‰$ whereas for "C4" carbohydrates, the range is $-17 < \delta^{13}C < -10‰$ (Meier-Augenstein, 1999). As lemons have a "C3" metabolism, only important amount of "C3" type compound could influence isotopic ratios. Therefore, isotope ratio mass spectrometry (IRMS) determination became a choice method for "C4" type citric acid and sugars adulteration (Doner, 1985; Jamin et al., 1998a). In order to improve the authenticity control, isotope ratio of fruit proteins were used as an internal reference (Jamin et al., 1998b). Combination of multi-isotope analysis also allowed the detection of "C3" type citric acid adulteration (Gonzalez et al., 1998; Jamin, Martin, Santamaria-Fernandes, & Lees 2005). In those studies, a prerequisite to obtain isotopic information is the

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http://dx.doi.org/10.1016/j.foodchem.2013.09.020

extraction and separation of each component from the fruit matrix, moreover for some experiments, citric acid needs to be derivated to be studied by ^{2}H-NMR. These purification steps could limit the use of those techniques in authenticity control routine applications. Moreover, the determination of carbon 13 isotope ratio of sugars is performed on the overall mixture (sucrose + glucose + fructose) and this global determination of isotope ratios could hide some sugars additions.

Recently, the link between high performance liquid chromatography (HPLC) and isotope ratio mass spectrometry (IRMS) was achieved through an interface insuring a chemical oxidation (*co*). A recent review (Godin & McCullagh, 2011) details some applications of this technique that allows δ^{13}C determination of single compounds coming from a complex mixture without any purification steps. Despite its potential uses in food authenticity, it has been scarcely applied for this type of application. To our knowledge, the link HPLC-*co*-IRMS has only been applied for honey, (Cabanero, Recio, & Rupérez, 2006; Elflein & Raezke, 2008) caffeine authentication, (Zhang, Kujawinski, Federherr, Schmidt, & Jochmann, 2012) and wine (Cabanero, Recio, & Rupérez, 2008; Cabanero, Recio, & Rupérez, 2010; Guyon, Gaillard, Salagoity, & Medina, 2011). As HPLC-*co*-IRMS allows δ^{13}C ratio measurement of major wine components, (Guyon et al., 2011) this technique has been applied to the authentication of lemon matrix which is essentially made up of water, acids and sugars, (Souci, Fachmann, and Kraut, 2000).

This study presents the application of HPLC-*co*-IRMS to the determination of carbon isotope ratio of major lemon components. An original strategy was set up to answer to the important difference in concentration between acids and sugars. Organic acids, glucose and fructose δ^{13}C values were determined on 35 authentic lemon or lime juices obtained from the fruits squeezed in the laboratory. Then 30 commercial lemon or lime juices were analysed and their δ^{13}C values confronted to authentic databank values.

2. Experimental

2.1. Chemicals

Ammonium persulfate and orthophosphoric acid (Fischer Scientific, Illkirch, France) are analytical reagent grade and used without any purification step. Carrier gas, helium (5.6 grade), and CO_2 (4.5 grade), reference gas, are Linde products (Bassens, France). Solutions and dilutions are realised with de-ionised water (Elga, Bucks, United Kingdom).

2.2. Samples

Databank is based on the results of the analysis of 35 authentic citrus fruits (10 limes and 25 lemons) bought at fruit-store stalls and squeezed in the laboratory. These 35 fruits come from various geographical origins: Argentina (5), Brazil (5), Chile (2), Italy (5), Mexico (5), Spain (12) and Turkey (1). 30 commercial lemon juices were analysed, 6 coming from concentrated juices (ABC) and 24 labelled as "pure juices". Before analysis, each juice is diluted (1/30) and filtered (0.45 µm), the results are the average of two measurements.

2.3. Instrumentation

High performance liquid chromatography (HPLC, Ultimate 3000, Dionex, Voisin-le-Bretonneux, France) is linked to the isotope ratio mass spectrometer (Elementar/IsoPrime, Villeurbanne, France. Analytical characteristics on http://www.isoprime.co.uk) via a liquid interface (Liquiface®, Elementar/IsoPrime) and symbolised by "*co*" in the acronym HPLC-*co*-IRMS as it ensures the chemical oxidation of organic matter. The system is fully described elsewhere (Guyon et al., 2011). Chromeleon® software runs the HPLC system, IRMS and isotope ratio computation is automatically performed with IonVantage (V-1.3.6) software.

2.4. Isotope ratio determination

25 µL of the diluted and filtered juices are automatically injected on the carbohydrate column 700 CH (Alltech, 300 × 6.5 mm, Epernon, France), warmed at 80 °C. Elution rate is set up at 0.4 mL min^{-1} (elution: de-ionised water). Eluted solution is homogenised, via a three-way Valco valve, with a solution of persulfate (15% in mass) and orthophosphoric acid (2.5% in volume) brought by a peristaltic pump at 0.6 mL min^{-1} flow rate; then the mixture is warmed at 93 °C leading to the oxidation of the eluted organic molecules. After solution cooling, the CO_2 resulting from the oxidation step is extracted from the aqueous solution using a high density porous PTFE tubing (60-8) (1 mm i.d., 0.4 mm wall thickness, 2 µm pore size; International polymer engineering, Tempe, Arizona, USA) flushed on the outside by helium. The mixture (helium, CO_2 and water vapour) passes through a Nafion® membrane for gas drying before IRMS analysis. Helium fluxes are fixed at 15 and 50 mL min^{-1} for CO_2 extraction and for gas drying, respectively. Eluting solutions are de-aerated during 1 h of helium bubbling and then kept under helium atmosphere. The overall duration of a single analysis is 1350 s. An increase in the filament current intensity (+300 µA) is performed automatically at 600 s, after CO_2 peak resulting of organic acids oxidation.

2.5. Calculation

Measured masses are *m/z* 44 and 45 corresponding to $^{12}C^{16}O_2$ and $^{13}C^{16}O_2$. Isotope ratios, $^{13}C/^{12}C$, are expressed as relative deviations δ ‰ against the international standard, V-PDB (Vienna-Pee Dee Belemnite) according to:

$$\delta^{13}C_s\ (‰) = [(R_s/R_{st}) - 1] \times 1000$$

where R is the $^{13}C/^{12}C$ isotope ratio of the sample (s) and the international standard (st). In the following, all isotopic data, δ^{13}C, are expressed in ‰ vs V-PDB.

3. Results and discussion

HPLC-*co*-IRMS system was applied to lemon juice authentication as HPLC allows compounds separation and IRMS, carbon 13 isotope ratio measurement (δ^{13}C), with a Liquiface® interface that insures eluted organic matter chemical oxidation (*co*). The main limitation of such a system results from the elution that needs to be performed with water: organic solvents cannot be used as they will be oxidised in the interface, hiding the requested information. Considering this, chromatographic column choice is restricted: a carbohydrate column, with water elution at 80 °C, offers a good carbohydrate separation but a relatively poor resolution for organic acid compounds. As a result, the peaks of the two main organic acids, malic and citric, are merged and dissymmetrical. In the following, the term "organic acids" corresponds to citric and malic acids even if for most of the samples the signal corresponds essentially to citric acid as its concentration can be up to 105 times higher (Table S-1); these values determined by capillary electrophoresis (experimental conditions in Chauvet, Poulit, & Medina, 2001; Chabreyrie et al., 2008) are consistent with previously described ratio citric/malic acids range (Souci et al., 2000; AFNOR, 1986). Previous experiments, conducted with water slightly acidified permanently modified column characteristic, therefore, for new column, a conditioning step (1 h elution with acidic solution [H_2SO_4] = 3 mmol) is a prerequisite to reproduce this study results. This conditioning modify the column properties as instant polyose hydrolysis in monosaccharide's is observed; as a result, sucrose signal is not observed on the chromatogram as it is hydrolyzed in glucose and fructose (Fig. 1). This is not a limitation as the analysis of commercial lemon juices with NH_2 column (Alltima 5 μ, 250 × 4.6 mm, elution acetonitrile-water, v/v 75/25) using a refractometer as a detector does not detect any sucrose as well: it reveals a complete sucrose hydrolysis in glucose and fructose during juice process conditioning. In Fig. 1, glucose and fructose signal is the superposition of glucose and fructose naturally synthesised and coming from hydrolyzed natural sucrose. $\delta^{13}C$ values repeatability and reproducibility, performed on organic acids peak, were found to be 0.27‰ and 0.41‰, respectively. Moreover, the method accuracy and linearity has been verified with the study of the impact of concentration variation on $\delta^{13}C$ ratios with a synthetic solution containing citric acid, glucose and fructose mixture (Fig. S-1). In the range 0.5–2.5 g/L, the $\delta^{13}C$ ratio of the three compounds corresponds (within experimental error) to the target value individually determined by EA-IRMS.

In food matrix, simultaneous measurement of $\delta^{13}C$ isotope ratios of every component can be arduous due to high differences in concentration ratio i.e., either a peak saturation or a too low signal intensity occurs. In lemon juice, concentration ratio citric acid/sugars is in the range 3–57 (Table S-1). A concentration ratio higher than 4 prevent from the simultaneous determination of $\delta^{13}C$ values because of citric acid peak saturation and/or a too low sugars signal intensity according to the dilution level and/or injected volume. This difficulty was overcome with the application of a so called "current jump" by analogy to the "peak jump" frequently used in other IRMS applications. A "peak jump" consists in a modification of the magnetic field and the current trap intensity allowing the study of different molecules (N_2 and CO_2, as an example). Similarly and for the first time in the HPLC-*co*-IRMS link, a "current jump" was applied during the chromatography step, consisting in an increase in the current trap intensity, only. During lemon juice elution, a 300 μA jump in the current trap intensity is applied to the filament, increasing the intensity from 200 to 500 μA. The magnet field is not modified as the isotope ratio of the same molecule, CO_2, is studied. This "current jump" is possible as the time scale between citric acid and first eluted sugar is large; it increases the IRMS sensitivity that compensates the differences between the concentrations and allows simultaneous determination of citric acid and sugars $\delta^{13}C$ (Fig. 1). As reference gas valve provides reference CO_2 pulses used for $^{13}C/^{12}C$ quantification, two pulses are performed at the beginning and at the end of the run in order to determine reference gas isotope ratio under the two measurement conditions, i.e. before and after the filament intensity increase. On Fig. 1 is presented a typical chromatogram of a lemon juice eluted in the conditions described in the experimental part, the interrupt in the baseline, at 600 s, results from the filament intensity jump.

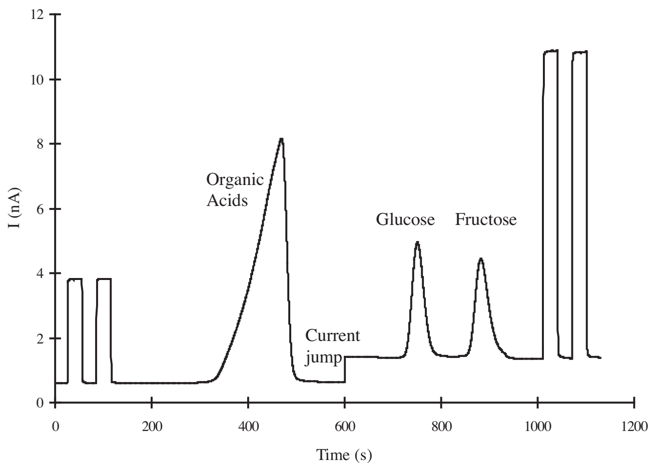

Fig. 1. Chromatogram obtained for lemon juices (dilution 1/30) analysed by HPLC-*co*-IRMS. The peak jump consists in the IRMS filament intensity increase (+300 μA).

Under these experimental conditions, 25 authentic citrus fruits, 8 limes and 17 lemons, bought at a local fruit-store stalls and squeezed in the laboratory, were analysed. These citrus fruits come from various geographical origins. For each sample, HPLC-*co*-IRMS technique was used to determine $\delta^{13}C$ isotope ratio of citric acid, glucose and fructose. Results of authentic citrus fruits are listed in Table 1. Organic acids $\delta^{13}C$ values are in the range −29.20‰ to −23.45‰ that is agreement with other studies (Jamin et al., 1998a; Jamin et al., 2005). Similar range of $\delta^{13}C$ values is found for the sugars, glucose (−27.20‰ to −21.02‰) and fructose (−29.22‰ to −22.81‰). A depletion of −1.8 ± 0.9‰ (computed from Table 1) is observable in carbon 13 concentration on fructose carbon skeleton. $\delta^{13}C$ value for "global sugar" was computed from individual ratio of glucose and fructose providing $\delta^{13}C$ values very close to values found in the literature (Jamin et al., 1998a; Jamin et al., 2005). Data are plotted on two graphs merged in Fig. 2, showing the relation between glucose and organic acids $\delta^{13}C$ ratios (Fig. 2A) and between glucose and fructose $\delta^{13}C$ values (Fig. 2B). From these data, a domain of lemon authenticity can be define, considering a confidence level of 95% and the equation $y = ax + (b \pm 2s)$: where "a" and "b" are the authentic samples linear

Table 1
$\delta^{13}C$ values of lemon and lime juices from different geographical origin and squeezed in the laboratory (‰ vs V-PDB).

Sample	Origin	Information	$\delta^{13}C$ (‰)		
			Organic acids	Glucose	Fructose
A1	Argentina	Lemon	−29.20	−27.20	−29.22
A2	‘’	‘’	−28.26	−26.10	−28.59
A3	‘’	‘’	−28.16	−25.92	−28.82
A4	‘’	‘’	−28.99	−25.87	−28.57
A5	‘’	‘’	−27.02	−25.18	−26.24
A6	Brasil	Lime	−23.45	−21.48	−24.06
A7	‘’	‘’	−24.76	−22.49	−24.88
A8	‘’	‘’	−23.75	−23.39	−23.56
A9	“	“	−24.53	−22.47	−23.85
A10	‘’	‘’	−24.22	−21.96	−24.07
A11	Chile	Lemon	−27.41	−25.17	−27.66
A12		“	−25.25	−23.74	−24.12
A13	Italy	Lemon	−24.53	−22.30	−24.83
A14	‘’	‘’	−25.81	−25.95	−28.43
A15	‘’	‘’	−26.11	−26.67	−27.12
A16	‘’	‘’	−26.57	−26.35	−27.05
A17	‘’	‘’	−25.11	−23.70	−25.28
A18	Mexico	Lime	−23.59	−21.02	−22.81
A19	‘’	‘’	−23.63	−21.78	−24.01
A20	‘’	‘’	−24.50	−22.97	−25.19
A21	‘’	‘’	−24.13	−21.87	−23.99
A22	‘’	‘’	−25.11	−22.19	−24.09
A23	Spain	Lemon	−24.33	−23.64	−25.85
A24	‘’	‘’	−25.05	−23.67	−25.74
A25	‘’	‘’	−24.37	−22.36	−25.01
A26	‘’	‘’	−24.45	−24.09	−25.62
A27	‘’	‘’	−23.71	−22.78	−25.08
A28	‘’	‘’	−24.85	−21.85	−25.23
A29	‘’	‘’	−25.24	−25.14	−25.72
A30	‘’	‘’	−25.27	−25.93	−26.44
A31	‘’	‘’	−24.62	−24.51	−24.69
A32	‘’	‘’	−24.30	−22.08	−24.46
A33	‘’	‘’	−24.34	−21.10	−23.94
A34	‘’	‘’	−28.21	−26.03	−27.82
A35	Turkey	Lemon	−26.66	−25.12	−26.54

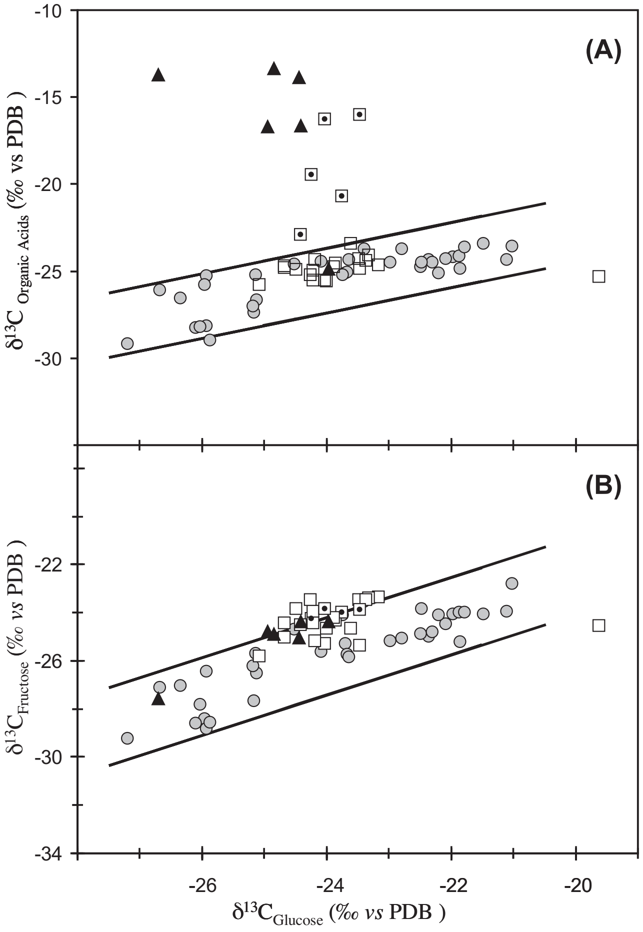

Fig. 2. $\delta^{13}C$ variability for lemon juices: representation of the relationship between glucose and citric acid (A) and glucose and fructose $\delta^{13}C$ ratios (B). symbols: authentic lemon lab-squeezed, □ commercial "pure juice", ▲ juices from concentrate. Dots allow to follow the position for some commercial samples. Lines correspond to 95% confidence limits.

Table 2
$\delta^{13}C$ values of commercial lemon and lime juices (in ‰ vs V-PDB).

Sample	Origin	$\delta^{13}C$		
		Citric acid	Glucose	Fructose
C1	Italy	−25.35	−19.62	−24.54
C2	"	−24.11	−23.32	−23.39
C3	"	−24.41	−23.37	−23.49
C4	"	−23.42	−23.61	−24.67
C5	"	−20.72	−23.75	−24.00
C6	"	−25.56	−24.02	−25.28
C7	"	−24.85	−23.47	−25.37
C8	"	−25.59	−24.00	−24.65
C9	"	−25.53	−24.21	−24.44
C10	"	−16.25	−24.02	−23.84
C11	"	−16.04	−23.46	−23.88
C12	Sicily	−19.47	−24.24	−24.24
C13	"	−24.67	−23.16	−23.35
C14	"	−24.31	−23.48	−23.48
C15	"	−22.89	−24.42	−24.50
C16	"	−24.75	−23.88	−24.22
C17	"	−24.37	−24.19	−25.18
C18	"	−24.92	−24.49	−23.85
C19	"	−24.97	−24.21	−23.94
C20	"	−25.23	−24.25	−23.49
C21	Unspecified	−24.79	−24.68	−25.05
C22	"	−24.54	−23.85	−24.32
C23	"	−24.69	−24.67	−24.45
C24	"	−25.81	−25.07	−25.80
ABC1	Italy	−13.72	−26.70	−27.56
ABC2	"	−24.85	−23.97	−24.37
ABC3	"	−16.67	−24.94	−24.78
ABC4	"	−16.65	−24.42	−24.35
ABC5	Sicily	−13.36	−24.85	−24.88
ABC6	Unspecified	−13.86	−24.44	−25.03

regression coefficients, "*s*" corresponding to the standard deviation of the difference between observed and reconstructed "*y*" values (Jamin, Guérin, Rétif, Lees, & Martin, 2003). Any sample with a $\delta^{13}C$ ratio outside these limits must have been supplemented with a "C4" type compound, sugar and/or organic acid.

$\delta^{13}C$ values of 30 commercial juices were determined and their isotope ratio confronted to authentic lemon one's. Among these 30 juices, 6 were elaborated from concentrate (ABC) and 24 were labelled as "pure juices". On their label, none of the 30 samples specified citric acid or sugars addition. $\delta^{13}C$ values of organic acids, reported on Table 2, are in the range −25.53‰ to −13.72‰. Some of these ratio are too high to be pure endogenous citric acid, i.e., an addition of "C4" type exogenous organic acids must have occurred. Glucose and fructose $\delta^{13}C$ values, for commercial juices, are in the range −27.56‰ to −19.62‰. The higher $\delta^{13}C$ isotope ratio value reveals a sugar addition. These commercial samples results are plotted in Fig. 2 together with authentic lemons data illustrating the impact of "C4" organic acids or sugar addition on isotope ratios. 5 "ABC" and 5 "pure juices" present an organic acids $\delta^{13}C$ ratio much higher than expected and way outside the 95% confidence level. This means that for these 10 samples, the specification of organic acids addition was omitted on the label. In Fig. 2A, one sample has a $\delta^{13}C$ ratio higher than expected. Added "C4" type sucrose can be suspected. But, in Fig. 2B, fructose $\delta^{13}C$ ratio of this sample is comparable with authentic lemon fructose isotope ratios. Thus, this lemon juice must have been supplemented with glucose syrup, addition not mentioned on the label. In Fig. 2B, most of commercial juices $\delta^{13}C$ values are grouped and 10 of them are slightly outside the confidence level. Small addition of fructose syrup can be suspected. This suspicion is backed up with the similarity in the $\delta^{13}C$ vales of glucose and fructose (Table 2) which is usually not observed for authentic juices that present a gap of 1.8 ± 0.9‰. This fructose addition is not noticeable on a glucose/fructose concentration ratio as the added amount is within the HPLC uncertainty (≈10%). This observation confirms the power of isotope measurement to detect "C4" type sugar addition even at low level. In Fig. 2, "pure juices" commercial samples supplemented with exogenous organic acids are highlighted with a dot in the symbols: it appears that among these 5 commercial samples, 2 of them seems to have been sugared as their fructose $\delta^{13}C$ ratios are slightly outside the confident level. A similar conclusion can be drawn for ABC juices as 2 of them are supplemented with organic acids and present a fructose $\delta^{13}C$ ratio slightly outside the expected range.

4. Conclusion

HPLC-*co*-IRMS link is a powerful tool for food authentication as this system combines product constituents separation by HPLC and their carbon 13 isotope ratio determination. This technique was applied to lemon juices to determine organic acids and sugars authenticity. As it happens frequently, concentration differences prevent from a simultaneous $\delta^{13}C$ ratio determination. To overcome this problem, a peak jump was applied during the chromatographic elution: a filament intensity increase was performed to enhance the sensitivity, allowing the simultaneous determination of $\delta^{13}C$ value of organic acids and sugars. A set of 35 lemons, squeezed in the laboratory, were used to elaborate a reference model. A correlation is found between the $\delta^{13}C$ values of organic acids and sugars, allowing the definition of a confident level for authentic lemon juices. 30 commercial samples with no labelled addition of citric acid or sugars were confronted to the model. If most of the samples are in the defined limits, for 10 of them, $\delta^{13}C$ values revealed a "C4" type organic acids and for a single juice, a "C4" glucose addition. Moreover, for 12 other samples, 2 ABC juices and 10 "pure juices", a suspicion of "C4" type fructose can be drawn up. Among them, 4 juices (2 ABC and 2 pure juices) appear to be supplemented with organic acids and sugar. All these samples are not in accordance with the EU regulation that requires the mention on the label of any additive supplementation and prohibits the simultaneous addition of acids and sugar. In front of these results, it appears important to control the possible addition of "C3" type compounds as well. This research requires other solutions than IRMS, like deuterium NMR measurements (Gonzalez et al., 1998). Nonetheless, HPLC-*co*-IRMS can be used as a screening method for a rapid estimation of added "C4" type products as it is easily applicable for routine control analysis.

Acknowledgment

The authors want to thanks all the DGCCRF officers for collecting lemon juices and lemon/lime fruits.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2013.09.020.

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