Economically Motivated Adulteration of Lemon Juice: Cavity Ring Down Spectroscopy in Comparison to Isotope Ratio Mass Spectrometry – Round-Robin Study

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ABSTRACT

Economically motivated adulteration (EMA) of foods is an increasing concern in the recent years. In this work, an optimized sample preparation procedure for the determination of lemon juice adulteration was validated using Elemental Analyzer – Isotope Ratio Mass Spectrometry (EA-IRMS); additionally, 69 imported lemon juice samples were evaluated using Combustion Module – Cavity Ring Down Spectrometry (CM-CRDS) and compared to the well-established EA-IRMS. Equivalency of CM-CRDS to EA-IRMS was further demonstrated by conducting a round-robin study involving eight laboratories throughout the United States, Canada, and New Zealand. Overall, the results obtained for CM-CRDS were statistically indistinguishable from the results obtained using EA-IRMS for EMA lemon juice analysis.

1 Introduction

Lemon juice is a one of the common targets of Economically Motivated Adulteration (EMA). Other commonly reported targets for EMA of foods include honey, olive oil, dairy products, spices and other citrus juices [1]. Available data shows that the US imported roughly 37% of the world's crop of lemons and limes [2], while Argentina and Italy are the world's largest suppliers of lemon juice [3]. This study focuses on carbon Stable Isotope Ratio Analysis (SIRA) measuring $\delta^{13}C_{VPDB}$ (‰) values of 69 imported lemon juice samples for detecting adulteration. $\delta^{13}C_{VPDB}$ (‰) is $\delta^{13}C$ values expressed in (units of %), defined as parts per thousand differences in the $^{l3}C/^{12}C$ ratio of a sample from that in standard Vienna PeeDee Belemnite (VPDB).[4-5]

Lemon juice consists of approximately 9% dissolved solids. The solids are approximately 60% citric acid, 20% sugars [4], minor amounts of malic acid and other components. The quality and price of lemon juice is directly related to the relative quantities of citric acid and sugars present in the juice.[6] There are two primary ways of lemon juice adulteration for economic gain, addition of inexpensive sweeteners, like cane sugar and/or high fructose corn syrup (HFCS), and/or the addition of

exogenous citric acid. Lemon trees follow a C3 photosynthetic pathway [4] with $\delta^{13}C_{VPDB}$ values typically ranging from -23.5 to -26.6‰ [6]; therefore both sugars and citric acid naturally produced by lemons exhibit similar $\delta^{13}C_{VPDB}$ values. In contrast, cane sugar and HFCS, are produced in a C4 photosynthetic pathway [4]. Commercial citric acid is commonly manufactured by fermenting some sugars that follow C4 photosynthetic pathway by certain *Aspergillus niger* strains; correspondingly, its carbon isotopic composition is close to that of C4 sugars. Sugars and citric acid derived from C4 plants have less negative (or more positive) $\delta^{13}C_{VPDB}$ values, ranging from -13.1‰ to -9.8‰. Addition of C4 derived sugar or citric acid to a C3 lemon juice, enriches the ^{13}C content and increases the natural $\delta^{13}C_{VPDB}$ of the lemon juice in the more positive direction, which is indicative of adulteration [4]. Note all subsequent reported $\delta^{13}C$ values imply $\delta^{13}C_{VPDB}$.

Applications of carbon SIRA using an elemental analyzer interfaced with an isotope ratio mass spectrometer (EA-IRMS) to determine the $\delta^{13}C$ values of various components of lemon juice [6-9] and other fruit juices [7, 9-12] related to EMA are reported in the scientific literature. In various reports, the mean and standard deviation of $\delta^{13}C$ values of citric acid isolated from lemon juice were -25.5 ± 1.2‰ (n=84) [6]; -24.8 ± 1.1‰ (n=10) [7]; -24.1‰, -25.1‰ and -24.2‰ (fresh squeezed lemons, n=3) [4]; and -25.8 ± 0.8‰ (fresh squeezed lemons, n=7) and -25.9 ± 0.7‰ (lemon juice concentrates, n=7) [9]. The reported averages ranged from 25.8‰ to -24.1‰, which corresponds well to the expected values for C3 plants. To our knowledge, no official regulations for adulteration of lemon juice with exogenous citric acid exist, therefore the data presented above was used to support the criterion for this study that a citrate value greater than -23.0‰ is indicative of adulteration. Commercial citric acid, potentially used for adulteration, is derived from fermentation of a variety of common carbohydrate sources. When produced by fermentation of corn syrup, $\delta^{13}C$ of -9.7 and -10.1‰ [4] have been reported, corresponding to the expected values for C4 plants. Other fermentation sources of citric acid include paraffin, petroleum, or beet molasses with reported $\delta^{13}C$ values ranging from of -27.2‰ to -25.2‰ [4], consisted with C3 plants. Citric acid from these other sources is not detectable as adulterant using the current conventional methods, including the method which is presented here.

A citric acid isolation method commonly utilized in the literature is based on work by Doner [4], in which organic acids are precipitated by adding excess calcium hydroxide. However, CO_2 may become trapped during the industrial production of $Ca(OH)_2$ [6] and potentially bias $\delta^{13}C$ values of the calcium citrate. Therefore, the precipitation procedure was optimized and validated within our laboratory using EA-IRMS for analysis. The standard method for carbon SIRA is EA-IRMS, a technique requiring an elevated degree of technical knowledge for operation as well as high cost of purchase and maintenance. To make this method more versatile, the same isolates were analyzed using another carbon SIRA technique, combustion module – cavity ring down spectrometry (CM-CRDS), which has lower operation costs, simpler analysis, increased robustness (further discussed elsewhere [13]). CM-CRDS has been used previously for detecting EMA in honey [13-16], but to our knowledge detection of EMA in lemon juice by CM-CRDS has not been reported. In this study, we report comparative analysis of calcium citrate precipitates from 69 lemon juice samples by EA-IRMS and CM-CRDS as part of a single laboratory validation for CM-CRDS applied to EMA of lemon juice. As a follow up, data from a round-robin study in which with eight participating laboratories analyzed citric acid isolated from six lemon juice samples by EA-IRMS with three laboratories also utilizing CM-CRDS.

2 Materials and Method

2.1 Reagents and Standards

Water used throughout the experiments was ultrapure deionized water (DIW) with resistivity of at least 18 MΩ·cm obtained from a Milli-Q system (Bedford, MA, USA) unless otherwise noted. Citrate was precipitated using various combinations of ammonium hydroxide (Fisher OPTIMA, Fair Lawn, NJ, USA), sodium hydroxide (Fisher Scientific), calcium chloride dihydrate (Sigma Aldrich, St. Louis, MO, USA), calcium hydroxide (Fisher and Acros Organics), calcium nitrate (Fisher Scientific). Validation experiments were carried out using two commercially available citric acids, citric acid monosodium salt ($C_6H_7NaO_7$), from Aldrich Chemical (St. Louis, MO, USA) ($\delta^{13}C$ -12.16‰,

designated Source A) and citric acid anhydrous (Acros Organics Fair Lawn, NJ, USA) (δ^{13}C -24.15‰, designated Source B). Three lemon juice samples (Brands 1, 2, and 3) purchased from local markets in Cincinnati, OH, along with a freshly squeezed lemon juice (referred to herein as "fresh lemon juice") from 20 locally purchased lemons (Cincinnati, OH) were also analyzed as part of the validation experiments as in-house controls. For both EA-IRMS and CM-CRDS analysis, citrate samples were placed in 5 x 9 mm tin foil capsules from Costech Analytical Technologies, Inc. (Valencia, CA, USA). Acetanilide (Costech Analytical Technologies, Inc) was used to condition the reactors, verify proper sample combustion, and as a quality control check for δ^{13}C values. Standards used for δ^{13}C normalization to the international Vienna Pee Dee Belemnite scale were purchased from NIST (Gaithersburg, MD, USA): NIST Reference Material (RM) 8542 (IAEA-CH-6, Sucrose, $\delta^{13}C_{VPDB}$ = -10.45 ± 0.07‰), 8573 (USGS40, L-glutamic acid, $\delta^{13}C_{VPDB}$ = -26.39 ± 0.09‰), 8543 (NBS 18, calcite, $\delta^{13}C_{VPDB}$ = -5.01 ± 0.07‰), and 8574 (USGS41, L-glutamic acid, $\delta^{13}C_{VPDB}$ = +37.63 ± 0.10‰). The linearity, sensitivity, and precision of CM-CRDS were determined by using citric acid (Fisher) [13]. All subsequent reported δ^{13}C values infer $\delta^{13}C_{VPDB}$.

2.2 Samples

Sixty-nine lemon juice samples were selected from a 2013 FDA import assignment for analysis by EA-IRMS and CM-CRDS. Due to the lack of certified lemon juice reference materials, freshly squeezed lemon juice from 20 locally purchased lemons was used as an in-house control, which was prepared and analyzed along with each analytical batch to verify consistent method performance. Two adulterant solutions of 6% (w/w) citric acid (from Source A or B) were prepared and mixed at proportions of 0%, 5%, 10%, 20%, 50% and 80% (w/w) to the locally purchased lemon juice, Brand #2 to serve as adulterated samples. Samples selected for the round-robin study were chosen from the 69 above, two adulterated, two inconclusive (< 0.4‰ from the cut off value of -23‰), and two unadulterated. In order to provide sufficient, well-homogenized material for analysis by eight laboratories, three individual preparations of each selected sample were combined, mixed with

157 spatula thoroughly, and split into eight portions for distribution. Each portion was homogenized and
158 prior to distribution, at least 3 portions of each sample were analyzed by the US FDA laboratory using
159 EA-IRMS to ensure adequate homogenization.
160 *2.3 Instrumentation and Operating Principles*
161 Lemon juice samples were analyzed using both EA-IRMS and CM-CRDS. The elemental analyzer for
162 the EA-IRMS system was the Costech Elemental Combustion System (ECS) model 4010 from
163 Costech Analytical Technologies, Inc. (Valencia, CA, USA) interfaced to a Thermo Delta V Advantage
164 (Thermo-Scientific, Waltham, MA, USA) with a Conflo IV gas flow controller (Thermo Fisher, Bremen,
165 Germany). For CM-CRDS, the samples were combusted in the Combustion Module, Model 02 by
166 Costech with the Liaison interface module, and CO_2 Cavity Ring Down Spectrometer analyzer, model
167 G2121-i, both from Picarro Inc. (Santa Clara, CA, USA). Nitrogen (99.9998%) was used as a carrier
168 gas for the CM-CRDS. The principle, operation and comparison of CM-CRDS with EA-IRMS are
169 discussed in detail in Mantha et al. [13], Balsley-Clausen et al.[17], and Crosson et al.[18].
170
171 *2.5 Sample Preparation Procedure*
172 The method for precipitation of citric acid from lemon juice was adapted from Doner et al. [4],
173 AOAC Official Method 981.09[19], and AOAC Official Method 982.21[20]. The procedure was optimized
174 as detailed in the method validation section and the finalized conditions are listed as follows.
175 Approximately 10 mL of lemon juice was poured into a 50 mL centrifuge tube and centrifuged for 10
176 minutes at 3000 rpm, discarding the precipitated pellet (consisting of pulp and extraneous material in
177 the lemon juice). The pH of the supernatant was adjusted to 8.5 or above using concentrated
178 ammonium hydroxide. Approximately 2 mL of 3M calcium chloride ($CaCl_2 \cdot 2H_2O$) was mixed with the
179 supernatant and heated in an oven at 60°C for at least two hours to precipitate the citrate (as calcium
180 citrate. The precipitate along the supernatant was centrifuged for 10 minutes at 3000 rpm and
181 vacuum filtered. The precipitate was washed twice with 5 mL of DIW, once with 5 mL of acetonitrile,
182 and finally with 5 mL of DIW. The precipitate was transferred into a petri dish and dried in an oven at

60°C for over two hours. The dried calcium citrate was gently pulverized and thoroughly homogenized. Triplicate portions of the precipitated calcium citrate (0.3– 2.0 mg for EA-IRMS and 0.7– 6.0 mg for CM-CRDS) were weighed into tin capsules for determination of $\delta^{13}C$ values.

2.6 Multi-Laboratory Round-Robin Study Parameters

Eight laboratories, four from Canada, three from the US and one from New Zealand volunteered to participate in the round-robin study conducted to evaluate the equivalency of CM-CRDS to EA-IRMS applied to EMA of lemon juice. The sample set provided to these laboratories (distributed by the US FDA laboratory) included calcium citrate samples isolated from two adulterated, two not adulterated, and two inconclusive juices out of the set of 69 previously described. Three of the eight laboratories conducted the study using both EA-IRMS and CM-CRDS; five laboratories used EA-IRMS only. Each laboratory used at least two standards for normalization of $\delta^{13}C$ values and at least one verification standard to check the stability of the run during the analysis sequence. Table 1 specifies the standards utilized by each laboratory for quality control. Laboratories were left to their own quality control guidelines to ensure their reported values were appropriate. All laboratories used normalization standards with $\delta^{13}C$ values which bracketed that of the sample range, and check standards with the normalization range. For laboratories that used both EA-IRMS and CM-CRDS, the same standards were used for both techniques.

Table 1: The isotopic standards used in the study

EA-IRMS/CM-CRDS								
	Normalization Standard - 1	Normalization Standard - 2	Normalization Standard - 3	Normalization Standard - 4	Check Standard - 1	Check Standard - 2	Check Standard - 3	Check Standard - 4
Lab 1	A	B	-	-	B	C	D	A
Lab 2	E	F	G	H	I	K	-	-
Lab 3	H	B	-	-	R	-	-	-
Lab 4	H	B	-	-	M	-	-	-
Lab 5	A	N	-	-	O	P	-	-
Lab 6	J	L	-	-	K	-	-	-
Lab 7	A	N	K	-	S	-	-	-
Lab 8	A	B	-	-	Q	-	-	-

A	NIST RM 8573 L-Glutamic Acid; -26.39 ‰	J	USGS 61, Caffeine; -35.05 ‰
B	NIST RM 8542 Sucrose; -10.45 ‰	K	USGS 62, Caffeine; -14.79 ‰
C	IAEA-CH3 Cellulose; -24.72 ‰	L	USGS 63, Caffeine; -1.17 ‰
D	EIL-72 Cellulose; -25.47 ‰	M	Fructose -1: -21.1 ‰
E	Fructose -ILS; -10.98 ‰	N	NIST RM 8574 L-Glutamic Acid; +37.63 ‰
F	Galactose ILS; -21.41 ‰	O	Acetanilide; -26.3 ‰
G	Sucrose ILS -26.02 ‰	P	Urea_UIN3; -11.7 ‰
H	NIST RM 8540 'Polyethelene Foil; -32.15 ‰	Q	Acetanilide; -28.32 ‰
I	Nicotinamide ILS; -22.95 ‰	R	HP-V3 (In-Lab Honey Check); -25.66 ‰
ILS	Internal Laboratory Standard	S	Cane Sugar; -11.83 ‰

3 Results and Discussion

3.1 Sample Preparation Optimization

The precipitation of citrate was initially carried out with calcium hydroxide ($Ca(OH)_2$) as described in Doner et al. [4] To assess the $\delta^{13}C$ values of the isolated citrates, the two citric acid sources (A & B) were dissolved in degassed water (~6% w/w, to mimic approximate citric acid levels in lemon juice), isolated, and analyzed by EA-IRMS. The resulting $\delta^{13}C$ values of the isolated citrate were compared to that of the respective neat form. When using the procedure from Doner and coworkers, the values were similar, however, the method blanks (water rather than lemon juice) used to test contribution from reagents exhibited an elevated CO_2 signal, indicating the presence of a carbonaceous impurity in the commercial attributed to dissolved carbonates in the calcium hydroxide [6]. Alternate sources from various vendors of calcium hydroxide were tested and all contained detectable carbon impurities. The observed amount of the carbon from calcium hydroxide had a negligible impact on the $\delta^{13}C$ values of the isolated citrate. However, due to an unpredictable quantity of such impurities, such interference should be avoided. Reducing the amount of calcium hydroxide resulted in an

unacceptably low yield of citrate. Similar experiments were carried out substituting calcium hydroxide with calcium chloride ($CaCl_2$) or calcium nitrate ($Ca(NO_3)_2$), or using sodium or ammonium hydroxides to adjust the pH. Precipitation of the citrates with $Ca(NO_3)_2$, in combination with either NaOH or NH_4OH freshly prepared solutions, resulted in a slight positive shift in $\delta^{13}C$ values relative to the neat citric acids. Precipitation of citrates with $CaCl_2$, in combination with either NaOH or NH_4OH, produced results consistent with the neat citric acids and with no measurable carbon signal for method blanks. Use of NH_4OH provided better control of the pH adjustment and produced more precipitate than was achieved with NaOH. For this study, $CaCl_2$ was used along with NH_4OH to precipitate citrate from lemon juice, lemon juice concentrate and citric acid samples, using the finalized conditions described in Sample Preparation Procedure.

3.2 Single Laboratory Validation Utilizing Modified Sample Preparation Procedure

The modified sample procedure was validated for both accuracy and precision by EA-IRMS analysis, using two commercially available citric acids (Source A and B), three locally purchased lemon juice (from concentrate) samples and freshly squeezed, locally purchased lemons.

3.2.1 Accuracy

Assessment of accuracy was based on a comparison of results obtained from testing the calcium citrate precipitated from solutions of two pure citric acid sources, to the results obtained from the neat citric acid by EA-IRMS. The mean $\delta^{13}C$ obtained from calcium citrate isolated from a 6% (w/w) aqueous solution of Source A (apparently derived from a C4 plant source) was -12.23 ± 0.7‰ (n=3, ±2σ) and that from the $\delta^{13}C$ obtained from the neat citric acid, -12.16 ± 0.04‰ (n=2, ±2σ). Similarly, the mean $\delta^{13}C$ obtained from calcium citrate isolated from a 6% (w/w) aqueous solution of Source B (apparently derived from a primarily C3 plant source) was -23.94 ± 0.02‰ (n=3, ±2σ) and that from the $\delta^{13}C$ obtained from the neat citric acid (-24.15 ± 0.02‰, n=3, ±2σ). These results demonstrated that the precipitation process does not induce significant isotopic fractionation to citric acid.

Accuracy of the procedure was further demonstrated by comparison of results from the multi-laboratory round-robin study presented in Section 3.4

3.2.2 Precision

The short term and intermediate precision of the modified procedure was demonstrated by comparison of the results obtained for three bottles of each of three brands of locally purchased lemon juice and juice from freshly squeezed lemons. Three different analysts performed triplicate citrate isolations on each of the three bottles and the fresh lemon juice; each analyst performed their analysis on a separate day. Each of the citrates were weighed in triplicate and analyzed by EA-IRMS. The results are summarized in Table 2. The mean standard deviation for thirty-six sets of triplicate analysis (triplicates from four lemon juice sources analyzed by three analysts) was 0.06‰ (max = 0.13‰). The mean standard deviation for 27 preparations from each brand (three preparations, three days, three analysts/brand) was 0.10‰ (max = 0.11‰). The results obtained for each brand by the three analysts agreed to within 0.2‰.

Table 2: Precision

Lemon Juice Type	Analyst 1 $\delta^{13}C$ (n=9, ± 2σ)	Analyst 2 $\delta^{13}C$ (n=9, ± 2σ)	Analyst 3 $\delta^{13}C$ (n=9, ± 2σ)
Brand 1	**-26.86** ± 0.04 ‰	**-26.95** ± 0.18 ‰	**-26.99** ± 0.14 ‰
Brand 2	**-26.79**± 0.12 ‰	**-26.95** ± 0.12 ‰	**-26.98** ± 0.08 ‰
Brand 3	**-24.44** ± 0.12 ‰	**-24.51** ± 0.28 ‰	**-24.60** ± 0.10 ‰
Fresh Lemon Juice	**-25.40** ± 0.10 ‰	**-25.39** ± 0.16 ‰	**-25.53** ± 0.10 ‰

The precision of the procedure was further demonstrated by comparison of results obtained for three brands of lemon juice from concentrate (three bottles each of brand, analyzed by three different analysts on three different days) using the modified sample preparation to historical results (past results produced by US FDA laboratory) derived from the $Ca(OH)_2$ based method. The mean $\delta^{13}C$ values obtained for the validation trials were not statistically distinguishable from the historical $\delta^{13}C$ values as seen on Table 3. The historical values for the lemon juices and the fresh lemon juice was obtained by using lemon juice from the same bottle, for each brand and fresh lemon juice for a period of 2 years.

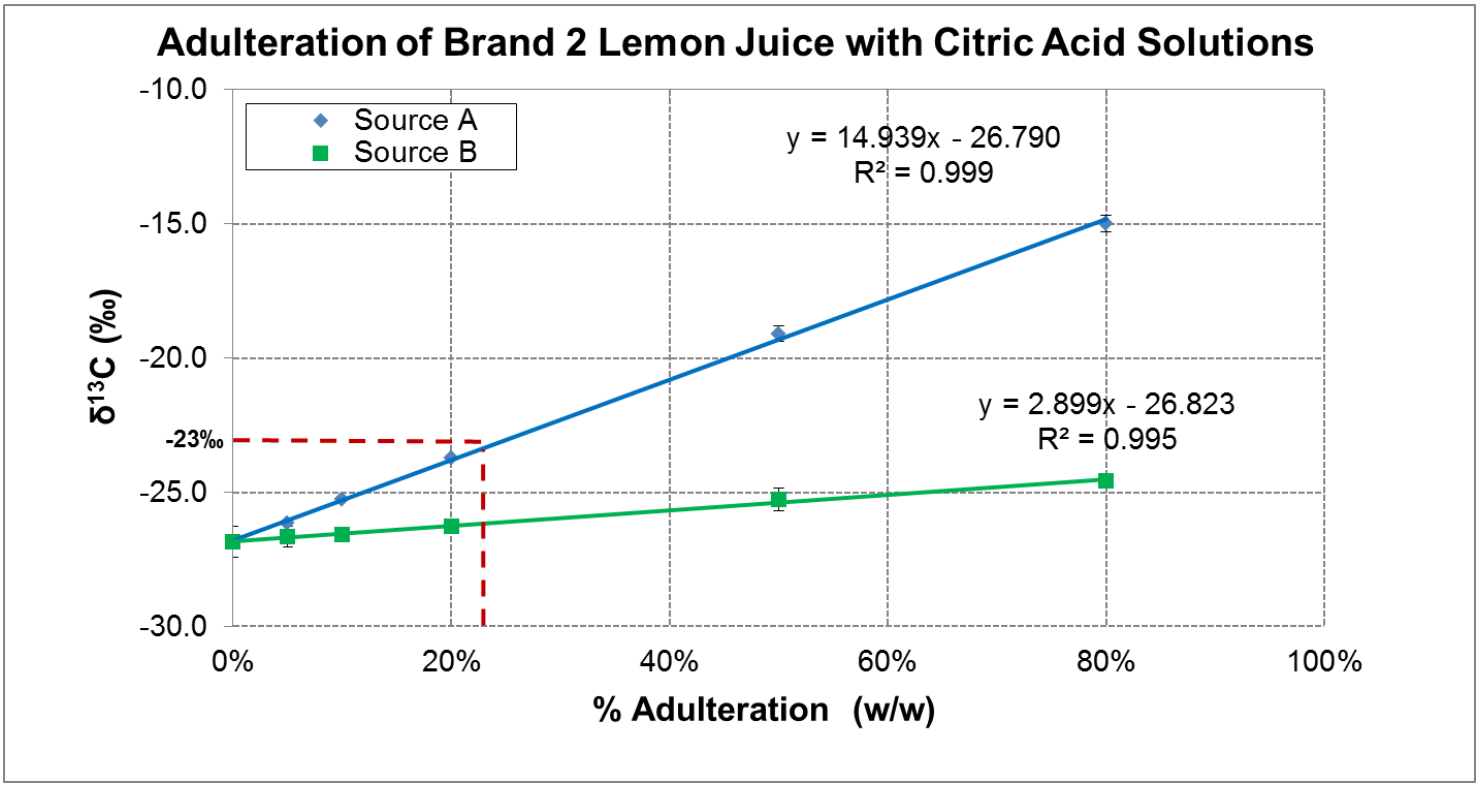
Table 3: Precision comparison with the historical results

Lemon Juice Type	Average $\delta^{13}C$ (n=27, ± 2σ)	Historical Average $\delta^{13}C$ (± 2σ)
Brand 1	**-26.96** ± 0.16 ‰	**-26.88** ± 0.52 ‰
Brand 2	**-26.91** ± 0.20 ‰	**-27.03** ± 1.52 ‰
Brand 3	**-24.52** ± 0.22 ‰	**-24.42** ± 0.64 ‰
Fresh Lemon Juice	**-25.44** ± 0.18 ‰	**-25.38** ± 0.26 ‰

3.2.3. Verification of Adulteration Detection Threshold

Brand #2 lemon juice was adulterated with 6% of two commercially available citric acids (Source A ($\delta^{13}C$ = -12.16‰) and Source B ($\delta^{13}C$ = -24.15‰)) by 0%, 5%, 10%, 20%, 50% and 80% (w/w). The citrate was precipitated and analyzed by EA-IRMS. The data is presented in Figure 1. In this study, lemon juice samples were considered adulterated when the $\delta^{13}C$ values of citrate were more positive than -23‰. The detection of adulteration is possible when citric acid derived from a C4 plant is added. The exact detection threshold is also dependent on the $\delta^{13}C$ of the original juice. In this particular example, an adulteration of lemon juice Brand #2 ($\delta^{13}C$ = -26.91 ± 0.07‰) with the addition of a C4 based citric acid (Brand A, $\delta^{13}C$ = -12.16 ± 0.22‰) would be interpreted as adulterated at approximately 25% w/w or greater (based on a citric acid $\delta^{13}C$ value of > -23‰). Adulteration with a citric acid source from a C3 source (Source B) is practically undetectable using the given methodology.

Figure 1: The change in $\delta^{13}C$ values of precipitated calcium citrate upon addition of solutions of 6% (w/w) citric acid from Source A (blue) and Source B (green).



3.2.4. Analyte Response Linearity and Sensitivity Determination

The IRMS linearity criteria used within our laboratory, based on manufacturer's recommendations, was a slope less than 0.066‰/V [5]; this criteria was routinely confirmed over a signal range from 500 mV to 10,000 mV for m/z 44 y [13]. The CM-CRDS was demonstrated to have a linear response over a $^{12}CO_2$ concentration range from 1,000 to 9,000 ppm (which corresponds to 0.25 mg to 2.25 mg of carbon) [13].

3.2.5. Analytical Working Range

The approximate amount of calcium citrate needed to produce EA-IRMS signals in the range of 1,000 mV to 10,000 mV for *m/z* 44 is 0.08 mg (at 0% sample dilution) to 10 mg (at 95% sample dilution). The typical analytical portion of calcium citrate used for EA-IRMS, in this study, was 0.3 mg to 2 mg. Similarly, for CM-CRDS, the amount of calcium citrate needed to produce signals in the range of 1000 ppm to 9,000 ppm $^{12}CO_2$ was 0.7 mg to 6 mg [13], which was the typical analytical portion of calcium citrate used in this study, expanding upon the manufacturer's recommended linearity range of 2000 to 5000 ppm

3.2.6 *Comparability of Accuracy*

The comparison of $\delta^{13}C$ values of calcium citrate isolated from the 69 imported lemon juice samples is shown in Table 4 and Fig. 2. The results are in a good agreement. The average difference between the measured $\delta^{13}C$ values was -0.14‰ with a range of -0.30‰ to 0.13‰. This represents a general bias of CM-CRDS values being slightly negative compared to EA-IRMS values. The average bias of -0.14‰ is less than the generally acceptable standard deviation of 0.2‰ for EA-IRMS and 0.3‰ for CM-CRDS, therefore it was deemed insignificant.

3.2.7 *Comparability of Adulteration Classification*

For the purpose of this study, lemon juice samples were classified as adulterated when the $\delta^{13}C$ values of citrate were greater than -23‰ and not adulterated otherwise. The results were considered inconclusive when the 2σ range around the mean overlapped the classification threshold of -23‰ (σ only includes analysis variability among replicates). The classification (not adulterated, inconclusive, or adulterated) based on the CM-CRDS results were in good agreement with the classification based on EA-IRMS results. Fifty-seven samples were classified as not adulterated, nine samples were classified as adulterated, and one sample classified as inconclusive by both techniques. Although the overall replicate variability has been shown to be smaller for EA-IRMS, the two samples (#36 and 38) classified as inconclusive based on EA-IRMS results, were classified as not adulterated based on CM-CRDS results, due a smaller replicate variation (±2σ).

3.2.8 *Comparability of Precision*

The mean standard deviation for 69 sets of triplicate analysis by CM-CRDS (0.06‰) compared well with the mean standard deviation (0.07‰) obtained for the same samples by EA-IRMS. The pooled standard deviations for samples classified as adulterated (0.05‰) and for samples classified as not adulterated (0.05‰) by CM-CRDS were similar to the pooled standard deviations obtained by EA-IRMS (0.06‰) and (0.04‰), respectively.

Sixty-five of the sixty-nine samples were analyzed in triplicates, each from three separate isolations of citrate from lemon juice. Four other samples were analyzed in triplicate, but from single isolation of citrate from the lemon juice. Standard deviations of the $\delta^{13}C$ values from both analysis methods were similar, which demonstrates the reproducibility of the precipitation method.

Figure 2: Comparison of 69 calcium citrate isolates as determined by IRMS and CRDS.

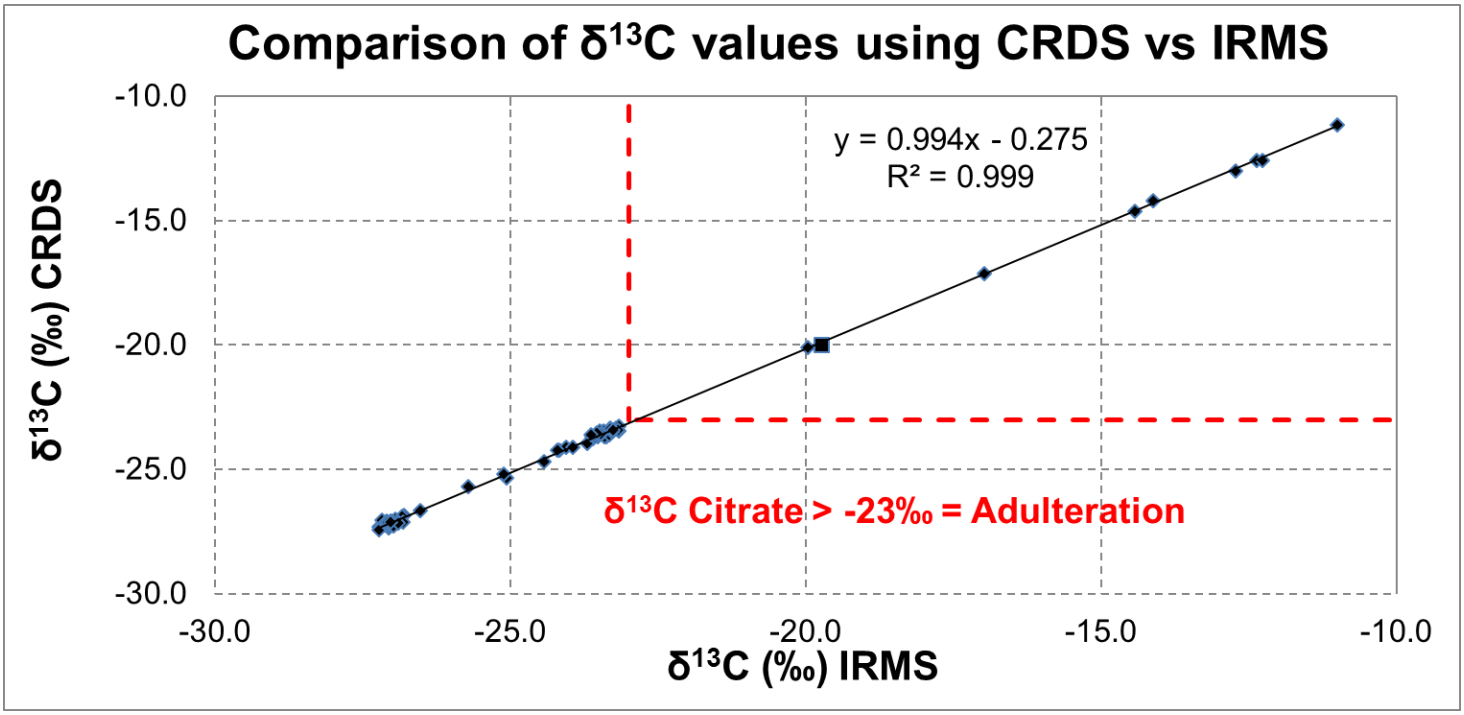


Table 4: $\delta^{13}C$ for 69 calcium citrate isolates as determined by IRMS and CRDS.

Sample No.	IRMS		CRDS		Sample No.	IRMS		CRDS	
	$\delta^{13}C \pm 2\sigma$, ‰	Results[1]	$\delta^{13}C \pm 2\sigma$, ‰	Results[1]		$\delta^{13}C \pm 2\sigma$, ‰	Results[1]	$\delta^{13}C \pm 2\sigma$, ‰	Results[1]

1	-26.80±0.04	NA	-26.86±0.02	NA	**36**	-23.18±0.22	I	-23.42±0.17	NA
2	-26.84±0.04	NA	-26.88±0.06	NA	**37**	-23.46±0.02	NA	-23.56±0.06	NA
3	-16.98±0.06	A	-17.13±0.06	A	**38**	-23.28±0.28	I	-23.49±0.08	NA
4	-19.73±0.12	A	-20.00±0.16	A	**39**	-23.70±0.14	NA	-23.97±0.30	NA
[2]5	-27.23±0.02	NA	-27.30±0.14	NA	**40**	-23.31±0.02	NA	-23.40±0.04	NA
[2]6	-24.17±0.14	NA	-24.22±0.14	NA	**41**	-23.49±0.32	NA	-23.47±0.14	NA
7	-12.72±0.12	A	-13.00±0.12	A	**42**	-23.47±0.24	NA	-23.57±0.10	NA
[2]8	-11.00±0.12	A	-11.14±0.08	A	**43**	-23.54±0.26	NA	-23.53±0.10	NA
9	-14.43±0.04	A	-14.64±0.12	A	**44**	-23.64±0.18	NA	-23.63±0.10	NA
10	-24.06±0.02	NA	-24.12±0.10	NA	**45**	-23.27±0.08	NA	-23.41±0.10	NA
[2]11	-24.20±0.24	NA	-24.22±0.12	NA	**46**	-25.71±0.38	NA	-25.70±0.08	NA
12	-25.06±0.04	NA	-25.34±0.12	NA	**47**	-27.02±0.08	NA	-27.13±0.20	NA
13	-23.52±0.06	NA	-23.69±0.18	NA	**48**	-27.07±0.22	NA	-27.34±0.20	NA
14	-23.21±0.18	NA	-23.35±0.12	NA	**49**	-27.10±0.26	NA	-27.09±0.04	NA
15	-23.16±0.22	I	-23.28±0.28	I	**50**	-26.98±0.22	NA	-27.16±0.26	NA
16	-23.31±0.04	NA	-23.35±0.12	NA	**51**	-27.12±0.04	NA	-27.24±0.16	NA
17	-23.41±0.12[3]	NA	-23.59±0.14	NA	**52**	-26.95±0.26	NA	-27.01±0.10	NA
18	-23.61±0.30	NA	-23.76±0.22	NA	**53**	-27.11±0.16	NA	-27.22±0.30	NA
19	-24.43±0.06	NA	-24.71±0.18	NA	**54**	-27.03±0.04	NA	-27.07±0.12	NA
20	-26.82±0.04	NA	-27.11±0.26	NA	**55**	-27.01±0.14	NA	-27.10±0.22	NA
21	-23.37±0.04	NA	-23.67±0.08	NA	**56**	-27.23±0.16[3]	NA	-27.43±0.18	NA
22	-12.36±0.22	A	-12.59±0.06	A	**57**	-27.08±0.02	NA	-27.26±0.16	NA
23	-12.27±0.08	A	-12.57±0.08	A	**58**	-27.07±0.10	NA	-27.24±0.12	NA
24	-14.12±0.08	A	-14.19±0.04	A	**59**	-27.04±0.06	NA	-27.21±0.02	NA
25	-25.12±0.12	NA	-25.19±0.20	NA	**60**	-27.17±0.08	NA	-27.04±0.08	NA
26	-19.96±0.14	A	-20.12±0.04	A	**61**	-27.12±0.04	NA	-27.11±0.08	NA
27	-23.94±0.20	NA	-24.12±0.16	NA	**62**	-26.97±0.04	NA	-27.23±0.10	NA
28	-23.51±0.04[3]	NA	-23.57±0.06	NA	**63**	-27.00±0.10	NA	-27.25±0.10	NA
29	-23.46±0.06	NA	-23.54±0.05	NA	**64**	-27.08±0.06	NA	-27.14±0.08	NA
30	-23.62±0.08	NA	-23.67±0.12	NA	**65**	-26.93±0.20	NA	-27.12±0.12	NA
31	-23.41±0.04	NA	-23.68±0.12	NA	**66**	-26.99±0.06	NA	-27.26±0.22	NA
32	-27.17±0.04	NA	-27.29±0.10	NA	**67**	-26.90±0.04	NA	-27.15±0.28	NA
33	-23.33±0.06	NA	-23.62±0.16	NA	**68**	-26.52±0.08	NA	-26.66±0.30	NA
34	-23.43±0.10	NA	-23.45±0.12	NA	**69**	-27.03±0.44	NA	-27.11±0.10	NA
35	-23.16±0.10	NA	-23.45±0.04	NA					

Unless otherwise noted, 2σ is based on analysis of one weighing for each of three individual isolation preparations
[1]NA = Not Adulterated, A = Adulterated, I = Inconclusive [2] Triplicate analyses of a single preparation. [3]n=2

3.3 *Analytical Results from the Round-Robin Study*

Reported averages (±2σ, ~95% confidence) provided by eight laboratories for the six citrate samples analyzed by EA-IRMS and CM-CRDS are presented in Table 5a and 5b, respectively. The overall average of the reported $\delta^{13}C$ values for each of the six citrate samples are not statistically

distinguishable when comparing those from each technique. The largest spread between reported average values was 0.48‰ for EA-IRMS and 0.37‰ for CM-CRDS. Additionally, the largest difference between a reported value from a laboratory and the overall average (using the same technique), was 0.36‰ (sample 23 by laboratory 4 using EA-IRMS), however, this was not a statistical outlier using the Grubb's test. For a given sample, the overall average of the reported citrate $\delta^{13}C$ values differed by <0.15‰ between CM-CRDS and EA-IRMS, which is within generally accepted analysis deviations of 0.2‰ and 0.3‰ for EA-IRMS and CM-CRDS, respectively. Repeatability (within laboratory, r) and reproducibility (among laboratories, R), were estimated using the AOAC International Interlaboratory Study Workbook for Blind (Unpaired) Replicates [21]. The average r and R for all six samples were 0.17‰ and 0.30‰, respectively, for IRMS, and 0.18‰ and 0.37‰, respectively, for CRDS. A comparable inter-comparison study was performed by Guillou et. al. [12], involving seventeen laboratories examining acids isolated from juices and analyzed by EA-IRMS. The resulting r and R values were 0.58‰ and 1.75‰, respectively. These relatively large values were attributed to the fact that each of the seventeen laboratories isolated the acids prior to analysis, whereas in this study, the samples were prepared in one laboratory, homogenized, and distributed for analysis. Perhaps a more comparable criterion derives from ten of the participants in the Guillou study that analyzed the reference material NBS 22 ($\delta^{13}C_{VPDB}$ = -29.73 ± 0.09‰) with an average $\delta^{13}C$ value of -29.8‰, with repeatability and reproducibility of 0.20‰ and 0.27‰, respectively, however this only represents the analysis of one sample rather than six in the presented study.

In our study, the results from each of the participating laboratories had allowed to correctly classify the previously determined non-adulterated and adulterated samples using both techniques. Only one result would have been reported as inconclusive (sample number 15, by laboratory 8 using CM-CRDS), the remainder of the "inconclusive" samples would be classified as not adulterated.

Table 5a: $\delta^{13}C$ values of Calcium Citrate determined by EA-IRMS. The overall average represents an unweighted average of the reported averages for each sample.

EA-IRMS ($\delta^{13}C \pm 2\sigma$ (‰))						
Samples	**Adulterated**		**Inconclusive**		**Not Adulterated**	
Lab #	**7**	**23**	**15**	**16**	**64**	**69**
Lab 1	-12.76 ± 0.18	-12.42 ± 0.08	-23.26 ± 0.06	-23.25 ± 0.08	-26.88 ± 0.08	-26.90 ± 0.04
Lab 2	-12.93 ± 0.04	-12.55 ± 0.08	-23.35 ± 0.02	-23.36 ± 0.02	-27.07 ± 0.08	-27.01 ± 0.08
Lab 3	-12.90 ± 0.06	-12.52 ± 0.06	-23.40 ± 0.10	-23.30 ± 0.04	-27.06 ± 0.04	-27.08 ± 0.16
Lab 4	-12.71 ± 0.16	-12.09 ± 0.12	-23.33 ± 0.06	-23.31 ± 0.06	-26.99 ± 0.02	-26.75 ± 0.16
Lab 5	-12.82 ± 0.08	-12.51 ± 0.06	-23.33 ± 0.02	-23.35 ± 0.14	-27.02 ± 0.44	-26.94 ± 0.14
Lab 6	-12.81 ± 0.44	-12.57 ± 0.30	-23.34 ± 0.08	-23.43 ± 0.32	-26.93 ± 0.32	-26.96 ± 0.18
Lab 7	-12.78 ± 0.04	-12.41 ± 0.06	-23.29 ± 0.04	-23.29 ± 0.04	-26.99 ± 0.04	-26.98 ± 0.04
Lab 8	-12.69 ± 0.12	-12.27 ± 0.08	-23.16 ± 0.22	-23.31 ± 0.04	-27.08 ± 0.06	-27.03 ± 0.44
Average	**-12.80 ± 0.16**	**-12.42 ± 0.33**	**-23.31 ± 0.15**	**-23.33 ± 0.11**	**-27.00 ± 0.14**	**-26.96 ± 0.20**

Table 5b: $\delta^{13}C$ values of Calcium Citrate determined by CM-CRDS. The overall average represents an unweighted average of the reported averages for each sample.

CM-CRDS ($\delta^{13}C \pm 2\sigma$ (‰))						
Samples	**Adulterated**		**Inconclusive**		**Not Adulterated**	
Lab #	**7**	**23**	**15**	**16**	**64**	**69**
Lab 1	-	-	-	-	-	-
Lab 2	-	-	-	-	-	-
Lab 3	-12.74 ± 0.18	-12.57 ± 0.18	-23.63 ± 0.20	-23.40 ± 0.20	-27.20 ± 0.12	-27.22 ± 0.34
Lab 4	-	-	-	-	-	-
Lab 5	-	-	-	-	-	-
Lab 6	-	-	-	-	-	-
Lab 7	-12.80 ± 0.24	-12.47 ± 0.16	-23.26 ± 0.20	-23.34 ± 0.26	-26.99 ± 0.24	-26.94 ± 0.16
Lab 8	-13.00 ± 0.12	-12.57 ± 0.08	-23.28 ± 0.29	-23.35 ± 0.12	-27.15 ± 0.08	-27.11 ± 0.10
Average	**-12.85 ± 0.27**	**-12.54 ± 0.12**	**-23.39 ± 0.42**	**-23.36 ± 0.06**	**-27.11 ± 0.22**	**-27.09 ± 0.28**

4. Conclusion

While readying our laboratory to determine lemon juice adulteration via exogenous citric acid addition, we improved a previously presented citrate isolation procedure to remove possible carbon contamination. After extensive testing of this procedure using EA-IRMS analysis, several imported samples were also analyzed by CM-CRDS. Both techniques showed excellent agreement in the

determination of $\delta^{13}C$ values of the calcium citrate precipitated from the 69 lemon juices using the improved methodology. A round-robin study involving eight laboratories was successful in assessing the accuracy of the CM-CRDS compared to EA-IRMS. Given that the data produced by CM-CRDS is statistically indistinguishable (<0.15‰ difference) from EA-IRMS, CM-CRDS could be implemented as an alternative analysis technique for the determination of adulteration in lemon juice. It should be noted that the overall standard deviations associated with replicate variability of CM-CRDS were slightly higher than those of EA-IRMS, which could potentially lead to more inconclusive results compared to EA-IRMS. Additionally, more citric acid is needed for analysis via CM-CRDS than for EA-IRMS. Although these issues are worth considering, they are minor and do not preclude CM-CRDS from this application. Furthermore, this study adds to the growing body of literature that supports CM-CRDS as a comparable technique for multiple matrices.

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