Determination of Chromium in Refined and Unrefined Sugars by Oxygen Plasma Ashing Flameless Atomic Absorption

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The interest in possible submarginal deficiencies of trace elements that may occur as a consequence of changing nutritional practices has led to the collection and measurement of the chromium content of refined and unrefined sugars from various countries. Analyses were carried out by graphite furnace atomic absorption. Cr content which in the less refined sugars and in the molasses amounts to 160 and 270 ng/g drops to one-tenth of these values when sugar is refined. Cr in sugar, as in many biological sources, may occur in different chemical forms. The biologically active form is most likely an organic complex whose chemical and analytical characteristics are poorly known. Erroneously low analytical results are obtained due to the loss of an organic Cr complex when sugar samples are introduced directly into the graphite furnace or ashed at 450° prior to analysis; added inorganic Cr is not lost. More Cr is lost from the unrefined sugars than from the refined suggesting a decrease in content of this organic Cr during refinement. To avoid this loss of Cr, it is essential that the sugar samples be ashed at a low temperature, *e.g.*, by the action of an activated oxygen plasma. It is likely that similar Cr analytical problems exist with other biological materials.

In an attempt to better understand the role of dietary factors in the etiology of certain diseases, attention is being focused on trace elements and particularly on submarginal deficiencies of these elements that may occur as a consequence of nutritional practices among highly industrialized population groups (Masironi, 1970). Within the framework of WHO activities aimed at elucidating the relationship existing between dietary habits and geographic distribution of cardiovascular diseases (mainly arteriosclerotic heart disease), it was thought necessary to initiate coordinated studies whereby widely used foodstuffs would be collected in various countries and analyzed for trace elements by using different techniques. Standardized protocols and intercomparison of results obtained by various laboratories are essential in these studies. This holds true particularly for chromium, the analysis of which still presents considerable uncertainty, as discussed at a recent IAEA/WHO research coordination meeting (WHO/IAEA, 1973).

Chromium is one of the trace elements that is being studied with increasing thoroughness because of its essentiality for insulin action and maintenance of normal glucose tolerance (Schwarz and Mertz, 1959; Mertz, 1969; Mertz and Roginski, 1971). Impaired glucose tolerance may develop into a diabetic state, which is an important risk factor in the etiology of atherosclerosis and myocardial infarction, and chromium deficiency may possibly play a harmful role. Chromium was also reported in some studies to exert beneficial effects against experimentally induced atherosclerosis and to decrease blood cholesterol levels in rats (Schroeder *et al.*, 1970; Staub *et al.*, 1969). Excessive consumption of refined sugar was found to be statistically associated with high cardiovascular mortality (Masironi, 1970), although a direct cause-to-effect relationship was not ascertained.

Tissues of elderly subjects sampled from more affluent countries where coronary heart disease is highly prevalent have very low Cr content while tissues of subjects from developing areas of the world, whose populations are less susceptible to this disease, have higher Cr content (Schroeder *et al.*, 1970). The above observations may possibly be linked together by the findings (Schroeder *et al.*, 1970) that refined sugar contains less Cr than brown and unrefined sugars and reports indicating that glucose increases mobilization and depletion of body Cr *via* the urine (Glinnsman *et al.*, 1966; Schroeder, 1968).

In order to evaluate precisely the extent of Cr loss in sugar as a consequence of refinement, WHO has collected various types of sugar from several countries and is coordinating and comparing analyses among various collaborating laboratories which use different analytical techniques. These samples have been analyzed at the Vitamin and Mineral Nutrition Laboratory, U. S. Department of Agriculture, by a flameless atomic absorption technique. Preliminary reports of this study (Wolf *et al.*, 1973; Masironi *et al.*, 1973) have indicated that the analytical response of Cr in sugar samples by using the graphite furnace atomic absorption is very dependent upon the method of preparation of samples before they are introduced into the furnace. In this paper we would like to describe and detail these studies and their implications for Cr analysis in biological samples in general.

MATERIALS AND METHODS

The unrefined sugars were hard blocks of solidified cane squeezings which had undergone no refinement. The brown sugars were, in general, produced by adding molasses or coloring to refined white sugar. Origins of the sugar samples are listed in Table I. Except for the beet sugar from the U. S. (no. 1), all others were cane sugars. Fourteen of the sugars (no. 2–6 and 12–20) were obtained with the help of the WHO Regional Advisors in Nutrition; the other six (no. 1, 7–11) and the three household molasses samples (no. 21–23) were bought in local U. S. stores. The samples were stored in plastic bags or original containers.

The sugars were then prepared for analysis in the following ways. (a) One gram of each sugar sample was dissolved in 10 ml of doubly distilled water (Cr < 0.5 ppb). The refined and brown sugar samples and the molasses dissolved readily but the unrefined sugars left a residue, apparently of vegetable fibers, which was centrifuged off. These residues represented 1.5 ± 0.2% of the sample weight.

(b) One milliliter of each water solution of (a) was dried under a heat lamp in a 5-ml Pyrex dish and ashed in an activated oxygen plasma low-temperature asher (LTA-505, LFE Corporation, Waltham, Mass.). This plasma is

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Table I. Types of Sugar Used in Present Study

	Code no.	Origin	Local name	Characteristics	Used mainly by
Refined	1	U. S. store (Grand Forks, N. D.)	Beet sugar	Free-flowing white crystals	All economic classes
	2	Rio de Janeiro	Diamantino Moido (Novia)	White fine powder, confectioner sugar	Higher and middle class of large cities
	3	Chiclayo, Peru	Azucar AAA Refinada	Free-flowing white crystals	Higher and middle class of large cities
	4	Lima, Peru	Azucar Refinada para Consumo Domestico	Free-flowing white crystals	Higher and middle class of large cities
	5	Lima, Peru	Azucar Industrial	Free-flowing white crystals	All classes (mostly in soft drinks)
	6	Chiclayo, Peru	Azucar Refinada	Free-flowing white crystals	Higher and middle class of large cities
	7	U. S. store	Superfine quality cane	Free-flowing, white microcrystals	All economic classes
Brown	8	U. S. store (Washington, D. C.)	Dark brown cane	Sticky brown crystals	All economic classes
	9	U. S. store (Washington, D. C.)	Dark brown cane	Sticky brown crystals	All economic classes
	10	U. S. store (Washington, D. C.)	Light brown cane	Sticky light brown crystals	All economic classes
	11	U. S. store (Washington, D. C.)	Light brown cane	Sticky light brown crystals	All economic classes
	12	Chiclayo, Peru	Cruda-B, exportacion	Free-flowing brown crystals	Mainly exported to U. S.
Unrefined	13	Guatemala City	Panela blanca	Dark brown, sticky, hard blocks	Middle and lower classes
	14	Guatemala City	Panela blanca	Dark brown, sticky, hard blocks	Middle and lower classes
	15	Guatemala City	Panela obscura	Very dark brown, sticky hard blocks	Lower class (rural areas)
	16	Guatemala City	Panel negra	Dark brown, sticky hard blocks	Lower class (rural areas)
	17	Cajamarca, Peru	Chancaca "Callacate"	Dark brown, sticky hard blocks	All classes—mainly for homemade sweets
	18	Cajamarca, Peru	Chancaca "Callayne"	Dark brown, sticky hard blocks	All classes—mainly for homemade sweets
	19	Cajamarca, Peru	Chancaca "Cochabamba"	Dark brown, sticky hard blocks	All classes—mainly for homemade sweets
	20	Cajamarca, Peru	Chancaca "Paccha"	Dark brown, sticky hard blocks	All classes—mainly for homemade sweets
Molasses	21	U. S. store (Washington, D. C.)	Sorghum and cane molasses	Thick, brown liquid	All economic classes
	22	U. S. store (Washington, D. C.)	Cane molasses	Thick dark-brown liquid	All economic classes
	23	U. S. store (Washington, D. C.)	Cane molasses, unsulfured	Thick dark-brown liquid	All economic classes

at a relatively low temperature, <150°. The residues from the unrefined sugar samples were also dried and ashed. The refined sugar samples ashed completely in about 1 hr, but the brown and the unrefined sugar samples, the residues, and the molasses took much longer. It was found useful, therefore, to interrupt the ashing after 3–4 hr. This time period was enough to char, although not to completely ash, the samples. Ashing was then completed in a muffle furnace at 450° where the previously charred samples would ash in about 0.5 hr. The brown sugar samples

Table II. Cr Content in Various Types of Sugars

Code no.	ng/g of sample: Oxygen plasma ashed (150°)	Muffle furnace ashed (450°)	Graphite furnace ashed (1000°)
Refined			
1	21	22	8
2	13	21	3
3	11		6
4	11		9
5	22		8
6	35		9
7	20	21	7
Brown			
8	154	141	34
9	56	32	27
10	53	68	35
11	74	58	33
12	71	56	26
Unrefined			
13	160	101	9
14	56	35	7
15	276	97	42
16	120	71	22
17	39	33	18
18	232	69	23
19	160	60	24
20	250	217	39
Molasses			
21	258	95	39
22	371	235	27
23	161	58	20

contained an average 1.6% ash, and the unrefined sugar samples 2.7% ash.

(c) Other aqueous samples of (a) were ashed only in the muffle furnace at 450° without being precharred in the low temperature asher. Under these conditions ashing took 3–4 hr.

(d) In other runs, 100-mg aliquots of unrefined sugar samples were ashed *in toto* either in the low temperature asher or in the muffle furnace at 450°. When ashing was completed by any of these methods, each sample was dissolved in 1 ml of 1.2 *M* HCl (Ultra High Purity, J. T. Baker Chemical Co., Phillipsburg, N. J.).

The solutions of each of the above preparations, *i.e.*, nonashed (a) and ashed (b,c,d), were analyzed by flameless atomic absorption by using a Model 303 double beam atomic absorption spectrophotometer (Perkin-Elmer Corporation, Norwalk, Conn.) equipped with an HGA-2000 Graphite Furnace and updated optical baffling. Signal output was connected *via* a recorder readout to a Perkin-Elmer Model 56 Strip Chart recorder. Nitrogen gas (Prepurified Grade, Air Products and Chemicals Co., Emmaus, Pa.) was used to purge the furnace and to provide an inert gas atmosphere. Fifty-microliter aliquots of the samples were introduced into the graphite furnace with an Eppendorf microliter pipet. The program cycle normally used was: sample drying, 30 sec at 100°; dry ashing, 30 sec at 1000°; and atomization, 15 sec at 2500°. Background correction with the deuterium arc was not used since preliminary studies showed no interferences in these relatively noncomplex carbohydrate matrices.

Analyses were done at least in duplicate by comparing the strip chart recorder response of the sample to that of inorganic chromium standards of increasing concentrations prepared in either dilute HCl or in a matrix of refined sugar solution in doubly distilled water. Appropriate calibration lines obtained with either water, HCl, or the sugar matrix solutions were used; these lines were not significantly different. Blanks were run throughout the whole ashing procedure. The sensitivity of this system was 0.05 ng of Cr for 1% absorption. Since the noise level of this system was approximately 0.3% absorption, the relative detection limit (twice the noise level) was approximately 0.03 ng of Cr. Based on a sample of 100 mg of sugar/ml of solution, a 50-μl aliquot would have a relative detection limit of 6 ng of Cr/g of sugar.

The emission spectrometer used was an Applied Research Laboratories Model 29,000, 1.5-m, diffraction grating, direct reading, optical spectrometer. The excitation chamber was a controlled atmosphere static argon arc chamber (Gordon, 1965, 1967, and 1968). Analytical conditions and electrode preparation were similar to those previously reported by Hambidge (1971). Twenty-microliter aliquots of the dissolved ash used for the AA analysis were placed on the prepared electrodes (Ultra F Purity C-7543, U-5, Ultracarbon Corporation, Bay City, Mich.) and arced at 15-A d.c.; the response was compared with responses of similar electrodes containing inorganic Cr standards.

RESULTS AND DISCUSSION

Cr contents for the individual samples analyzed with each of the ashing methods are summarized in Table II. The mean Cr content for each type of sample (Table III) shows the wide difference in results obtained by the different types of sample ashing. One of the apparent advantages of the graphite furnace atomizer is the potential of charring or ashing the sample in the furnace without any preanalysis treatment. This would eliminate extensive sample preparation and the concomitant potential for contamination which is a constant possible source of error in trace element analysis. However, results in Table III for

Table III. Mean Cr Content in Different Types of Sugars

Type	No. of samples	ng/g of sample[a]: Oxygen plasma ashing (150°)	Muffle furnace ashing (450°)	Graphite furnace ashing 1000° (direct anal.)
Molasses	3	266 ± 50	129 ± 54	29 ± 5
Unrefined	8	162 ± 36	88 ± 20	37 ± 13
Brown	5	64 ± 5	53 ± 8	31 ± 2
Refined	7	20 ± 3	25 ± 3	<10

[a] Values listed are averages of means of multiple determinations of each type ± standard mean error of this average.

Table IV. Cr Content of Water-Soluble and Residue Fractions in Oxygen Plasma Ashed Unrefined Sugars

Sample no.	ng/g of sample		
	Water-soluble fraction	Residue fraction	Total soluble + residue fractions
13	55	77	132
14	61	19	80
15	128	30	158
16	72	69	141
17	39	37	76
18	125	48	173
19	55	86	141
20	51	142	193
Mean ± SEM	74 ± 12	63 ± 14[a]	137 ± 14[b]

[a] Equivalent to 4.2 μg of Cr/g of residue. [b] Not significantly different from analysis of total Cr without fractionation (see Table III).

Table V. Comparison of Cr Content in Residue Fraction of Unrefined Sugar Samples by Atomic Absorption and Emission Spectrometry

Sample no.	ng of Cr/g of sample	
	AA	ES
13	77	58
17	37	17
18	48	60
19	86	78
20	142	65
Mean ± SEM	78 ± 18.3	56 ± 10.3

Cr in the sugar samples upon direct introduction into the graphite furnace appear to be approximately the same for the molasses, unrefined, and brown sugar samples.

If the samples are pre-ashed in the activated oxygen plasma, the Cr content is seen to be highest in molasses, followed by the content in unrefined sugars, and then in brown sugars; the least content is in the refined sugars. This pattern follows the refinement process, with the Cr of the unrefined sugars being removed and concentrated in the molasses, leaving the refined sugars low in this trace element. This pattern agrees on the whole with findings of Schroeder *et al.* (1970) and would suggest that these values are the ones representative of the Cr content of sugar, not the values obtained by direct analysis. For the unrefined sugar samples the contributions of the water-soluble and residue fractions (74 and 63 ng of Cr/g of sample, respectively) to the total content are similar (Table IV). The mean sum of these two fractions was not significantly different from the value from the analysis of the unrefined sugar *in toto* (137 ± 14 SEM *vs.* 162 ± 31). When referred to their own weights, the insoluble residues of the unrefined sugar samples appear to be rich in Cr, *i.e.*, an average of 4.2 μg/g of residue. The results on several of the ashed samples were checked by emission spectrometry (ES) (Table V). It was not possible to do the unashed samples by ES due to the matrix effects of organic material in this method (Hambidge, 1971). Analysis by both ES and AA of solutions of the ashed residues of the unrefined sugars with added Cr at the levels of 13–350 μg/g of residue were correlated by using linear regression. A correlation coefficient of 0.990 for the eight samples with a slope of 0.98 showed the good agreement between the two methods (Figure 1), which confirms the values of the Cr in the ashed solutions.

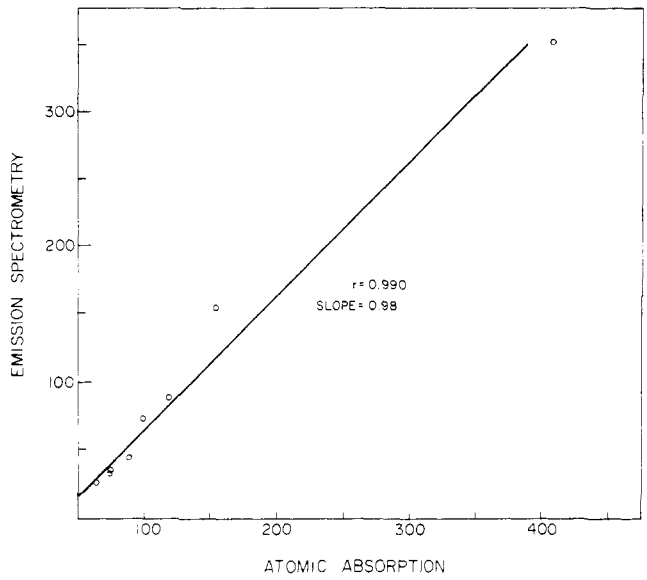

Figure 1. Comparison of atomic absorption and emission spectrometry analysis of Cr content of solutions of ashed residues (micrograms of chromium/gram of residue).

The possibility that the increase in Cr during the ashing procedures was due to contamination was very carefully considered. Laboratory procedures were carried out to minimize airborne, sample container, and ashing dish contamination. Sample transfer was kept at a minimum. Reagent blanks for the acids and water used were carried through the entire procedure for each set of two or three samples and results were corrected for blank values. A Dry Ice (CO_2) trap was placed in the vacuum line between the pump and the low-temperature asher to prevent back flow of Cr containing vapors from the vacuum pump. The low-temperature asher is an enclosed, all-glass system and would be expected to contribute less contamination than a muffle furnace. Since the results were higher with the low-temperature asher than the muffle furnace, this would suggest that the difference was not due to contamination. The strongest evidence against extraneous contamination during the analytical procedure is the data for the refined sugar samples. If these are considered as a blank then the maximum contamination introduced by ashing would be approximately 10 ng/g of sample, much below the differences seen for the brown sugar, unrefined sugar, and molasses samples.

Further experiments were carried out to determine the cause of the loss of Cr upon introduction of sugar samples directly into the graphite furnace. Prolonged ashing of these samples in a muffle furnace at 450° also led to loss of Cr compared to the oxygen plasma ashing method (Tables II and III). In both muffle furnace ashing and graphite furnace ashing, the higher the Cr content of the sugars the greater the percentage Cr loss compared to oxygen plasma ashing (Table VI). The percentage of Cr lost in the muffle ashing at 450° was less than in the direct addition ashing at 1000°, suggesting a temperature-dependent relationship and the possibility that the loss is due to a

Table VI. Per Cent Cr Lost Relative to Oxygen Plasma Ashing

Type of sugar	Graphite furnace ashing	Muffle furnace ashing
Refined	0	63
Brown	13	62
Unrefined	47	86
Molasses	52	89

Table VII. Recovery of Inorganic Cr in Refined Sugar Solution and HCl Solution

	Cr^{3+}		Cr^{6+}	
Ashing method	1.0 *N* HCl soln	Sugar soln	1.0 *N* HCl soln	Sugar soln[a]
Oxygen plasma	1.23[b]	1.03	1.03	1.03 ± 0.03 SEM
Muffle furnace	1.01	1.13	1.01	0.65 ± 0.06 SEM
Graphite furnace	0.98	1.20	1.07	0.99 ± 0.04 SEM

[a] Cr^{6+} in sugar, $N = 6$, all others $N = 2$ or 3. [b] ng of Cr/50 μl, expected 1.10 = 100% recovery.

naturally occurring volatile Cr compound. A previous report on the analysis of different types of foods by neutron activation analysis (Maxia *et al.*, 1972) has suggested possible losses of Cr content through volatilization of Cr in some unidentified form during drying of samples at 60° under vacuum.

Recovery of added inorganic Cr was used to check the possibilities of interactions with the sugar during ashing. Solutions of Cr^{3+} as $CrCl_3 \cdot 6H_2O$ (Baker Analyzed, J. T. Baker Chemical Co., Phillipsburg, N. J.) and Cr^{6+} as $K_2Cr_2O_7$ (Certified A.A. Standard, Fisher Scientific Co., Fair Lawn, N. J.) were made to give 20 ng of Cr/ml in 1 *N* HCl with and without 0.10 g of refined sugar/ml (*i.e.*, 200 ng of Cr/g of sugar). These solutions were then analyzed either directly or aliquots of 1.0 ml were dried, ashed, and redissolved in 1 *N* HCl. Aliquots (50 μl) were analyzed; expected response for each aliquot was 1.10 ng (1.0 ng added + 0.10 ng of Cr present in the refined sugar). The data in Table VII show that recovery was complete (within experimental error ± 10%) for Cr^{3+} in all cases and for Cr^{6+} with graphite furnace ashing or oxygen plasma ashing. Muffle furnace ashing of Cr^{6+} in a sugar matrix resulted in a significant loss of 42%. This suggests that in the direct method and oxygen plasma ashing the inorganic Cr does not react with the sugar to form a complex that is lost.

The fact that Cr^{6+} in the sugar matrix is lost when ashed at 450° rather than at 1000° in the graphite furnace can possibly be explained as follows. (a) Cr^{6+} is known to be a very good oxidizing agent for organic material and carbohydrates in particular. The added Cr^{6+} is reduced to Cr^{3+} by the sugar and in the process forms a complex which is lost either during the ashing at 450° or subsequently upon introduction into the furnace. (b) In the 450° ashing, the samples were placed in the muffle furnace at room temperature and allowed to heat gradually. The kinetics of formation of Cr^{3+} complexes are known to be relatively slow and the conditions of direct addition (small sample size (5 mg), rapid heating to 1000°, and short time at elevated temperature) may not be sufficient for formation of the complex during the ashing cycle in the graphite furnace.

Another explanation for loss other than naturally occurring volatile complexes of Cr is the formation of a decomposition product of the complex that is itself either: (a) volatile, (b) not dissociated to atomic Cr during atomization, or (c) refractory and not atomized. Cr is known to form complexes with both oxygen and nitrogen donor ligands so that oxides or nitrides that could be formed may volatilize or come off the graphite tube as molecular complexes during atomization. Another possibility is the formation of carbides by reaction with the graphite tube (Talmi and Morrison, 1972). A very volatile Cr compound (chromyl chloride, CrO_2Cl_2) is known; however, conditions do not appear to be favorable for the formation of this compound in the experiments described herein. Also experiments using 1 *N* HNO_3 (subboiling distilled, National Bureau of Standards, Gaithersburg, Md.) solutions of these sugars did not show any difference from the ones with 1 *N* HCl. Initial attempts to isolate or identify the complexes or decomposition products of Cr naturally occurring in these sugars have not been successful. Further investigations are underway in this laboratory.

During the course of this study several sugar samples were given to us by the Carbohydrate Nutrition Laboratory, Nutrition Institute, U. S. Department of Agriculture, Beltsville, Md., for Cr analysis. These samples—pure sucrose; the monosaccharides, glucose and fructose; "blackstrap" molasses; and honey—are listed in Table VIII with Cr content by direct addition and oxygen plasma ashing.

The results for the refined sugars and the honey are in general agreement with those found for the WHO samples (Table II). The data for the "blackstrap" molasses seem to be higher than for the molasses samples listed in Table III, but the difference between the results by direct analysis and oxygen plasma ashed analysis (Table VIII), 190 ng/g of sample, is approximately the same as the previous samples, 230 ng/g as shown in Table III. This can possibly be explained by the fact that "blackstrap" molasses is an earlier refinement product than the household molasses. The "blackstrap" molasses may contain more impurities in the form of inorganic Cr which would be detected by both procedures. Thus, the difference between the two procedures, 190 or 230 ng/g, would reflect the organic Cr content. The numbers in parentheses in Table VIII are the values for the same brand of sugar collected in the WHO survey. The good agreement for the white sugar (sucrose) sample and the slightly poorer agreement for the light brown sugar may reflect the use of a different batch of molasses used to put the brown color back into the sugar. The honey sample is essentially a refined sugar as far as Cr is concerned.

CONCLUSIONS AND NUTRITIONAL SIGNIFICANCE

The conclusions drawn from these data are: (a) inorganic chromium in a sugar matrix can be determined by direct addition of the sample to the graphite furnace; (b) chromium naturally present in sugar and probably in other foodstuffs and biological materials occurs in an organically bound complex that is lost to detection by atomic absorption upon direct placement of the sample in the graphite furnace, *i.e.*, it behaves differently from inorganic chromium; (c) in order to determine organically bound Cr by graphite furnace atomic absorption it is necessary to convert it to inorganic chromiun by oxygen plasma ashing before introduction of the sample into the furnace.

The difference in analytical behavior of organically bound chromium and inorganic chromium has significant consequences from a nutritional viewpoint. It is known that the active form of chromium, the glucose tolerance factor, is a naturally occurring organic complex of chromium which has an influence in the insulin-glucose metabolic system (Mertz, 1969). Inorganic chromium has very little biological activity. Thus, the biologically active portion of chromium present in foodstuffs is probably that

Table VIII. Cr Content of Various Sugar Samples

Type	Source	ng of Cr/g of sample: Graphite furnace ashing[a]	ng of Cr/g of sample: Oxygen plasma ashing[b]
Molasses no. 1	"Blackstrap"	270	460
Molasses no. 2	"Blackstrap"	209	393
Sucrose	Domino	10.7 (7.3)[c]	19.2 (20.1)[c]
Sucrose	Nutritional Biochemical	8.8	29.5
Sucrose	Aristar	2.9	44.3
Glucose	Nutritional Biochemical	2.9	16.0
Fructose	Nutritional Biochemical	4.9	34.4
Light brown	Domino	23.0 (34.8)[c]	110 (53.3)[c]
Honey	Billybee	10.3	42.9

[a] 100 mg dissolved in 1.0 ml of 0.3 *N* HNO_3 (Subboiling Distilled, NBS), inject 50-μl aliquots into graphite furnace. [b] Solutions from (a) dried overnight at 60° in vacuum oven, ashed, dissolved in 1.0 ml of 0.3 *N* HNO_3, analyzed 50-μl aliquots. [c] Values for same brand, although different box, from Table II.

which is susceptible to the losses in analysis discussed above. Therefore, it is imperative to have a full understanding of these possible losses or analytical problems before interpreting the results of chromium analysis in biological samples.

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