

## DETECTION OF LOW-LEVEL C-4 SUGAR ADULTERATION OF HONEY

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Use of analysis of variance to compare results of internal standard stable carbon isotope ratio analysis of 5 samples from the same commercial origin with the standard database of 50 pure honeys reduces the detectable level of C-4 sugar (corn and cane) from 7% to 4.2%.

The internal standard stable carbon isotope ratio (ISIRA) procedure to indicate the presence of C-4 plant sugars (e. g. cane or corn) in honey (1) uses the isotope ratio value of the isolated and purified protein of a honey as a standard to which the isotope ratio value of the whole honey is compared. It was shown that for a pure honey the values for these entities do not differ. This has considerably reduced the statistical area of uncertainty in interpreting data regarding intentional adulteration of honey. The original isotope ratio method (2) compared the  $\delta^{13}\text{C}$  value of a sample with the mean of  $-25.4\text{‰}$  ( $s = 0.98\text{‰}$ ) which was obtained by analysis of 119 authentic honey samples<sup>1</sup>. Only if the value of a sample differed from the mean by  $4s$  (equivalent to  $\delta^{13}\text{C} = -21.5\text{‰}$ ) was it considered undoubtedly adulterated (probability of error = 0.00004). This limiting value unfortunately allowed a "gray area" equivalent to about 20% added material. This made necessary the use of a confirmatory TLC test (3) for samples in this range. This test is useful for indicating the possible presence of both C-3 and C-4 starch-derived syrups.

Occasional problems have since arisen with visual interpretation of the TLC test; for C-4 sugars these were eliminated by the development of the ISIRA test.

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<sup>1</sup>The validity of the mean value for honey and the distribution has since been authenticated by data from SIRA for 9217 honeys (4); mean  $-25.4\text{‰}$ ,  $s = 0.91$ .

However, use in this test of the same statistical approach as previously (setting the limit at the mean value for the difference + 4s) permits the presence of *ca.* 7% of added C-4 sugar before reaching the 4s limit for any given sample.

It has been alleged by honey packers that this "cushion" is in some cases being exploited by routinely adding about 5% of a sugar to all honey packed by an individual processor, thus remaining generally within the 7% detectable limit. This addition, though relatively minor, cannot be demonstrated by ISIRA, yet provides enough cost saving to allow its sale sufficiently under the market to obtain many contracts.

The objective of this paper is to examine another statistical basis for evaluating honey purity by ISIRA analysis which would reduce this area of uncertainty. When a single sample is compared with a database, the approach based on the normal distribution curve is used. However, if an average of several samples having a common origin is used, a t-test or an analysis of variance may be applied. With the comparison database of 50 or more samples, highly significant F values can result from analysis of variance comparing as few as 5 samples of suspected material of common origin. We have simulated such materials by comparing by ANOVA the 50-member database used earlier with random sets of from 5 to 20 samples from a 93-member database, but incrementally changed by between -0.2 to -0.8‰ to simulate consistent addition of small amounts (less than 7%) of C-4 sugars.

## EXPERIMENTAL

Materials and methods.-

*Pure honey database:* The 93 samples consisted of (a) the 50 samples of certified pure U. S. honey used to establish the ISIRA method (Table 4, ref. 1), (b) 15 samples from Table 2 (1) excluding those obviously impure (#6, 13, 15, 17), and (c) data from 28 samples of authentic honeys from Germany and world sources (excluding U. S.) described in their Table 1 by Rossman, Lüllman and Schmidt (4).

In evaluating the differences between the  $\delta^{13}\text{C}$  of the honey protein and the whole honey, values in the negative direction only are significant (1). Positive differences may for practical purposes be taken to indicate the absence of adulteration, hence they may be replaced by zero for calculation. Therefore all positive differences in the pure honey databases have been replaced by zero in the databases used here. The characteristics of the various published pure honey difference values are shown in Table 1 and are those use for the evaluations described here.

*Statistical evaluation:* The Minitab software (Release 6.1), Minitab Inc., 3081 Enterprise Drive, State College PA 16801) was used for ANOVA calculations. This program provides probability values for F only to 0.000. Since a literature search for F values at lesser probability was not fruitful, F values for lesser probabilities ( $10^{-4}$  and  $10^{-5}$ ) were obtained<sup>2</sup> elsewhere.

### RESULTS AND DISCUSSION

Twenty-four five-member subsets were selected from the 93-member database using a table of random numbers. By adding  $-0.4\text{‰}$  to each the presence of *ca* 2.4% of C-4 sugar is simulated. The addition of  $-0.5\text{‰}$  approximates the presence of *ca*. 3.5% C-4 sugar. Table 2 shows the analysis of variance for one of these subsets *vs.* the 50-member certified pure US honey database. In Table 3 are results of the analysis of the 24 subsets with the two simulated additions. Another group of 20 randomized 5-sample subsets was used similarly by addition of  $-0.3\text{‰}$  (*ca*. 2.8% C-4 sugar),  $-0.4\text{‰}$ , and  $-0.5\text{‰}$ . These data are not included in Table 3, but were combined with them to produce Figure 1 and for the regression calculation therein. Ten of the 108 values (4 with mean difference less than  $-0.4\text{‰}$  and 6 with F values over 40) were not included in Figure 1 but included in the regression calculation.

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<sup>2</sup>Provided by John Phillips, statistician for the AOAC Committee on Food Nutrition.

The regression line intersects the probability value of  $4 \times 10^{-5}$  at approximately  $-0.60\text{‰}$ , equivalent to 4.2% added c-4 sugar. This probability level is that used in the original SIRA paper to indicate positive adulteration. Using the same procedures, with six sets containing six and seven samples with additions of  $0.5\text{‰}$ , it is estimated that sensitivity of the test at the  $4 \times 10^{-5}$  probability level for six samples is  $0.55\text{‰}$ , equivalent to *ca.* 3.8% added c-4 sugars and is  $0.50\text{‰}$  or 3.5% added sugar for 7 samples.

Rossmann *et al.* (5) added 5% of 4 different high fructose corn syrups to a honey and determined  $\delta^{13}\text{C}$  of the honey and the protein. Since all of the differences were less than  $-1.0\text{‰}$ , he correctly pointed out that this level of admixture is not detectable by the ISIRA procedure. Using the ANOVA test as described herein, *i. e.* comparing the four difference values with their database of 28 pure honeys listed in their Table 1, with the positive differences as 0, an F value of 20.40 (30 DF) was obtained.

For a practical test of the procedure, a set of 5 samples was obtained from a trade source, from an enterprise thought to be adding sub-detectable amounts (*i. e.*  $<7\%$ ) of C-4 sugar to all honey sold. Table 4 shows the result of the analyses. ANOVA comparing this set with the 50-sample database gave  $F = 32.66$ , well beyond the  $10^{-5}$  probability of error.

#### Acknowledgements

The cooperation of Ken Winters, Coastal Science Laboratories, and John Phillips, statistician for the AOAC Committee on Food Nutrition is greatly appreciated.

## REFERENCES

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- (3) Kushnir, I. (1979) *J. Assoc. Off. Anal. Chem.* 62, 917-920
- (4) Winters, K. Personal communication
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## Legend for Figure 1:

To subsets of 5 difference values ( $\delta^{13}\text{C}$  of the protein -  $\delta^{13}\text{C}$  of the honey) selected at random from a database of ISIRA analyses of 93 pure honeys were added increments of -0.3, -0.4, -0.5‰. Each subset was compared with the difference values from 50 pure honeys (1) by the analysis of variance. Each of the 104 point represents ANOVA of a 5-member subset with the 50-member database. All positive difference values were recorded as zero (see text).

TABLE 1. Pure honey databases used <sup>a</sup>

Description & reference	Number of samples	Mean, ‰	S.D.
Certified U. S. honey (1)	50	-0.14	0.22
ASCS honey (1)	15	-0.21	0.29
German & import (4)	28	-0.20	0.29
All of above	93	-0.155	0.25

<sup>a</sup> All positive values for  $\delta^{13}\text{C}$  protein -  $\delta^{13}\text{C}$  honey entered as zero (see text).

TABLE 2. Analysis of variance for one 5-member subset.

Source	DF	SS	MS	F	p
Factor	1	1.1364	1.1364	22.88	0.000 <sup>a</sup>
Error	53	2.6320	0.0497		
Total	54	3.7684			

Individual 95% CI's for mean based on  
pooled Std. Dev.

Level	N	Mean	St.Dev.	
Subset	5	-0.6400	0.2608	(-----X-----)
Certified	50	-0.1400	0.2195	(-X--)
Pooled St. Dev.		0.2228		-----+-----+-----+-----+-----

-0.75   -0.50   -0.25   0.00

<sup>a</sup>See text.

Table 3. ANOVA comparing random 5-member subsets of 93-member database with 50-sample U. S. database.

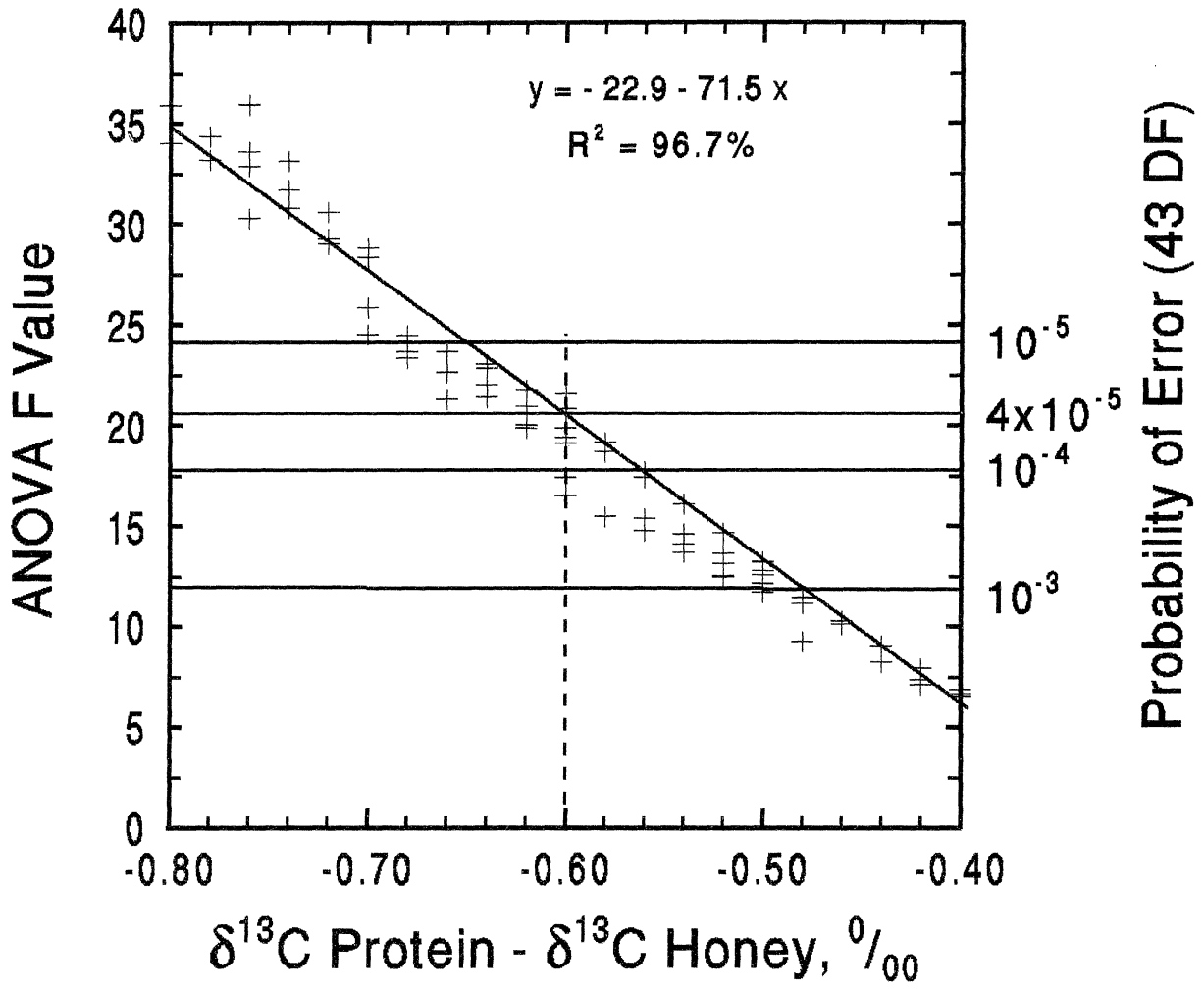
Subset No.	Mean difference after addition, ‰	F	Subset No.	Mean difference after addition, ‰	F
Addition of -0.40 ‰ (Equivalent to 2.8% C-4 sugar)			Addition of -0.50 ‰ (Equivalent to 3.5% C-4 sugar)		
1	-0.46	10.14	1	-0.56	17.47
2	-0.68	23.67	2	-0.78	33.25
3	-0.40	6.90	3	-0.50	13.23
4	-0.52	13.65	4	-0.62	21.78
5	-0.42	7.98	5	-0.52	14.69
6	-0.56	14.80	6	-0.66	22.68
7	-0.68	24.49	7	-0.78	34.41
8	-0.42	7.98	8	-0.52	14.69
9	-0.48	11.19	9	-0.58	18.75
10	-0.50	12.59	10	-0.60	20.55
11	-0.58	15.51	11	-0.68	23.35
12	-0.62	20.96	12	-0.72	30.60
13	-0.46	10.31	13	-0.56	17.77
14	-0.74	30.84	14	-0.84	44.85
15	-0.76	32.93	15	-0.86	44.41
16	-0.64	23.06	16	-0.74	33.20
17	-0.48	11.47	17	-0.58	19.21
18	-0.40	6.90	18	-0.50	13.25
19	-0.52	13.65	19	-0.62	21.78
20	-0.50	12.59	20	-0.60	20.55
21	-0.50	12.20	21	-0.60	19.91
22	-0.40	6.90	22	-0.50	13.25
23	-0.76	36.01	23	-0.86	48.56
24	-0.54	14.64	24	-0.64	22.88

For 53 DF, Probability 0.0001, F = 17.71; Probability 0.00001, F = 23.85

Table 4. ISIRA analysis of set of five market honeys from same packer.

No.	$\delta^{13}\text{C}$ honey ‰	$\delta^{13}\text{C}$ protein, ‰	Difference <sup>a</sup> , ‰
1	-24.9	-25.5	-0.6
2	-23.6	-25.6	-2.0
3	-23.6	-24.6	-1.0
4	-26.5	-25.7	+0.8
5	-24.4	-25.4	-1.0

<sup>a</sup>  $\delta^{13}\text{C}$  protein -  $\delta^{13}\text{C}$  honey.



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The regression equation is  
anovaf = - 22.9 - 71.5 diff

Predictor	Coef	Stdev	t-ratio	p
Constant	-22.8863	0.7666	-29.86	0.000
diff	-71.521	1.306	-54.76	0.000

s = 1.595      R-sq = 96.7%      R-sq(adj) = 96.7%

Analysis of Variance

SOURCE	DF	SS	MS	F	p
Regression	1	7624.8	7624.8	2998.97	0.000
Error	102	259.3	2.5		
Total	103	7884.1			

Unusual Observations

Obs.	diff	anovaf	Fit	Stdev.Fit	Residual	St.Resid
35	-0.760	36.010	31.470	0.288	4.540	2.89R
37	-0.740	33.200	30.040	0.267	3.160	2.01R
88	-0.600	16.550	20.027	0.160	-3.477	-2.19R
89	-0.300	2.610	-1.430	0.391	4.040	2.61RX
98	-0.800	40.050	34.331	0.333	5.719	3.67R

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