

Rapid Trichloroacetic Acid Extraction and Liquid Chromatography Method for Determination of Nicotinamide in Commercial Cereals

Denis E. LaCroix,¹ Wayne R. Wolf,^{1,2} and Albert L. Kwansa³

ABSTRACT

Cereal Chem. 82(3):277–281

Determination of niacin in fortified infant and dairy products has been accomplished using a variety of analytical liquid chromatography (LC) methods. Applications of these LC techniques to other food matrices suffer due to the presence of endogenous absorbing peaks at 260 nm that co-chromatograph with the nicotinic acid and nicotinamide vitamers. We have successfully adapted the LC method of Woollard and Indyk for the determination of nicotinamide in reference and commercial cereal products. Unbound nicotinamide in fortified cereal was extracted with 0.6*M* trichloroacetic acid and chromatographed on a C₁₈ reversed-phase column using a mobile phase of 75% methanol and water (pH 2.8, with formic acid) with sodium dioctylsulfosuccinate (0.1%) as the ion-pairing agent. Using Spectral Analysis ChromQuest software, a three-dimensional view

showed only nicotinamide under the LC peak. Similarity index spectral matches of nicotinamide standard and the LC peak were ≈100%, indicating the absence of interferences. Nicotinamide recoveries for the reference cereals of VMA195 and VMA 399 (from AACC International, St. Paul, MN) and GM 19B (from General Mills, Medallion Laboratories, Minneapolis, MN) were 90–103% of assigned value. Experimental values for oat, corn, rice, and bran cereals showed that actual niacin content in commercial cereals may be significantly above (111–170%) declared label values. Because manufacturers may fortify at a level higher than the declared label level to ensure shelf life compliance, these data do have significant implications when making precise estimates of niacin intake based on label claims.

Niacin (vitamin B-3) occurs in foods as either nicotinic acid or as nicotinamide, which is the most commonly used form for enriching foods for dietary supplementation. Niacin measurement in food matrices for food labeling regulatory purposes is generally done using either microbiological (Official Method of Analysis, AOAC International 2000) or chemical analysis methods (Eitenmiller and Landen 1999).

More robust liquid chromatography (LC) methods have been reported as replacement of the labor-intensive microbiological methods for these determinations. LC methods for nicotinic acid determination usually involve detection in the ultraviolet (UV) part of the spectrum at 260 nm (Tyler 1980; Krishan, et al 1999). Application of LC for niacin analysis has been reported in supplemented infant formulas and cereals (Tyler et al 1980; Woollard and Indyk 1997, 2002; Krishnan 1999). Niacin analysis by LC in complex food matrices is complicated by the presence of endogenous compounds that absorb at 260 nm and may interfere with the purity of the LC peak. We have employed solid phase extraction (SPE) as a sample clean-up procedure to remove these materials before analysis of infant formula and cereal samples for nicotinic acid by LC (LaCroix et al 1999, 2001, 2002a). The SPE/LC method was successfully applied for determination of niacin in wheat flour reference materials, but was unsuccessful for commercial all-purpose wheat flour due to the co-elution of endogenous absorbing LC peaks at 260 nm (LaCroix 1999).

Because nicotinamide is the main form of niacin added to formulated or supplemented products, Woollard and Indyk (1997, 2002) have developed a trichloroacetic acid (TCA) extraction of nicotinamide in milk products prior to LC analysis. Our objective in this study was to investigate the application of Woollard's TCA/LC

method for the qualitative and quantitative analysis of commercial cereal products for nicotinamide. Qualitative evaluation of the purity of the nicotinamide LC peak is done by spectral analysis. Verification and validation of the quantitative determination of nicotinamide content is done by using statistical protocols such as linear regression analysis of samples spiked with nicotinamide using the method of standard additions (MOSA) (Cardone 1983a,b; Mishalanie 1996).

Evaluation of the purity of the chromatographic peak is usually done by visual inspection of the single component LC peak as a 2-D (single wavelength absorbance vs. time) parameter. Use of a multiwavelength photodiode array detector (PDA), combined with appropriate computer software (manual A009651, ThermoQuest, San Jose, CA), allows for scanning the entire spectrum at 190–800 nm. During the course of an LC run, these systems allow for repetitive, multiwavelength spectral scans of the desired wavelength range for niacin at 220–300 nm to be obtained and processed. Spectral analysis of the subsequent views of the spectral scans allows definitive visual information of the presence of one or more compounds under an LC peak. These spectral analysis features can examine how clean the LC peak is (LaCroix et al 2002b).

LC methodology, combined with the use of appropriate matching reference materials (Tanner et al 1993; Wolf et al 2001) can provide high-quality data for nutrient content of foods that can be confidently incorporated by the end user in food compositional tables, to satisfy food labeling requirements, and to prepare formulated diets (Mishalanie 1996; Wolf and Iyengar 1997).

MATERIALS AND METHODS

Materials

Three types of cereal reference materials were used in this study. Two cereals (VMA 195 and VMA 399) were obtained from AACC International (St. Paul, MN) and one cereal (GM19B) was obtained from Medallion Laboratories (General Mills, Minneapolis, MN). Samples representing a variety of commercial cereal matrices, including four manufacturers' brands, were purchased from local supermarkets before their expiration dates. A total of seven samples including two separate brands of oat and rice cereals, and one brand each of corn, wheat, and bran cereal products were chosen. The cereals were ground dry in a blender and stored in plastic bags with no further storage precautions taken.

¹ Food Composition Laboratory, Beltsville Human Nutrition Research Center (BHNRC), United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Building 161, Beltsville, MD 20705-2350. Names are necessary to report factually on available data; however, the USDA neither guarantees nor warrants the standard of the product, and the use of the name by the USDA implies no approval of the product to the exclusion of others that may also be suitable.

² Corresponding author. Phone: 301-504-8927. Fax: 301-504-8314. E-mail: Wolfw@ba.ars.usda.gov

³ Student intern, Eleanor Roosevelt High School, Greenbelt, MD. Current Address: University of Wisconsin, Madison, WI.

Sample Extraction for LC Analysis

The dry cereal powders were accurately weighed to give a final concentration of 100–200 μg of nicotinamide per analysis based on the declared label value. Sampling constant studies show that significant analytical errors can result from using sample sizes below these levels (Wolf and LaCroix 1998). The weighed dry cereal powders were suspended in 0.6M TCA (15.0 mL), and the nicotinamide was extracted into the supernatant by shaking for 15 min using a mechanical wrist shaker. The suspension was then centrifuged for 20 min at $3,000 \times g$. An aliquot of the supernatant was filtered using a 0.45- μm syringe filter disk to remove minute particles before chromatography and made up to a volume of 30 mL. A calibrating standard solution of nicotinamide (99+% purity, Sigma Chemical Co) was prepared in the same manner as the cereal products with each batch of samples (Woollard 2002).

LC Instrumentation

An LC (ThermoSeparation Products [TSP]) system equipped with a photodiode array detector (PDA) (UV6000LP) and Thermo Quest software was used. Nicotinamide was separated from endogenous 260 nm absorbing components using a mobile phase of methanol (250 mL) and formic acid (10 mL) diluted to 1.0L with demineralized water and adjusted to pH 2.7–2.9 with potassium hydroxide (KOH). Dioctyl sulfosuccinate (1.0 g) was added to the mobile phase solution as an ion-pairing agent (Sood 1977; Woollard 2002). Reagents were obtained from Aldrich Chemical Co. Mobile phase flow rate was 0.6 mL/min, with an injection of 100 μL sample size. The LC column used was a Vydac 201TPC₁₈ reversed-phase (150 \times 4.6 mm, particle size 5) (The Separations Group, Hesperia, CA). Quantitative analysis of nicotinamide was determined at the LC peak maximum of 260 nm using a PDA.

Spectral Analysis

The spectral analysis feature of the ChromQuest software (LaCroix et al 2002b) was used to determine the purity of the LC analyte peak by comparison to the standard nicotinamide peak. A

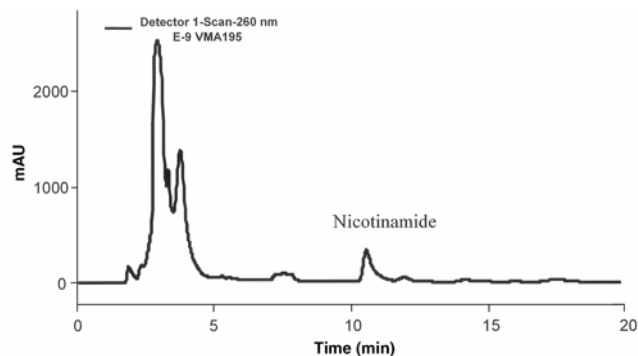


Fig. 1. Liquid chromatography results for trichloroacetic acid (TCA) extract of reference cereal VMA-195.

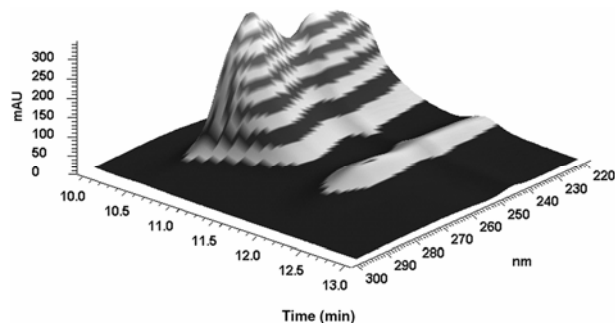


Fig. 2. 3-D view of nicotinamide liquid chromatography peak from trichloroacetic acid (TCA) extract of reference cereal VMA-195.

three-dimensional configurational analysis (wavelength, absorbance, and time) is obtained by means of a generated 3-D spectral scan over the spectral range of 220–300 nm. A similarity index (SI) is a spectral match of the library nicotinamide standard with the LC analyte peak of interest. A peak purity index (PPI) is the algorithm of the internal symmetry of the LC peak shape relative to the apex of the LC peak.

Statistical Analysis

Analytical response characteristics of the nicotinamide standard curves were determined by linear regression analysis (Wilson 1970; Cardone 1983a; Mishalanie 1996). Nicotinamide content in cereal reference materials and commercial cereal products was determined by measuring the peak area at 260 nm and by using linear regression analysis (LaCroix and Wolf 2001, 2002). All assumptions of linear regressions analysis were met. The nicotinamide data obtained was then examined for outliers using the outlier statistical criteria for extreme values (Dixon and Massey 1957).

Method performance characteristics examined by the method of standard additions (MOSA) for the VMA reference samples and for the commercial Oat-1 sample were 1) instrument response as a function of concentration, and 2) ratio of found to expected amount of nicotinamide added to the cereal matrix. MOSA involves the addition of increasing amounts of the analyte to equivalent samples of the test matrix. The slope of the resulting plot of the addition-response curve of the analyte (nicotinamide) in the cereal matrix gives information about the presence or absence of errors of the analytical system due to matrix effects (Cardone 1983a, 1986; Mishalanie 1996).

RESULTS AND DISCUSSION

Spectral Analysis

Visual 3-D inspection of multiple wavelength scans of an LC peak combined with spectral analysis software is a useful tool to identify presence or absence of components with retention times similar to that of the analyte (LaCroix et al 2002b). The software program also allows for calculation of the SI by means of a library spectral match that compares the spectra of an LC peak of the analyte with that of the standard. Figures 1–3 are representative of the data obtained for all samples examined in this study. Figure 1 is the LC chromatogram of the reference cereal VMA-195. The nicotinamide peak is a single peak at 260 nm, which is resolved from potentially interfering endogenous compounds that also absorb at 260 nm. Other samples showed similar chromatograms.

Figure 2 is a multiwavelength spectra of the nicotinamide peak of the VMA-95 sample. Visual inspection of the 3-D spectra of the nicotinamide LC peak obtained from both cereal matrices

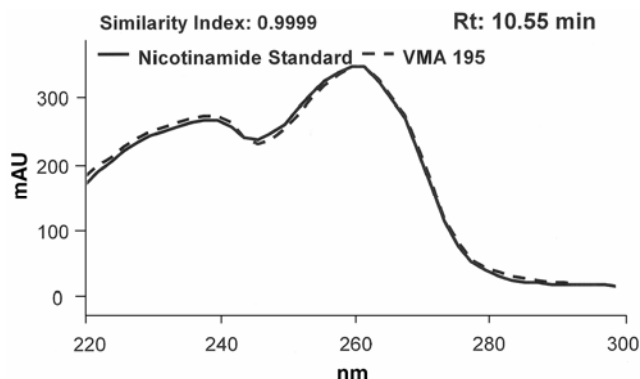


Fig. 3. Similarity index (SI) for nicotinamide standard and reference cereal VMA-195.

clearly show that the nicotinamide peak is free from interfering endogenous peaks at 260 nm. Other reference and commercial samples showed similar spectra.

Figure 3 shows a spectral library SI match of 0.999 for the VMA-195 reference sample relative to the spectra of the nicotinamide standard.

Performance Characteristics

The performance characteristics of an analytical method are parameters for assessing the suitability of a method for any given purpose based on experimentally determined quantitative values (Wilson 1970). Thus, performance characteristics refer to the quality of the data obtained by the method of Mishalanie (1996). The method performance characteristics examined in this study were 1) effect of instrument response as a function of increased nicotinamide concentration in the matrix as determined by the method of standard additions, and 2) the ratio of nicotinamide found to the amount of nicotinamide added to the cereal matrix (Cardone 1983b).

Analytical Response as a Function of Concentration

The method of standard additions (MOSA) was used to evaluate and minimize systematic error due to proportional matrix bias. The resulting addition response curve is plotted and represents the analyte in the presence of the matrix, offset by the amount of endogenous analyte in the sample matrix. The linearity of the curve is then obtained by the linear regression analysis equation of $y = mx + b$, where y is the peak area, m is the slope, and b is the intercept of the curve on the y -axis. The linearity and slope of this curve in relation to an equivalent standard curve in a matrix-free solution gives information about the presence or absence of matrix effects. MOSA calibrations are dependent on the matrix effect being constant over the range of the analyte used for spiking of the matrix examined (Cardone 1983a, 1986; Mishalanie 1996). Figure 4 is a typical dose response curve of the MOSA, identity, and nicotinamide standard in the reference VMA-195 sample. Linear regression analyses of the dose response for found peak

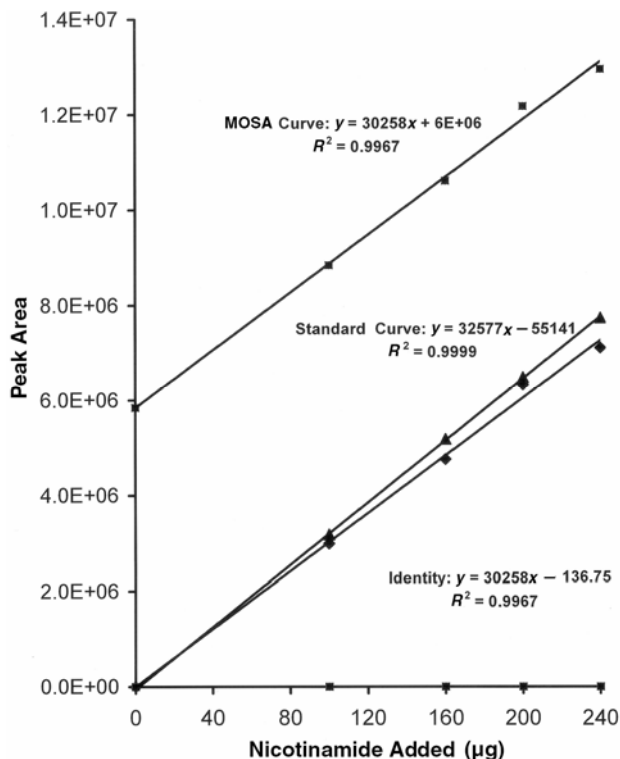


Fig. 4. Method of standard additions (MOSA), identity, and standard curve for reference cereal VMA-195.

area to the added nicotinamide shows a linear response close to unity ($R^2 = 1$).

The MOSA curve is used to diagnose any bias errors due to test portion extraction and interference by endogenous materials in the matrix complex. The identity curve is obtained by subtracting the peak area of the naturally occurring nicotinamide in the unspiked cereal from the peak areas of the spiked cereal samples. In the absence of bias errors, the identity curve should be parallel (same slope, m) to the dose-response curve of the nicotinamide standard in the analytical solution, and have an intercept of 0. For the reference cereal VMA 195 shown in Fig. 4, the unweighted regression analysis of nicotinamide added versus instrument response indicates an absence of matrix bias. Within analytical error, the slopes of the MOSA (and identity) curves are parallel (identical) to the standard curves, with the intercepts ≈ 0 . These data show that the instrument response is proportionally due only to the added nicotinamide (Wilson 1973; Cardone 1983a, 1986; Mishalanie 1996). MOSA of the commercial oat sample showed similar results.

Recovery

MOSA gives information on recovery of added analyte over a range of analytical concentrations and also allows for correction of proportional error if the curve is linear over the entire concentration range. The found-to-added ratio from the recovery curve of the added nicotinamide is shown in Fig. 5 for the reference cereal VMA 195. Linear regression analysis of the recovery curve is linear ($R^2 > 0.99$) with the slope (found-to-added ratio) very near unity. This means that $>99\%$ of the variation in the found amount is accounted for by the variation in the added nicotinamide over the range of the analyzed concentration. Deviation from the theoretical slope ($m = 1$) and intercept ($b = 0$) are attributable to experimental variability (Mandel and Linning 1957) or to corrigible error, which consists of both constant and variable errors (Cardone 1983b). Thus, our experimental recovery of the added nicotinamide is consistent over the dynamic range of the added nicotinamide investigated (Mishalanie 1996, 1997).

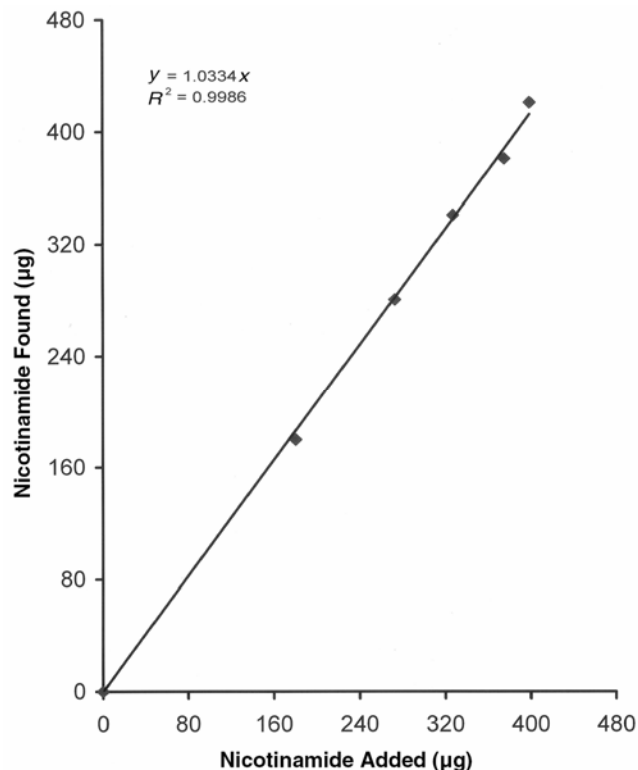


Fig. 5. Ratio of found to added nicotinamide for reference cereal VMA-195.

TABLE I
Nicotinamide Values Obtained from Reference Cereals

Reference Cereal	Reference Value (µg/g) ^a	Analyzed Value Avg. (µg/g)	Standard Deviation	% Relative SD	% Assigned Value	Similarity Index	Peak Purity Index	No. of Analyses
VMA-195	180 ^b ± 20.2	176.8 ^d	9.64	5.45	98.2	0.949	0.980	24
VMA-399	749.6 ^b ± 6.54	777.6 ^d	55.92	7.19	103.7	0.998	0.760	19
GM-19B	220.2 ^c ± 15.5	199.5	20.2	10.44	90.7	0.998	0.892	7

^a Microbiological method.

^b Assigned value, AACC International, St. Paul, MN.

^c Analyzed value, Medallion Laboratories, Minneapolis, MN.

^d MOSA value VMA-195 = 181.2; VMA-399 = 749.4.

TABLE II
Nicotinamide Values Obtained from Commercial Cereals

Commercial Cereal	Label Value ^a (µg/g)	Analyzed Value Avg. (µg/g)	Standard Deviation (µg/g)	% Relative SD	% of Label Value	Similarity Index	Peak Purity Index	No. of Determinations
Oat-1 ^b	167	188.8	31.49	16.68	113.1	0.957	0.993	17
Oat-2	167	199.0	19.75	9.93	119.2	1.000	0.957	12
Corn	180	306.1	29.92	9.77	170.1	0.998	0.888	13
Wheat	667	809.8	33.41	4.13	121.4	1.000	0.763	9
Rice-1	167	205.9	12.65	6.14	123.3	0.997	0.794	10
Rice-2	152	169.2	17.81	10.53	111.3	0.996	0.484 ^c	10
Bran	167	202.3	24.09	11.91	121.2	0.997	0.384 ^c	10

^a Niacin value on cereal box label based per Code of Federal Regulations (CFR): 1.01.9 at 20 mg/day Recommended Daily Allowance (RDA).

^b MOSA value for oat-1 = 191.3 µg/g.

^c Asymmetrical peak shape.

Experimental Niacinamide Levels

Using this LC method, nicotinamide content of the three reference cereals and seven commercial cereals are listed in Tables I and II, respectively.

The nicotinamide values were calculated from the daily standard linear calibration curves of the peak area of the chromatographic peak. Recovery values of nicotinamide from the reference cereals were ≈100% (Table I) and are within the assigned values that were obtained by microbiological assay for these samples. Earlier research (Lacroix et al 1999) has shown that there is no statistical difference in niacin values as determined by HPLC and microbiological assay. The %RSD of the analytical method at ≤10% are indicative of the robustness of the method. Spectral analysis shows that the SI for each nicotinamide chromatographic peak was >95% and approached unity. This parameter, along with the 3-D configurational visualization, clearly showed that only nicotinamide exists under the LC peak and it is completely resolved from endogenous 260 nm absorbing peaks. The PPI parameter is indicative of the symmetry of the LC peak shape. The data in Table I show generally symmetric chromatographic peaks (PPI ≈ 1.0) for the reference cereals, with some slight tailing for the VMA-399 sample.

The nicotinamide contents of the commercial cereals (Table II) are higher (11–70%) than the value declared on the cereal box label. This is not surprising because manufacturers may fortify some nutrient supplements in foods at a level higher than the declared label amount (FDA 1993) to ensure compliance with shelf life. The %RSD are in the vicinity of 10%, which are similar to those obtained for the reference cereals, with the exception of Oat-1 cereal, which is slightly higher but still acceptable at these levels of concentration.

The SI (Table II) for each of the types of commercial cereals examined in this study approached unity. Visual examination of the 3-D profile of the LC peak shows that only nicotinamide is present and completely separated from endogenous 260 nm peaks. PPI values showed a range of symmetrical chromatographic peaks from high to low symmetry for several of the samples. In general, we found that the PPI was not a useful parameter for the determination of the quality of the LC peak. It is very sensitive to slight tailing and seemed to have little effect on resulting quantitation.

CONCLUSIONS

A rapid TCA extraction and LC method for the determination of the nicotinamide content of a variety of commercial cereals was evaluated. The inclusion of reference materials with an assigned nicotinamide content as an integral part of the method verification and validation process gives the user confidence in the quality of the data obtained. Spectral analysis over a wavelength range of 220–300 nm and visual 3-D configurational analyses provides further definitive information that only nicotinamide exists under the LC peak. The spectrum library match (SI) of nicotinamide in standard and cereal matrices clearly shows that the LC peak obtained is completely resolved from interfering endogenous compounds at 260 nm. The MOSA and recovery curves of nicotinamide added to the cereal matrix demonstrate that the instrument response is solely due to the proportional increase of the added analyte. Use of the combination of these tools of statistical and spectral analysis generates information as to the robustness of the method and to the quality of the data obtained.

Experimental values for oat, corn, rice, and bran commercial cereals were 111–170% of label value, showing that actual niacin content in commercial cereals may be significantly above declared label values. These levels are not surprising because manufacturers may fortify some added nutrients in foods at a level higher than the declared label level to ensure compliance over a shelf life. These data do have significant implications when making precise estimates of niacin intake based upon label claims.

ACKNOWLEDGMENTS

We gratefully acknowledge the generous gift of the GM-19B reference cereal from Jon Devris and Brad Aeberg, Medallion Laboratories, Minneapolis, MN.

LITERATURE CITED

- AOAC International. 2000. Official Methods of Analysis, 17th Ed. The Association: Gaithersburg, MD.
- Cardone, M. J. 1983a. Detection and determination of error in analytical methodology. I. In the method verification program. *J. AOAC Int.* 66:257-1282.

- Cardone, M. J. 1983b. Detection and determination of error in analytical methodology. II. Correction for corrigible error in the course of real sample analysis. *J. AOAC Int.* 66:1283-1294.
- Cardone, M. J. 1986. New technique in chemical assay calculations. 2. Correct solution to the model problem and related concepts. *Anal. Chem.* 58:438-445.
- Dixon, W. J., and Massey, F. J., Jr. 1957. *Introduction to Statistical Analysis.* McGraw-Hill: New York.
- Eitenmiller, R. R., and Landen, W. O. 1998. *Vitamin Analysis for the Health and Food Sciences.* CRC Press: Boca Raton, FL.
- FDA. 1993. *Nutrition Labeling Manual: A Guide for Developing and Using Databases.* U.S. Food and Drug Administration: Washington DC.
- Krishnan, P. D., Mahmud, I., and Mathees, D. 1999. Postcolumn fluorometric LC procedure for determination of niacin content of cereals. *Cereal Chem.* 76:512-518.
- LaCroix, D. E., and Wolf, W. R., 2001. Determination of niacin in infant formula by solid-phase extraction and anion-exchange liquid chromatography. *J. AOAC Int.* 84:789-804.
- LaCroix, D. E., Wolf, W. R., and Vanderslice, J. T., 1999. Determination of niacin and wheat flour by anion-exchange liquid chromatography with solid-phase extraction cleanup. *J. AOAC Int.* 82:128-132.
- LaCroix, D. E., Wolf, W. R., and Chase, G. W., Jr. 2002a. Determination of niacin in infant formula by solid-phase extraction/liquid chromatography: Peer-verified method performance-interlaboratory validation. *J. AOAC Int.* 85:654-664.
- LaCroix, D. E., Wolf, W. R., and Hindsley, T. H. 2002b. Evaluation of niacin LC methods by diode array/spectral analysis. *Anal. Lett.* 35:2187-2198.
- Mandel, L., and Linning, F. J. 1957. Study of accuracy in chemical analysis using linear calibration curves. *Anal. Chem.* 29:743-749.
- Mishalanie, E. A. 1996. *Intralaboratory Analytical Method Validation.* AOAC International: Gaithersburg, MD.
- Mishalanie, E. A. 1997. *Basic Statistics for Analytical Science.* AOAC International: Gaithersburg, MD.
- Sood, S. P., Wittmer, D. P., Ismaiel, S. A., and Haney, W. G. 1977. Simultaneous high-pressure liquid chromatographic determination of niacin and nicotinamide in multivitamin preparations: Reversed-phase, ion pairing approach. *J. Pharm. Sci.* 66:40-42.
- Tanner, J. T., Wolf, W. R., and Horwitz, W. 1993. Nutritional metrology: The role of reference materials in improving quality of analytical measurements and data on food components. Pages 99-104 in: *Quality and Accessibility of Food-Related Data.* H. Greenfield, ed. AOAC International: Gaithersburg, MD.
- Tyler, T. A., and Shrago, R. R. 1980. Determination of niacin in cereals by LC. *J. Liquid Chromatogr.* 3:269-277.
- Wilson, A. L. 1970. The performance characteristics of analytical methods. I. *Talanta* 17:21-29.
- Wolf, W. R., and Iyengar, V. 1997. Food-based reference materials. *Food Testing Anal.* 2:27-31.
- Wolf, W. R., and LaCroix, D. E. 1998. Sampling constants for niacin content in standard reference material 1846 infant formula. *Fresenius J. Anal. Chem.* 360:459-464.
- Wolf, W. R., DeVries, J., and Ikens, W. 2001. Matching reference materials with AOAC International methods of analysis. *Fresenius J. Anal. Chem.* 371:300-306.
- Woollard, D. C., and Indyk, H. E. 2002. Rapid determination of thiamine, riboflavin, pyridoxine, and nicotinamide in infant formulas by liquid chromatography. *J. AOAC Int.* 85:945-951.

[Received December 30, 2003. Accepted December 2, 2004.]