Food Chemistry **43** (1992) 79–82
Analytical Methods Section

TECHNICAL NOTE

Comments on the Standard Fluorometric Determination of Riboflavin in Foods and Biological Tissues†

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(Received 27 March 1990; revised version received 29 January 1991; accepted 6 February 1991)

The vitamin B_2 content of foods has historically been determined as total riboflavin (TRF), and the most common method of TRF analysis has been the AOAC standard fluorometric procedure. A modification of this method to permit the use of flow injection analysis (FIA) is reported here. A number of foods were analyzed and the results generally agreed with the published values. However, the standard method was not found to be universally suitable for all types of samples.

INTRODUCTION

Although vitamin B_2 occurs naturally in three principal forms, riboflavin (RF), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), it has historically been determined as total riboflavin (TRF) by converting the FMN, and the FAD to RF prior to quantitation (Association of Vitamin Chemists, 1966; Cerletti & Giordano, 1971; Foy & Mbaya, 1977; Roy, 1979; Lumley & Wiggins, 1981; Ashoor *et al.*, 1983). Since the AOAC fluorometric method (Association of Official Analytical Chemists, 1984) has become the 'standard' means for determining TRF, the majority of the published data on the vitamin B_2 content of foods was generated by this method. In fact, the published tables of food composition (United States Department of Agriculture, 1976–1988) list only the TRF content of foods.

The suitability of the standard AOAC method for use as a reference procedure has been questioned (Roy & Conetta, 1976). It has been criticized for being time-consuming and tedious, and for requiring considerable sample preparation including purification of the extracts and/or conversion of the analytes of interest (Williams *et al.*, 1973; Pelletier & Madère, 1975; Roy & Conetta, 1976; Kirk, 1977; Roy, 1979; Dunbar & Stevenson, 1979; Kamman *et al.*, 1980; Lumley & Wiggins, 1981; Ashoor *et al.*, 1985). In order to increase precision and throughput, the analytical portion of the manual method has been adapted for use with the AutoAnalyzer (method 43.045; Association of Official Analytical Chemists, 1984), which is based on continuously flowing, air-segmented streams of reagents. In the present study, the AOAC standard manual method was modified to permit more convenient quantitation by flow injection analysis (FIA); Stewart (1983) has published a comprehensive discussion of FIA as an analytical technique.

Several weaknesses in the standard fluorometric method have been documented. The manual and semi-automated methods share a common chemical basis, and are prone to overestimation of the TRF content due to interference from fluorescing impurities (Haworth *et al.*, 1971; Ismaiel & Yassa, 1973; Bamji *et al.*, 1973; Richardson *et al.*, 1978; Macpherson & Ottaway, 1978; Wittmer & Haney, 1979; Wehling & Wetzel, 1984). There have also been reports of RF dimerization and destruction by some of the reagents used in the standard method (Woodrow *et al.*, 1969; Roy, 1979). Additional pitfalls in the extraction portion of the standard method, and its applicability to certain samples were uncovered in the course of the present study.

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Food Chemistry 0308-8146/91/$03·50 © 1991 Elsevier Science Publishers Ltd, England. Printed in Great Britain

MATERIALS AND METHODS

The TRF content of a variety of foods was determined using a modification of the standard fluorometric method (43.039–43.042, Association of Official Analytical Chemists, 1984). All reagents were certified ACS grade (Fisher Scientific, Springfield, NJ). Water from a Milli-Q Water Purification System (Millipore Corp., Bedford, MA) was used throughout the study. Standard solutions were freshly prepared daily using RF (Sigma Chemical Co., St Louis, MO). All extracts were filtered through Millex-GV 0·22 μm disposable filters (Millipore Corp.) before injection into the FIA system. Yellow lights were installed throughout the laboratory, and low actinic glassware was used to protect the RF from photolysis.

Foods that constituted a significant dietary source of TRF (Block *et al.*, 1985) were analyzed. All samples were purchased from major retail grocery chains with outlets located in the Beltsville, MD area. Only the edible portion of the food was sampled. Foods were prepared using common cooking techniques such as pan-frying ground round (beef), and round steak (beef) on a kitchen range until well-done (Granseth, 1981). Milk was sampled directly from the retail carton at the time of analysis. Raw beef liver was cut into 1 g pieces, and rapidly frozen to −30°C until required for extraction and analysis. Frozen samples were not thawed prior to extraction.

The standard fluorometric TRF method of the AOAC was followed, except that the fluorescence was measured via FIA on 100 μl aliquots delivered by an ISS-100 autosampler connected to a Series 4 HPLC pump operating at a flow rate of 1·0 ml min^{-1}, and an LS-4 fluorescence detector set at 440/565 nm (excitation/emission), with slit widths of 10 nm (Perkin-Elmer Corp., Norwalk, CT). Peak heights were recorded on a CR-1A Chromatopac recording integrator (Shimadzu Corp., Kyoto, Japan). The sensitivities of the detector and the integrator were adjusted so the height of the largest peak was 75–95% of full scale. Distilled water was used as the mobile phase or carrier. For the blank determination, sodium hydrosulfite ($Na_2S_2O_4$) was dissolved in 0·4% sodium acetate as specified in the AOAC semi-automated method for TRF (AOAC method 43.045; Association of Official Analytical Chemists, 1984), and added to the autosampler vials just before aspiration of the sample into the sample loop.

RESULTS AND DISCUSSION

Table 1 lists the TRF levels generated by the FIA method, and those published in the USDA Handbook No. 8 Series (United States Department of Agriculture, 1963, 1976–1988). The experimental TRF values for raw beef liver, raw ground round steak (beef), cooked round steak (beef), and pasteurized whole milk showed reasonable agreement with the published data. The other experimental values all exceeded the published TRF levels. The variability of the experimental values agreed with that obtained by Pelletier and Madère (1977) for the manual AOAC method, but did not approach their values for the semi-automated procedure. The samples analyzed in the present study were selected to test the method, and should not be construed as being representative of such foods due to the limited nature of the sampling. The differences between the experimental and the published values for TRF were most likely a result of a limited sampling on the part of the present study, and some questionable published values. For instance, the published value for the ready-to-eat breakfast cereal was the concentration needed to meet the label declaration exactly. However, over-fortification is the norm in the food industry, in order to ensure compliance with the label declaration. It should be noted that the high levels of TRF in the eggs and breakfast cereal were confirmed by recent HPLC studies (Russell & Vanderslice, in press).

Table 1. Comparison of Experimental and Published TRF Values

Type of sample	Total riboflavin concentration (mg per 100 g)	
	Published[a]	Experimental[b]
Raw beef liver	2·780	2·92 ± 0·183 (8)
Raw ground round (beef)	0·210	0·21 ± 0·012 (6)
Raw round steak (beef)	0·188	0·23 ± 0·009 (3)
Cooked round steak (beef)	0·278	0·31 ± 0·055 (6)
Raw skinless boneless chicken breast	0·092	0·13 ± 0·011 (5)
Pasteurized whole milk	0·162	0·19 ± 0·007 (3)
Raw whole egg	0·301	0·43 ± 0·048 (3)
Ready-to-eat breakfast cereal	1·5	3·70 ± 0·247 (3)
Hamburger buns	0·23[c]	0·37 ± 0·011 (3)

[a] United States Department of Agriculture, 1976–1988.
[b] Expressed as mean ± standard deviation (number of analyses).
[c] United States Department of Agriculture, 1963.

Several additional problems in the AOAC method were uncovered during the course of this study. The Polytron homogenizer (Brinkmann Instruments, Westbury, NY) was not suitable for use with AOAC extracts because sample material trapped around the rotor shaft could not be recovered. Since there was no internal standard in the AOAC method, this represented an outright loss of flavins that could not be recovered or accounted for. The design of the Omnimixer permitted more efficient washing of the mixer blades and cup, and reduced this type of loss to a minimum. However, the Omnimixer required a much longer mixing time, and produced a less homogeneous slurry than the Polytron homogenizer. A noticeable increase in the temperature

Table 2. Recovery of Riboflavin Spikes added to Raw Liver Samples at the Outset of the Extraction Procedure and After Autoclaving

	Spiked at the start of extraction	Spiked after autoclaving
Concentration of RF spike added ($\mu g\ g^{-1}$ liver)	33·44	43·86
TRF content of ($\mu g\ g^{-1}$ liver)		
Raw liver		28·95
Raw liver	28·21	28·06
Raw liver	27·94	30·83
Spiked raw liver	54·21	71·21
% Recovery	78%	96%

of the extract also accompanied grinding with the Omnimixer. Due to the lack of an internal standard in the AOAC method, there was also no means of accounting for any flavin losses that occurred during the numerous sample manipulations inherent in this method.

Raw liver samples were spiked with known concentrations of RF before, and immediately after autoclaving. The recovery from the samples spiked after autoclaving was 96%, while that of the samples spiked at the outset was only 78%, indicating that indigenous enzymes were not inactivated quickly enough to prevent some RF degradation (Table 2). This observation may be of particular significance to the analysis of biological tissue samples.

Analyses using model systems that contained known amounts of RF standards in the presence and absence of tallow, at the levels in which it occurs in raw ground beef, indicated that recoveries of 99% in the absence of the fat were reduced to 87% when the tallow was present (Table 3). The problem appeared to result from physical occlusion of the flavins within fat adhering to the mixing blades, glassware, and centrifuge tubes.

Table 3. Recovery of Riboflavin Spikes Added to Model Systems Containing Varying Levels of Fat (Tallow). Model Systems: 10 ml of the Standard Solution Prepared According to the AOAC Standard Fluorometric Method (43.040) ± 25 ml 0·1 N HCl

Weight of fat added (g)	Calculated quantity of RF added in the spike (μg)	Total quantity of RF by experiment (μg)	Recovery of RF spike (%)
0	1·00	1·04	104
0	1·00	1·01	100
0·5	1·00	0·90	91
0·5	1·00	0·86	86
1·0	1·00	0·90	90
1·0	1·00	0·87	87

Since the AOAC method contained no internal standard, and made no provision for defatting, it was impossible to compensate for these losses. Despite these observations, the experimental results for both raw beef liver and raw ground beef either agreed with, or overestimated the published values for TRF. This raises questions concerning what is actually being quantitated by the AOAC method. Are there enough fluorescent artifacts present in the extracts to fortuitously compensate for the flavin losses?

In conclusion, the standard method did not appear to be suitable for use with samples containing active enzyme systems, or high concentrations of fat. Based on past and present observations, the use of the standard fluorometric procedure for a reference method does not appear to be advisable for all samples.

ACKNOWLEDGEMENTS

This research was funded in part by Agriculture Canada through its Educational Leave program. This paper was taken from one of the authors' (L.F.R.) doctoral dissertations, submitted to the Graduate Program in Nutritional Sciences, University of Maryland.

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