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Analytical Methods Section

Non-degradative extraction and simultaneous quantitation of riboflavin, flavin mononucleotide, and flavin adenine dinucleotide in foods by HPLC

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The published tables of nutrient composition only list values for the total riboflavin (TRF) content of foods and much of this data was generated by methods of questionable accuracy. There are no published methods for simultaneously determining the three principal forms of vitamin B_2, i.e. riboflavin (RF), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD), in foods.

A simultaneous high performance liquid chromatographic (HPLC) separation of RF, FMN and FAD has been developed on polymer-based columns, using a mobile phase gradient of acetonitrile in citrate–phosphate buffer, pH 5·50. The natural fluorescence of the flavins was used for detection, and a RF analog, 7-ethyl-8-methyl-riboflavin, was used as the internal standard.

A two-step extraction procedure, using methylene chloride, methanol, and the citrate–phosphate buffer, was also developed. The recoveries from spiked food samples demonstrated that the flavins were stable under these extraction conditions.

The commercial FMN and FAD standards were found to contain significant quantities of impurities, and the appropriate correction factors were developed and applied to the HPLC results.

This method was successfully applied to a variety of foods that represented significant dietary sources of vitamin B_2, including raw and cooked meats, dairy products, eggs, and cereal products. The results of the HPLC analyses generally agreed with published values for TRF.

INTRODUCTION

Vitamin B_2 occurs naturally as riboflavin (RF), flavin mononucleotide (FMN), and flavin adenine dinucleotide (FAD). Unfortunately, the published tables of nutrient composition, such as USDA Handbook No. 8 (United States Department of Agriculture, 1976–1988), presently list only the total riboflavin (TRF) content of foods, and much of these data have been generated by methods of questionable accuracy.

Historically, the principal TRF determination has been the standard chemical method (Association of Vitamin Chemists, 1966; American Association of Cereal Chemists, 1983; Association of Official Analytical Chemists, 1984) which has repeatedly been reported to overestimate the TRF content of foods, due to interference by fluorescing artifacts (Woodrow *et al.*, 1969; Haworth *et al.*, 1971; Bamji *et al.*, 1973; Ismaiel & Yassa, 1973; Macpherson & Ottaway, 1978; Richardson *et al.*, 1978; Roy, 1979; Wittmer & Haney, 1979; Wehling & Wetzel, 1984). Although simultaneous HPLC determinations of RF, FMN and FAD have recently been developed for clinical applications (Ohkawa *et al.*, 1982; Pietta *et al.*, 1982; Speek *et al.*, 1982; Ohkawa *et al.* 1983; Lopez-Anaya & Mayersohn, 1987; Seki *et al.*, 1987), biological applications (Ichinose *et al.*, 1985; Oka & McCormick, 1985; Park, 1985; Ichinose & Adachi, 1986; Ogura *et al.*, 1986; Vastano *et al.*, 1987), and chemical applications (Light *et al.*, 1980; Hausinger *et al.*, 1986; Nielsen *et al.*, 1986) there are no HPLC methods for quantifying RF and its two coenzymes in foods.

Due to the lability of the flavins to light, phosphatase enzymes, and alkaline or extremely acidic pH,

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special nonhydrolytic extraction conditions are required in order to quantitate RF and its coenzymes (Cairns & Metzler, 1971; Fazekas & Kokai, 1971; Koziol, 1971; Wagner-Jauregg, 1972). Although a number of these extractions have been published, little attempt has been made to measure the stability of the individual flavins during extraction. There is evidence of coenzyme instability, incomplete extraction, and RF destruction under the published nonhydrolytic extraction conditions (Yagi, 1951; Haenel & Gassman, 1963; Massey & Swoboda, 1963).

Despite increasing analytical automation, the extraction continues to be a manual operation, and can be the source of significant errors. These errors can be minimized by incorporating an internal standard into the method at the earliest possible stage of the extraction (Haefelfinger, 1981; Lambert *et al.*, 1985; Vanderslice *et al.*, 1985). Although several clinical and pharmaceutical applications have incorporated internal standards into HPLC analyses for TRF, only the method of Pietta *et al.* (1982) used this type of calibration for the simultaneous determination of RF, FMN and FAD. None of the HPLC determinations for vitamin B_2 in foods has included an internal standard. The suitability of most of the compounds used as internal standards has been questioned due to lack of structural similarity with RF (Lambert *et al.*, 1985). Only one recent clinical TRF determination made use of a structural isomer, isoriboflavin, for the internal standard (Lambert *et al.*, 1985).

The presence of significant quantities of fluorescing artifacts in commercial preparations of the flavin coenzymes has been reported (Moonen & Müller, 1982; Entsch & Sim, 1983; Nielsen *et al.*, 1983). Although a number of purification procedures have been proposed (Yagi *et al.*, 1967; Mayhew, 1971; Mayhew & Strating, 1975; Scola-Nagelschneider & Hemmerich, 1976; Johnson *et al.*, 1978; Walsh *et al.*, 1978; Massey & Mendelsohn, 1979; Light *et al.*, 1980; Merrill & McCormick, 1980; Ohkawa *et al.*, 1982; Entsch & Sim, 1983; Nielsen *et al.*, 1983; Ohkawa *et al.*, 1983; Hausinger *et al.*, 1986; Nielsen *et al.*, 1986), at present there is no rapid means of producing reasonable quantities of pure FMN and FAD.

Of 27 published HPLC methods for the separation of one or more coenzymes, only seven made any attempt to compensate for the lack of purity of the commercial coenzyme preparations. Four of the seven methods in this latter group (Entsch & Sim, 1983; Nielsen *et al.*, 1983; Hausinger *et al.*, 1986; Nielsen *et al.*, 1986), were designed specifically for purity determination, rather than analytical quantitation per se. Ogura *et al.* (1986) purified the commercial flavins by HPLC, although the exact method used is unclear, and the purity of the products generated by the methods of Ohkawa *et al.* (1982, 1983) has been questioned (Entsch & Sim, 1983). In the majority of the published HPLC separations, it appears that the impurities in the commercial flavins were coeluted with the flavins of interest, and were not observed.

At present, very little information is available on the individual B_2 vitamers that occur in foods. Therefore this research was designed to develop a reliable nonhydrolytic extraction procedure, and a reproducible high performance liquid chromatography (HPLC) separation for the three principal forms of vitamin B_2, including quantitation by internal standard.

MATERIALS AND METHODS

Materials

All HPLC separations were carried out on a Series 4 liquid chromatograph equipped with quaternary gradient and helium solvent-degassing systems (Perkin-Elmer Corp., Norwalk, CT). Aliquots (100 μl) were injected manually using a model 7125 syringe loading injection valve with a 100 μl loop (Rheodyne, Colati, CA), or automatically using an ISS-100 autosampler (Perkin-Elmer). An LS-4 fluorescence detector (Perkin-Elmer) was used at wavelengths of 360/550 nm (excitation/emission) or at 450/522 nm (excitation/emission), with slit widths of 10 nm. A CR-1A Chromatopac recording integrator (Shimadzu Corp., Kyoto, Japan) was used to record the chromatograms, integrate the areas under the peaks, and calculate flavin concentrations by the internal standard method. The temperature of the HPLC columns was controlled by immersion in a constant temperature water bath (model FS or FK, Haake Inc., Saddle Brook, NJ).

Samples were homogenized using an R-2 commercial food processor (Robot Coupe USA, Ridgeland, MS), and a Polytron PT10/35 homogenizer (Brinkmann Instrument Co., Westbury, NY) equipped with a PTA 10 TS or a PTA 20 TS rotor. All centrifugation steps were carried out at 42000 × g and 4°C in a J2-21 refrigerated centrifuge (Beckman Instruments Inc., Pala Alto, CA). Measured aliquots of the extraction reagents were added to the samples using Repipet dispensers (Labindustries, Berkeley, CA). Yellow lights were installed throughout the laboratory, low actinic glassware was used, and the autosampler was completely covered with a black drape to protect the flavins from photolysis.

HPLC grade solvents (Fisher Scientific, Springfield, NJ) were used. All other reagents were certified ACS grade from Fisher Scientific. Aqueous solutions were prepared with demineralized water that had been passed through an all-glass still (model AG-11, Corning Glass Works, Corning, NY), and collected in glass bottles.

Standard solutions were prepared fresh daily using RF (Sigma Chemical Co., St. Louis, MO), FMN and FAD (Fluka Chemical Corp., Ronkonkoma, NY), and 7-ethyl-8-methyl-riboflavin (7-Et-8-Me-RF; Lambooy, 1958; gift from Dr John Lambooy, Professor Emeritus, Biochemistry Department, University of Maryland

School of Dentistry). Spiking and standard solutions were prepared with 50% methanol in the buffer used for extraction, and the concentrations of the flavins were adjusted to approximate the levels in the food samples being analyzed. All standards and extracts were filtered through Millex-GV 0·22 μm disposable filters (Millipore Corp.) before injection into the HPLC.

Preparation of food samples

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 used for analysis. Foods were prepared using common cooking techniques such as: hard cooking the eggs, or pan-frying ground round and round steak 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 in a Revco Ultra-Low freezer (Fisher Scientific). All other foods were ground in the food processor to prepare a homogeneous sample. Aliquots (approximately 0·5–4·0 g) were then weighed into the extraction tubes, and stored at −30°C until required for analysis. Frozen samples were not thawed prior to extraction.

Non-degradative extraction method

Aliquots of methanol (9·0 ml) and of methylene chloride (10·0 ml) were dispensed into the extraction tube containing the sample. An aliquot (2 ml) of the internal standard solution (7-Et-8-Me-RF), or the spiking solution (RF, FMN, FAD and 7-Et-8-Me-RF) was immediately pipetted into the extraction tube, and the sample was homogenized for 75 s at 50% of full speed on the Polytron homogenizer. An aliquot (9·0 ml) of 100 mM citrate–phosphate buffer, pH 5·50 (McIlvaine, 1921) containing 0·1% sodium azide (NaN_3) was added, and the extract was homogenised for an additional 30 s. The extracts were centrifuged for 10 min, and an aliquot of the aqueous phase was decanted, filtered, and injected into the HPLC. All extractions were carried out in a darkened fume hood to prevent photodegradation of the flavins. Recoveries were calculated based on the differences in the mean concentrations of the individual flavins in a spiked sample, and at least three unspiked samples of the same type of food.

HPLC separation methods

The conditions used for the HPLC separations of the B_2 vitamers in foods are summarized in Table 1 and Fig. 1. In all food analyses, the RF, FMN and FAD concentrations were corrected for the impurity of the

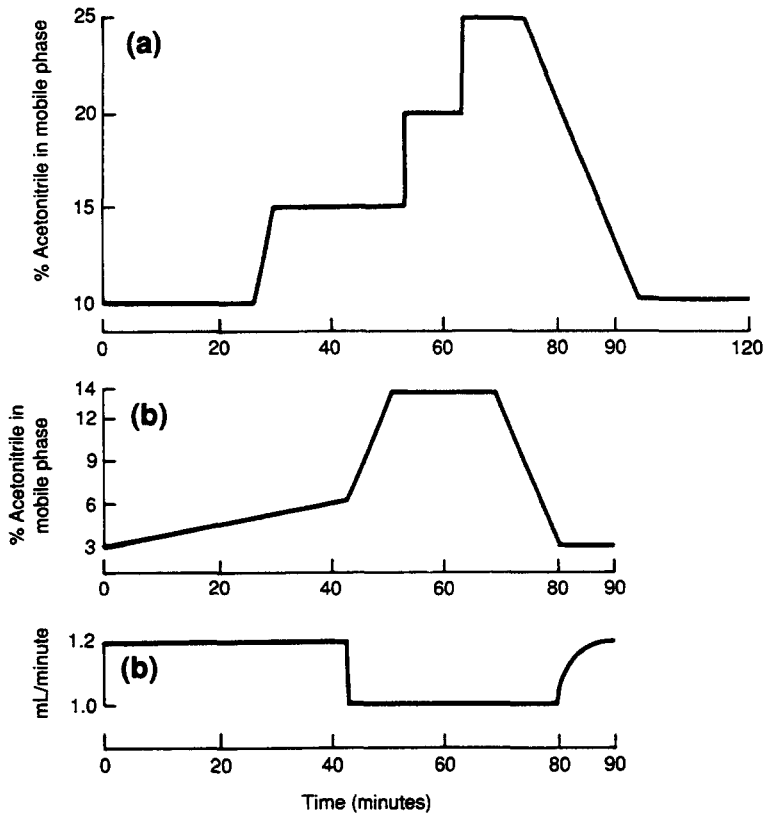

Fig. 1. Gradients used in the HPLC separations. (a) Mobile phase composition gradient used in HPLC method I. Flow rate = 0·5 ml min^{-1} throughout. (b) Mobile phase composition gradient (upper) and flow rate gradient (lower) use in HPLC method II.

Table 1. HPLC Conditions for the simultaneous quantitation of the RF, FMN and FAD in foods

Method number	Guard column	Analytical column		Mobile phase		Detector wavelengths (fluorescence)
		Type	Temperature	Composition	Flow rate	
I	Act-1 (Interaction) 2 cm × 4·6 mm (10 μm macroporous polymer-based C_{18}, 80 Å pore size)	Act-1 (Interaction) 15 cm × 4·6 cm (10 μm macroporous polymer-based C_{18}, 80 Å pore size)	10°C	Acetonitrile/10 mM citrate–phosphate buffer, pH 5·50: — 10:90, v/v at 0 min — isocratic at 10:90, v/v to 26·0 min — convex gradient[a] to 15:85, v/v at 27·0 min — isocratic at 15:85, v/v to 53·0 min — convex gradient[a] to 20:80, v/v at 54·0 min — isocratic at 20:80, v/v to 64·0 min — convex gradient[a] to 25:75, v/v at 65·0 min — isocratic at 25:75, v/v to 75·0 min — linear gradient to 10:90, v/v at 95·0 min — isocratic at 10:90, v/v to 115·0 min (See Fig. 1(a))	0·5 ml min^{-1}	360/550 nm (ex/em)
II	PLRP-S (Polymer Laboratories Inc., Amherst, MA) 5 mm × 3 mm (macroporous polystyrene/divinylbenzene resin)	2 PLRP-S 100 Å columns (Polymer Laboratories) in series 25 cm × 4·6 mm + 15 cm × 4·6 mm (5 μm polystyrene/divinylbenzene resin, 100 Å pore size)	40°C	Acetonitrile/0·1% sodium azide in 10 mM citrate–phosphate buffer, pH 5·50: — 3:97, v/v at 0 min — linear gradient to 6:94, v/v at 43·0 min — linear gradient to 14:86, v/v at 51·0 min — isocratic at 14:86, v/v to 70·0 min — linear gradient to 3:97, v/v at 80·0 min — isocratic at 3:97, v/v to 90·0 min (See Fig. 1(b))	— 0 to 43 min at 1·2 ml min^{-1} — 43 to 80 min at 1·0 ml min^{-1} — change to 1·2 ml min^{-1} at 90 min using a convex gradient Fig. 1(b)	450/522 nm (ex/em)

[a] Convex gradient: curve defined by the equation $P(t) = P(i) + [P(f) - P(i)] \times (t/T)^{1/5}$ where
A & B = % composition of solvents acetonitrile and buffer, respectively,
$P(t) = A/(A + B)$ at time, t
$P(i)$ = the initial $A/(A + B)$,
$P(f)$ = the final $A/(A + B)$,
t = elapsed time (min),
T = total time required to complete the curve (min).

Table 2. Molar correction factors to be applied to the uncorrected concentrations in pMol ml^{-1} for FMN and FAD taken from the chromatogram

Commercial flavin	Molar concentration of flavins present
FAD	76·0% FAD 1·5% FMN 0·7% RF
FMN	62·0% FMN 5·5% RF

commercial standards (Table 2), and expressed in nMol flavin per g food. The individual contents were totaled and expressed as mg TRF per 100 g food.

RESULTS AND DISCUSSION

Development of the HPLC separations

Preliminary HPLC separations confirmed published reports of short column lifetime and irreversible changes in column performance in silica-based HPLC columns used for vitamin B_2 determinations (Van De Weerdhof *et al.*, 1973; Reyes *et al.*, 1988). HPLC separations were therefore developed on polymer-based columns, and the retention times of the standards were found to be much more reproducible. The citrate–phosphate buffer at pH 5·50 was used to achieve the optimum resolution of the flavins on the polymer-based columns within the pH range required for coenzyme stability.

Although isoriboflavin has been used as the internal standard in a TRF determination (Lambert *et al.*, 1985), it was available in such limited quantities that its routine use was not practical. Another RF analog, 7-Et-8-Me-RF, was found to meet all the criteria for an internal standard (Haefelfinger, 1981; Vanderslice *et al.*, 1985), was available in reasonable quantities, and could be separated from RF, FMN and FAD by gradient elution. An HPLC separation using an acetonitrile/citrate–phosphate buffer gradient at 40°C (HPLC method I) was adopted in order to maintain column pressure within acceptable limits and provide baseline resolution of RF, FMN, FAD and 7-Et-8-Me-RF.

While the present study was in progress, the manufacturer modified the Act-1 column packing. Although this necessitated changes in the HPLC conditions, comparable results were achieved on the original and the modified columns. The analytical results for a variety of foods are presented in Table 3. Raw beef liver contained approximately 70% FAD, 5% FMN, and 25% RF (Fig. 2). On a molar basis, raw chicken breast contained substantially more FAD (80–90%), and less RF (<10%). However the TRF content of chicken breast was about 25 times less than that of the beef liver. Ground beef contained 80–90% FAD, and 5–10% each of RF and FMN. Raw whole egg, and egg yolk contained only RF. No coenzymes were detected. Pasteurized whole milk contained 85% RF and 15% coenzymes on a molar basis. This HPLC method was also applicable to fortified foods, such as ready-to-eat breakfast cereals. Only RF, at about 2·5 times the label declaration, was detected in a corn-based breakfast cereal.

The chromatograms of cooked beef and bread obtained from the Act-1 columns exhibited multiple artifact peaks. The largest of these eluted shortly after the void volume, and tailed seriously enough to coelute with the RF peak and interfered with its integration. Due to the limitations on the operating conditions for the Act-1 columns, HPLC separation of the flavins and the artifacts on these columns was impossible. Attempts to clean up these extracts using a variety of solid phase

Table 3. The RF, FMN, FAD and TRF contents of foods determined by HPLC method I

Type of sample	Number of analyses	Concentration of individual flavins (nMol g^{-1})			TRF concentration (mg per 100 g)
		RF	FMN	FAD	
Raw beef liver	3	15·14 ± 0·788[a]	4·96 ± 1·426	41·72 ± 4·518	2·33 ± 0·191
Raw top round steak (beef)	3	0·63 ± 0·015	0·19 ± 0·012	4·05 ± 0·015	0·18 ± 0·001
Cooked top round steak (beef)	3	0·51 ± 0·039	0·25 ± 0·023	4·14 ± 0·080	0·19 ± 0·006
Raw ground round (beef)	3	0·23 ± 0·013	0·12 ± 0·019	3·23 ± 0·021	0·14 ± 0·001
Raw skinless boneless chicken breast	3	0·14 ± 0·010	0·12 ± 0·003	2·47 ± 0·010	0·10 ± 0·001
Pasteurized whole milk	3	3·84 ± 0·027	0·33 ± 0·116	0·32[b]	0·16 ± 0·010
Raw whole egg	3	12·30 ± 0·283	N/D[c]	N/D	0·46 ± 0·011
Cooked whole egg	3	12·46 ± 0·301	N/D	N/D	0·47 ± 0·011
Raw egg yolk	3	18·32 ± 1·310	N/D	N/D	0·69 ± 0·049
Ready-to-eat breakfast cereal	3	102·52 ± 5·318	N/D	N/D	3·86 ± 0·200

[a] Expressed as mean ± standard deviation.
[b] FAD peak too small to be integrated on one of the chromatograms.
[c] N/D = peak not detected.

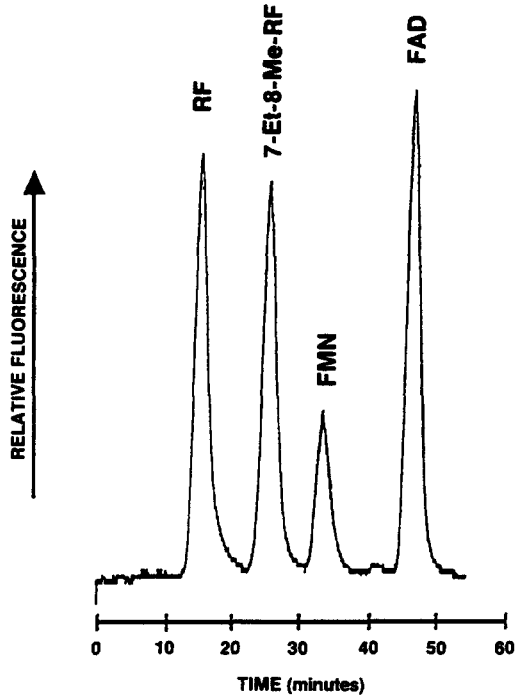

Fig. 2. Chromatogram of an extract of raw beef liver using HPLC method I.

extractions were unsuccessful.

A new on-column separation was therefore developed, using 5-μm polymer-based PLRP columns with no organic functional group (HPLC method II). Peak shape and resolution on these columns was superior to that of the preceding method. Independent scans of the excitation and emission monochromators indicated that the maximum fluorescence under the conditions of this HPLC separation occurred at 450 nm and 522 nm for all flavins of interest.

This method was successfully used to quantitate the flavins in cooked round steak, hamburger buns, and fast food hamburgers (Table 4). All peaks were baseline resolved (Figs 3 and 4). At a signal-to-noise ratio of 3:1, the limits of detection for all of the flavins was in the pMol or ng range (0·55 pMol or 0·21 ng RF; 1·96 pMol or 0·89 ng FMN; 14·19 pMol or 11·15 ng FAD). The overall variability of the HPLC separations was found to be within acceptable limits (0·5–3·0% CV), based on the results from multiple injections of standard solutions. Lopez-Anaya and Mayersohn (1987) have reported no loss of column efficiency after 4 months of determining the RF, FMN and FAD in clinical samples on a similar polymer-based column.

Correction for the impurities in the commercial coenzymes

The increased sensitivity of HPLC method II permitted the detection of substantial quantities of fluorescing artifacts in the commercial flavin preparations. Depending on the supplier, the purity of the coenzymes varied from 53% to 71%, based on the areas under the peaks. However, a simple correction based on peak areas was not feasible because all of the flavins do not fluoresce to the same extent on a molar basis (Koziol, 1971; Spencer & Weber, 1972; Marletta & Light, 1985), and values for their molar fluorescence responses under conditions similar to the HPLC mobile phase were not available. HPLC analysis of each of the flavins individually indicated that the artifacts were confined to the commercial coenzyme preparations.

A solution of RF (2·5 nM), 7-Et-8-Me-RF (3·0 nM), and commercial FAD (3·5 nM, by weight) was prepared in 10-mM, citrate–phosphate buffer, pH 5·50. These flavins were separated using a mobile phase buffer at pH 1·4 (HPLC method III, Table 5), where the molar fluorescence responses of RF and FAD are reported to be equal (Weber, 1950).

To determine the purity of FAD, the following assumptions were necessary: (i) the commercial RF was pure; (ii) the flavins were stable for short periods in the mobile phase at pH 1·4; (iii) the chromatographic response factors for RF and FAD were equal in mobile phases below pH 1·5; and (iv) the ratio of the peak areas in the presence and absence of acetonitrile using HPLC is equal to the ratio of the peak heights in the presence and absence of acetonitrile using flow injection analysis (FIA, i.e. analysis on the HPLC system with the column removed, as described by Stewart, 1983).

Correction for the effect of acetonitrile on the fluorescence of the flavins, was accomplished using FIA with the combination of analytes and mobile phases given in Table 6. The ratios of the FIA peak heights with and

Table 4. The RF, FMN, FAD and TRF contents of foods determined by HPLC Method II

Type of sample	Number of Analyses	Concentration of individual flavins (nMol g^{-1})			TRF concentration (mg per 100 g)
		RF	FMN	FAD	
Cooked round steak (beef)	3	0·63 ± 0·053[a]	0·56 ± 0·009	8·13 ± 0·169	0·35 ± 0·008
Hamburger buns (enriched)	3	5·47 ± 0·053	0·42 ± 0·004	0·77 ± 0·027	0·25 ± 0·003
Fast food hamburgers	27	3·27 ± 0·801	0·38 ± 0·092	2·42 ± 0·410	0·23 ± 0·041

[a] Expressed as mean ± standard deviation.

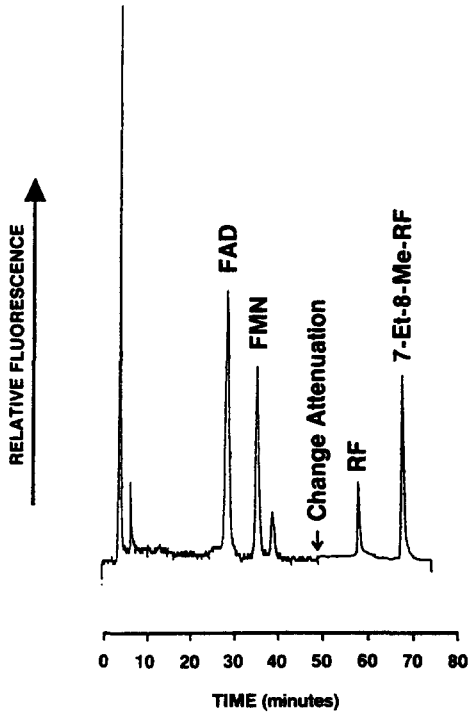

Fig. 3. Chromatogram of an extract of cooked top round steak (beef) using HPLC method II.

without acetonitrile were calculated, and used to correct the fluorescence response of each flavin on the chromatogram from HPLC method III to its response in 100% phosphate buffer, pH 1·4. The corrected HPLC peak areas for RF and 7-Et-8-Me-RF, and their concentrations by weight were then used to calculate a corrected chromatographic response factor for RF at 100% phosphate buffer, pH 1·4. Since Weber (1950) has shown that the molar fluorescence responses for RF and FAD were equal in solutions below pH 1·5, the corrected RF response factor was used as the FAD response factor. The FAD response factor and the corrected areas for the 7-Et-8-Me-RF and FAD peaks, were used to calculate the actual concentrations of FAD, and determine the purity of the commercial standard (Table 2).

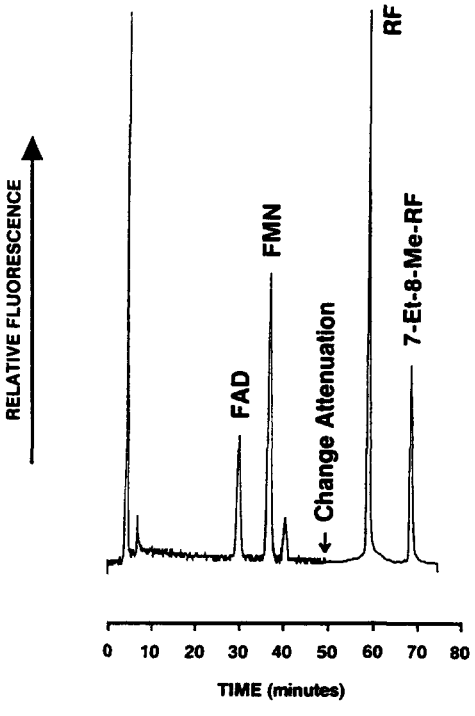

Fig. 4. Chromatogram of an extract of fast food hamburger using HPLC method II.

The purity of FMN was determined from its chromatogram at pH 5·50 (HPLC method II). Assumptions similar to those for FAD were necessary. Because RF and FMN were not eluted at the same mobile phase composition on the HPLC gradient, all values were corrected to reflect the equivalent responses in 100% citrate–phosphate buffer, pH 5·50. FIA was again used to determine the correction factors for the effect of acetonitrile on the fluorescence of the flavins (Table 7). Since the molar fluorescence responses of RF and FMN are considered to be equal (Koziol, 1971), a corrected chromatographic response for RF was determined and used, along with the peak areas corrected to 100% citrate–phosphate buffer, to calculate the actual concentration of the FMN peak. The purity of the commercial FMN was then determined (Table 2).

The areas of the FMN and the 7-Et-8-Me-RF peaks on the chromatogram of commercial FAD at pH 5·50 (HPLC method II) were similarly corrected, and applied, along with the corrected response factor for RF in 100% citrate–phosphate buffer at pH 5·50, to the determination of the actual concentration of the FMN peak in pMol FMN ml^{-1} standard solution. The molar content of FMN in commercial FAD was calculated by taking the ratio of the actual [FMN] in pMol ml^{-1} to the uncorrected [FAD] in pMol ml^{-1} based on the weight of commercial FAD used (Table 2).

Since the commercial RF was assumed to be pure, the RF concentration in pMol ml^{-1} was read directly from the chromatograms of commercial FMN and FAD at pH 5·50 (HPLC method II). The molar content of RF in the commercial coenzymes was based on the ratio of the [RF] determined from the chromatogram, to the uncorrected [coenzyme] in pMol ml^{-1} based on the weight of the commercial coenzyme preparation (Table 2).

These calculations would have to be repeated for each new batch of commercial coenzymes used. The purity values used to correct the flavin contents in this study generally agreed with the published values, given the variability of the coenzyme purities from different suppliers (Entsch & Sim, 1983; Nielsen *et al.*, 1983).

Development of a non-degradative extraction method

Raw beef liver was used as the test substrate during development of the extraction procedure because it was a major dietary source of TRF (Block *et al.*, 1985), a

Table 5. HPLC conditions for determining the purity of commercial FAD

Method number	Guard column	Analytical column		Mobile phase		Detector wavelengths (fluorescence)
		Type	Temperature	Composition	Flow rate	
III	PLRP-S (Polymer Laboratories) 5 mm × 3 mm (macroporous polystyrene/ divinylbenzene resin)	2 PLRP-S 100 Å columns (Polymer Laboratories) in series 25 cm × 4·6 mm + 15 cm × 4·6 mm (5 μm polystyrene/ divinylbenzene resin, 100Å pore size)	40°C	Acetonitrile/0·1% sodium azide in 50 mM phosphate buffer, pH 1·4: — 6:94, v/v at 0·0 min — linear gradient to 10·7:89·3, v/v at 30·0 min — linear gradient to 14:86, v/v at 60·0 min — linear gradient to 6:94, v/v at 70·0 min — isocratic at 6:94, v/v to 80·0 min	1·0 ml min^{-1}	450/522 nm (ex/em)

Table 6. Determination of the purity of commercial FAD. Flow injection analysis conditions used to determine the effect of acetonitrile on the fluorescence of the flavins

Flavin	Mobile phase for flow injection analysis
7-Et-8-Me-RF	0·1% NaN_3 in 50 mM phosphate buffer, pH 1·4 Acetonitrile + 0·1% NaN_3 in 50 mM phosphate buffer, pH 1·4 (12·5:87·5, v/v)
RF	0·1% NaN_3 in 50 mM phosphate buffer, pH 1·4 Acetonitrile + 0·1% NaN_3 in 50 mM phosphate buffer, pH 1·4 (10·7:89·9, v/v) Acetonitrile + 0·1% NaN_3 in 50 mM phosphate buffer, pH 1·4 (9·1:90·9, v/v) — HPLC elution conditions for FAD
FAD	0·1% NaN_3 in 50 mM phosphate buffer, pH 1·4 Acetonitrile + 0·1% NaN_3 in 50 mM phosphate buffer, pH 1·4 (9·1:90·9, v/v)

good source of the coenzymes, and contained active enzyme systems, such as the phosphatases, which are known to degrade the flavin coenzymes (Koziol, 1971).

Early attempts to use cold trichloroacetic acid extractions (Park, 1985) resulted in low recovery rates, possibly due to a short-term degradation of the flavins, as suggested by Massey and Swoboda (1963). Subsequently, a series of extraction media were evaluated by comparing the relative percentages of RF, FMN and FAD extracted from one piece of raw beef liver on the same day. The extraction media varied between 0 and 100% methanol in a 100 mM phosphate or citrate–phosphate buffer at pH 5·50. The buffer was needed to maintain the extracts within the pH range of flavin stability, and to provide phosphate anion, a known inhibitor of the phosphatase enzymes (Vanderslice *et al.*, 1981).

Table 7. Determination of the purity of commercial FMN. Flow injection analysis conditions used to determine the effect of acetonitrile on the fluorescence of the flavins

Flavin	Mobile phase for flow injection analysis
7-Et-8-Me-RF	0·1% NaN_3 in 10 mM citrate–phosphate buffer, pH 5·50 Acetonitrile + 0·1% NaN_3 in 10 mM citrate–phosphate buffer, pH 5·50 (14:86, v/v)
RF	0·1% NaN_3 in 10 mM citrate–phosphate buffer, pH 5·50 Acetonitrile + 0·1% NaN_3 in 10 mM citrate–phosphate buffer, pH 5·50 (14:86, v/v) Acetonitrile + 0·1% NaN_3 in 10 mM citrate–phosphate buffer, pH 5·50 (5·6:94·4, v/v) — HPLC elution conditions for FMN
FMN	0·1% NaN_3 in 10 mM citrate–phosphate buffer, pH 5·50 Acetonitrile + 0·1% NaN_3 in 10 mM citrate–phosphate buffer, pH 5·50 (5·6:94·4, v/v)

Methylene chloride was added to the extraction medium to remove lipid artifacts, and RF photodegradation products, such as lumiflavin and lumichrome. Lopez-Anaya and Mayersohn (1987) reported that undetectable amounts of FMN and FAD, and small quantities of RF (less than 2% of the added spike) were present in the chloroform phase of their extraction procedure. The methylene chloride used in the present study is expected to behave similarly.

The chromatographic system that was used throughout the development of the non-degradative extraction procedure consisted of an isocratic separation with a mobile phase of methanol, 2-propanol, and a citrate–phosphate buffer, pH 5·50 at ambient temperature and a flow rate of 0·5 ml min^{-1} on an Act-1 polymer-based C_{18} column (15 cm × 4·6 mm). The eventual change to an acetonitrile-based mobile phase in the chromatographic system did not necessitate any change in the extraction procedure.

The chromatographic response factors for the flavins in standard solution that had been diluted to volume were comparable with those for flavins that were extracted from standard solutions using the non-degradative extraction procedure. This indicated that the internal standard, 7-Et-8-Me-RF, and the flavins were behaving similarly toward the separation and extraction methods, and that the standard solutions used for calibration could simply be diluted to volume in all future analyses.

Due to the presence of highly active enzyme systems in raw beef liver, sample manipulation prior to extraction had to be minimized to prevent flavin degradation. However, preliminary grinding of raw muscle meats and cooked samples in the food processor, to obtain a more homogeneous sample before weighing the analytical aliquots, produced no deleterious effects on the flavin contents.

The efficiency of the non-degradative extraction method was determined based on the recoveries of individual flavins from spiked samples. The recovery values obtained from a variety of foods (Table 8) indicated that coenzyme degradation was minimal, even in samples with highly active enzymes, such as raw liver. In all cases, the samples were spiked with known concentrations of flavin standards at the beginning of the extraction procedure, and the spikes were carried with the sample throughout the entire extraction and separation. Consequently, some variation in the recovery values was unavoidable because the spiked and unspiked values could not be determined on the same extract. This was particularly true of the ground beef samples. The variability of the macro- and micronutrients in ground beef has been documented (Holden *et al.*, 1986).

The variation in the TRF contents determined by HPLC was generally within acceptable limits (less than ±10% of the mean). The increased variation in the hamburger data may simply be a reflection of the inherent variability of ground beef.

Table 8. Recoveries of RF, FMN and FAD from spiked food samples

Type of sample	HPLC method	% Recovery		
		RF	FMN	FAD
Raw beef liver	I	96	101	100
	I	105	104	101
Raw ground round (beef)	I	110	99	111
Raw round steak (beef)	I	107	106	94
Cooked round steak	II	100	90	101
Raw skinless boneless	I	102	98	104
chicken breast	I	115	105	101
Pasteurized whole milk	I	103	104	97
	I	102	110	99
Raw whole egg	I	103	104	92
	I	113	111	115
Cooked whole egg	I	112	113	109
Ready-to-eat breakfast cereal	I	111	100	94
Hamburger buns	II	98	100	102
Fast food hamburgers	II	102	93	93

In general, the HPLC data for TRF agreed well with the published values (United States Department of Agriculture, 1963, 1976–1988). The exceptions were the published values for eggs, which consistently underestimated the HPLC levels by 35–40%, the breakfast cereal, which was underestimated by 60%, and the published data for ground beef, and fast food hamburgers, which exceeded the HPLC levels. Because the food analyses in this study was designed specifically to test the HPLC method, none of the foods were sampled extensively, and the HPLC values should not be construed as being representative of such foods.

Table 9. Comparison of TRF values obtained by HPLC and the AOAC standard fluorometric method

Type of sample	HPLC method number	Total riboflavin concentration (mg per 100 g)	
		HPLC	AOAC
Raw ground round (beef)	I	0·17 ± 0·007[a]	0·20 ± 0·003
Raw round steak (beef)	I	0·18 ± 0·001	0·23 ± 0·009
Cooked round steak (beef)	II	0·35 ± 0·008	0·36 ± 0·012
Raw skinless boneless chicken breast	I	0·10 ± 0·001	0·13 ± 0·093
Cooked skinless boneless chicken breast	I	0·14 ± 0·001	0·19 ± 0·012
Pasteurized whole milk	I	0·16 ± 0·010	0·19 ± 0·007
Raw whole egg	I	0·46 ± 0·011	0·43 ± 0·048
Ready-to-eat breakfast cereal	I	3·86 ± 0·200	3·70 ± 0·247
Hamburger buns	II	0·25 ± 0·003	0·37 ± 0·011

[a] Expressed as mean ± standard deviation, $n = 3$.

Food analyses were also conducted by the AOAC standard fluorometric method (Association of Official Analytical Chemists, 1984) with modified detection as described in a companion paper (Russell & Vanderslice, 1991). Table 9 compares the TRF results obtained from the same samples by HPLC and by the AOAC standard method. In general, the AOAC values exceeded the HPLC levels for TRF. This is hardly surprising since the AOAC method has been widely reported to overestimate TRF due to the presence of interfering artifacts. The AOAC results confirmed the high levels of TRF in the eggs and breakfast cereal obtained by HPLC.

CONCLUSIONS

Simultaneous HPLC separations have been developed for RF, FMN and FAD, and were successfully tested on a variety of foods. A RF analog, 7-Et-8-Me-RF, was used as the internal standard. FAD was found to be the most abundant of the B_2 vitamers in all the foods tested, except milk and enriched cereal products. The TRF results by HPLC generally agreed well with the data in the published tables of nutrient composition.

The PLRP-S polymer-based columns provided a more sensitive separation of the flavins and were more robust than the Act-1 columns. Silica-based columns were found to be unsuitable for the separation of the flavins.

A 2-step, non-degradative, extraction method was developed, based on the use of methanol, methylene chloride, and citrate–phosphate buffer at pH 5·50. Since the internal standard and the spikes were added at the beginning of the extraction procedure, it was possible to demonstrate that the flavin coenzymes were extracted without hydrolysis, on the basis of their recoveries from spiked food samples.

The commercial coenzyme preparations were shown to contain significant quantities of fluorescing impurities, and correction factors were developed to compensate for these impurities.

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