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# Development of a robotic–HPLC determination of riboflavin vitamers in food

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The need for laboratory automation is fuelled by the demands for better nutrient composition values based on large numbers of representative food samples. A fully automated method for the simultaneous analysis of the individual riboflavin vitamers in foods has been developed. It combines robotic extraction with HPLC quantitation. The robotic method was compared with a similar manual extraction using a variety of unfortified foods that are significant dietary sources of total riboflavin (TRF). The polymer-based columns used in the HPLC separation were found to be sensitive to the total organic carbon (TOC) content of 'HPLC-grade' water. Several procedures of varying complexity were successfully applied in order to remove interfering artifacts from the water. © 1998 Elsevier Science Ltd. All rights reserved

## INTRODUCTION

Reports of correlations between dietary intake and prevention or modulation of disease (National Academy Press, 1982) have increased demands for detailed nutrient composition data and nutritional labelling of foods. Due to the inherent variability in natural products, large numbers of samples are generally required to generate representative nutrient composition data for a particular food. Computerized control of scientific instrumentation and the use of autosamplers has led to the automation of a variety of analyses, but extraction of vitamins from foods has remained a time-consuming, error-prone manual procedure. Using robotics for both the extraction and analysis of vitamins results in improved throughput and efficiency of this analytical procedure.

The pharmaceutical industry has developed a number of robotic applications, including some for vitamin analysis (Yoshida *et al.*, 1987; Millier, 1987). Specific robotic analyses for vitamins in foods include: determinations of the C vitamers, the thiamine vitamers, and total vitamin C (Higgs *et al.*, 1985; Higgs and Vanderslice, 1987; Vanderslice and Higgs, 1989). These methods combine robotic extraction with analysis by HPLC or flow injection analysis. Our objective was to develop a fully automated determination of the individual riboflavin vitamers in foods by combining robotic extraction with HPLC separation and quantitation. This automated procedure is based on a published HPLC method (Russell and Vanderslice, 1992*a*), which has been tested on a variety of foods, including those of this study.

The three principal forms of the riboflavin vitamers that occur naturally in foods are: riboflavin (RF) and its coenzymes, flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). Published tables of nutrient composition data generally list only the total riboflavin (TRF) content of foods (United States Department of Agriculture, 1976–1988), much of which is of questionable accuracy (Russell and Vanderslice, 1992*a*,*b*). Many HPLC determinations for total riboflavin in foods have been published, but only three determine individual riboflavin vitamers. Of these, two were developed specifically for milk (Kanno *et al.*, 1991; Bilic and Sieber, 1990), while the third tested a variety of food products (Russell and Vanderslice, 1992*a*).

In analysis, the photosensitivity of the riboflavin vitamers is of primary concern. Exposure to ultraviolet and visible light causes irreversible degradation to lumiflavin and lumichrome, resulting in loss of vitamin activity. The riboflavin coenzymes are also susceptible to progressive hydrolysis of their phosphate groups, which converts FAD to FMN, and FMN to RF. Coenzyme hydrolysis can be induced by the endogenous phosphatase enzymes in many foods. Thermal processing generally inactivates these enzymes, so they are a

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factor only in the riboflavin analysis of fresh or unprocessed foods. All riboflavin vitamers are unstable at alkaline pH; the coenzymes are also susceptible to hydrolysis below pH 5.0. This necessitates that extraction and analysis of the individual vitamers be conducted between pH 5.0 and 7.0, and under subdued light (Russell and Vanderslice, 1990).

## EXPERIMENTAL

### Reagents

All solvents were HPLC grade. All other reagents were certified ACS grade. Aqueous solutions were prepared with glass-distilled water that was further treated to reduce the total organic carbon (TOC) load using one of the following methods:

1. 500 ml of glass-distilled water was gently stirred with 5 g of PLRP-S bulk HPLC column packing (20 $\mu$m, 100 Å polystyrenedivinylbenzene; Polymer Laboratories, Amherst, MA) for 85 min, after which the water was filtered through 0.45 $\mu$m filters. The packing material was wetted with acetonitrile immediately before treating the water. After use, it was regenerated by stirring for 30 min with 150 ml of warm acetonitrile;
2. glass-distilled water was filtered through an Empore SDB-XC extraction disk (polystyrenedivinylbenzene; 3M, St. Paul, MN) that had been conditioned according to the manufacturer's instructions;
3. glass-distilled water was treated with hydrogen peroxide and UV photolysis according to the method of Malaiyandi *et al.* (1980); or
4. glass-distilled water was passed through an Easy-Pure UV water purification system (model D7401; Barnstead/Thermolyne, Dubuque, IA).

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); supplied by Dr J. Lambooy, Professor Emeritus, Biochemistry Department, University of Maryland School of Dentistry). The internal standard included in all standard solutions and all samples was 7-Et-8-Me-RF. Spiking and standard solutions were prepared according to the method of Russell and Vanderslice (1992*a*).

### Sample preparation

All food samples were procured and prepared as outlined by Russell and Vanderslice (1992*a*). Samples were purchased from three major retail grocery chains located in the Ottawa, ON area. Only the edible portion of the food was used for analysis. Raw beef liver was cut into 1-g pieces. Milk was sampled directly from the retail carton. All other samples were prepared according to common cooking practices; meat samples were pan-fried until well-done and eggs were hard-cooked. Cooked samples were ground in a DLC-10E food processor (Cuisinart) to prepare homogeneous composite samples. Sample aliquots were weighed into 50 ml polypropylene Falcon tubes (Becton Dickinson Labware, Lincoln Park, NJ) and then frozen.

### HPLC analysis

All HPLC analyses were conducted using method II of Russell and Vanderslice (1992*a*), which is summarized in Table 1. The concentrations of RF, FMN, and FAD were corrected for impurities in the commercial standards, according to the procedure described by Russell and Vanderslice (1992*a*); these correction factors are listed in Table 2. Contents of the individual vitamers were expressed in nmoles vitamer per g food. Individual vitamer contents were totalled and expressed as mg TRF per 100 g food.

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

Guard column:
- PLRP-S (Polymer Laboratories Inc., Amherst, MA)
- 5 mm×3 mm
- Macroporous polystyrenedivinylbenzene resin

Analytical column:
- Two PLRP-S columns in series
- (Polymer Laboratories Inc., Amherst, MA)
- 15 cm×4.6 mm + 25 cm×4.6 mm
- 5 $\mu$m macroporous polystyrenedivinylbenzene resin, 100 Å pore size
- Temperature: 40°C

Mobile phase:
- Composition:
  - 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 min
    - —linear gradient to 14:86 (v/v) at 51 min
    - —isocratic at 14:86 (v/v) until 70 min
    - —linear gradient to 3:97 (v/v) at 80 min
    - —isocratic at 3:97 (v/v) until 90 min
- Flow rate:
  - —0–43 min at 1.2 ml/min
  - —43–80 min at 1.0 ml/min
  - —change to 1.2 ml/min at 90 min using a convex gradient[a]

Detector:
- Fluorescence: 450/522 nm (excitation/emission)

[a]Convex gradient: curve defined by the equation

$$P(t) = P(I) + [P(f) - P(I)] \times (t/T)^{1/5}$$

where $A$ and $B$ = % 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 for FMN and FAD**

| Commercial vitamers | Molar concentration of vitamers present |
|---|---|
| FAD | 72.41% FAD<br>1.00% FMN<br>0.05% RF |
| FMN | 43.07% FMN<br>1.93% RF |

All HPLC separations were conducted on a Series 4 liquid chromatograph which was equipped with quaternary gradient and helium solvent degassing capabilities (Perkin Elmer, Montreal, PQ). The temperature of the HPLC columns was regulated by immersion in a 40°C constant temperature bath (Haake D8-L, Karlsruhe, Germany). The vitamers were detected using an RF-551 fluorescence detector (Shimadzu Corp., Kyoto, Japan) set at 450/522 nm (excitation/emission). Data were collected and integrated using a Star Workstation (version 4.0, Varian Canada) loaded on a 386DX personal computer. The Star 9100 autosampler (Varian Canada, Mississauga, ON) was used for manual analyses, and a Zymate XP robotics system (Zymark Canada, Mississauga, ON) for robotic determinations.

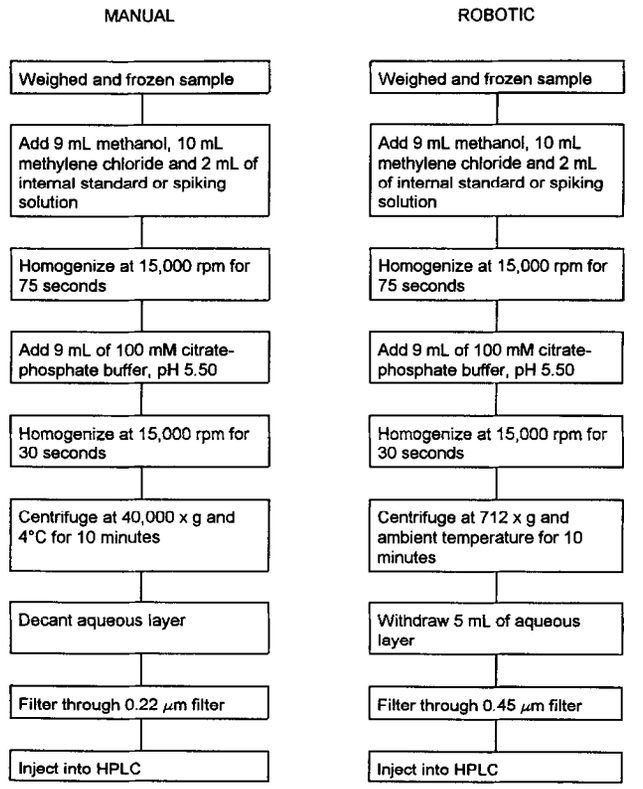


**Fig. 1.** Summary of manual and robotic extraction methods.

### Manual extraction

The riboflavin vitamers were extracted from the samples according to the non-degradative extraction method of Russell and Vanderslice (1992*a*; Fig. 1). Methanol, methylene chloride, and either the internal standard solution or the spiking solution were added to the tube of frozen sample. Homogenization was carried out using a Polytron PT3000 homogenizer equipped with a PT-DA-3012/2M generator (Brinkmann Instruments, Inc., Rexdale, ON), which was operated at 15 000 rpm. Citrate–phosphate buffer was added to the sample and it was homogenized again. The sample was centrifuged using a RC-5B refrigerated centrifuge (Sorvall Instruments) set at 20 000×*g* and 4°C. The aqueous layer was decanted, filtered, and injected into the HPLC. All extractions and analyses were carried out under yellow light, using low actinic glassware.

### Robotic extraction

The robotic method of vitamin extraction duplicated the manual extraction conditions as closely as possible (Fig. 1). The lay out of the robotic system is summarized in Figs 2 and 3. Specific differences in manual and robotic extraction conditions are outlined in Table 3.

For robotic analysis, frozen samples were placed in the refrigerated rack. The gripper hand was used to retrieve a sample, dispense reagents, homogenize the sample, and then place it in the centrifuge. After centrifuging, the syringe hand withdrew an aliquot of the aqueous layer, that was filtered and injected into the HPLC. Operation of the Series 4 HPLC pump, the RF-551 fluorescence detector, and the Star Workstation integrator were controlled by the robotics system. While one sample was being centrifuged the next was prepared ready for centrifuging, so that two samples were processed concurrently. All analyses were carried out in the dark.

Both the robotic hardware and software were modified. The refrigerated rack was insulated to improve temperature control. The gripper fingers and the cap parking pedestals of the Fully Automated Capping Station were customized to accommodate Falcon tubes. To prevent splashing during homogenization, caps with

**Table 3. Comparison of manual and robotic extraction methods**

| Manual extraction | Robotic extraction |
|---|---|
| Centrifuged:<br>for 10 min<br>at 20 000×*g*<br>at 4°C | Centrifuged:<br>for 10 min<br>at 712×*g*<br>at ambient temperature |
| Samples kept in an ice bath before and after centrifugation | Samples kept at ambient temperature before and after centrifugation |
| Batch centrifugation | Samples centrifuged immediately after homogenization |
| Conducted under yellow light | Conducted in dark |

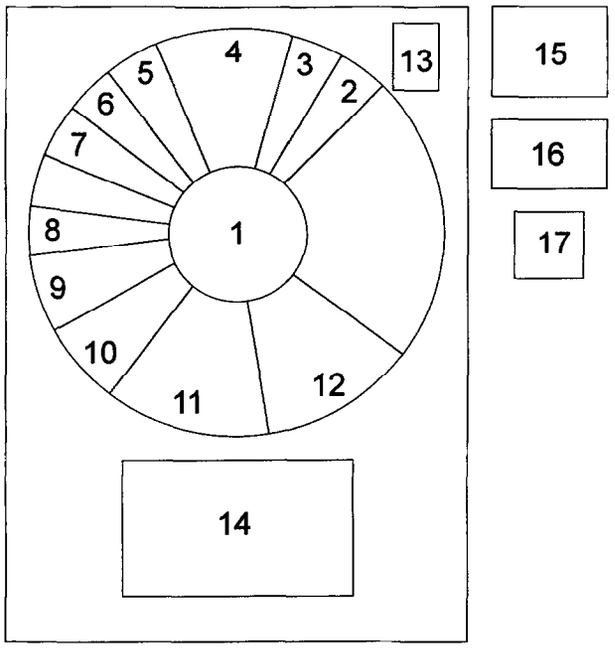


**Fig. 2.** Lay out of robotic system for $B_2$ vitamers. Table top level. **1.** Zymate XP robot; **2.** LC Sipping Injection; **3.** Membrane Filtration; **4.** Rack, 50 ml centrifuge tube; **5.** Syringe Hand G and Pipette Tip Rack, 1 ml; **6.** Fully Automated Capping Station, modified for 50 ml Falcon tubes; **7.** General Purpose Hand B, 20–30 mm container and auxiliary tip rack, 1 ml; **8.** Disposal; **9.** Dilute and Dissolve, 50 ml centrifuge tube; **10.** Polytron PT3000 Homogenizer, PT-DA-3012/2M Generator (Brinkmann Instruments); **11.** Z710 Centrifuge, 50 ml centrifuge tube; **12.** Refrigerated Rack, 50 ml centrifuge tube; **13.** 40°C constant temperature bath (Haake D8-L); **14.** System V Controller (version 1.51) and 486 AT personal computer; **15.** Series 4 Liquid Chromatograph (Perkin Elmer); **16.** RF-551 fluorescence detector (Shimadzu Corp.); **17.** Star Workstation (version 4.0, Varian Canada).

holes of 1.59 cm (diameter) were placed on the Falcon tubes. Software was adjusted accordingly.

### Comparison of manual and robotic methods

Both methods were directly compared over a range of 12 different samples for each food type (Table 4). On samples, one for each source, were prepared for each of the four replicates. Four analytical aliquots were taken from each sample; two were assigned to the manual method and two to the robotic. At least one additional aliquot was included each day as a spiked sample. Aliquots were randomly assigned, as were methods to 'days'. Analyses of the aliquots for each replicate were completed within 48 h. Therefore, the individual vitamers and TRF were determined on a total of 48 aliquots for each type of food.

The statistical analysis of the data was carried out by analysis of variance (ANOVA), using Genstat 5.3.1 (Genstat Committee, 1993). Four sources of variation (blocking strata) are identified (Table 5): among replicates (3 degrees of freedom (df)), among samples within replicates (8 df), between test-days within samples (12 df), and between aliquots within methods. The experimental factor of 'method' and the blocking factor of 'age' are estimated in the third blocking stratum. Variation between aliquots within 'method' and 'day' form the fourth stratum and represent the method error plus the variation between aliquots of the product, noted as 's' in Table 6. Statistical comparisons between methods were made with the F-statistic. Means and standard errors for all trials are reported.

**Table 4. Example showing the aliquots chosen in the sampling plan that compared manual and robotic methods**

| Replicate | Age (day) | Method | Sample | | | | | |
|---|---|---|---|---|---|---|---|---|
| [1] | 1 | Robotic | 1 | 1 | 2 | 2 | 3 | 3 |
| | 2 | Manual | 1 | 1 | 2 | 2 | 3 | 3 |
| [2] | 1 | Manual | 1 | 1 | 2 | 2 | 3 | 3 |
| | 2 | Robotic | 1 | 1 | 2 | 2 | 3 | 3 |
| [3] | 1 | Manual | 1 | 1 | 2 | 2 | 3 | 3 |
| | 2 | Robotic | 1 | 1 | 2 | 2 | 3 | 3 |
| [4] | 1 | Robotic | 1 | 1 | 2 | 2 | 3 | 3 |

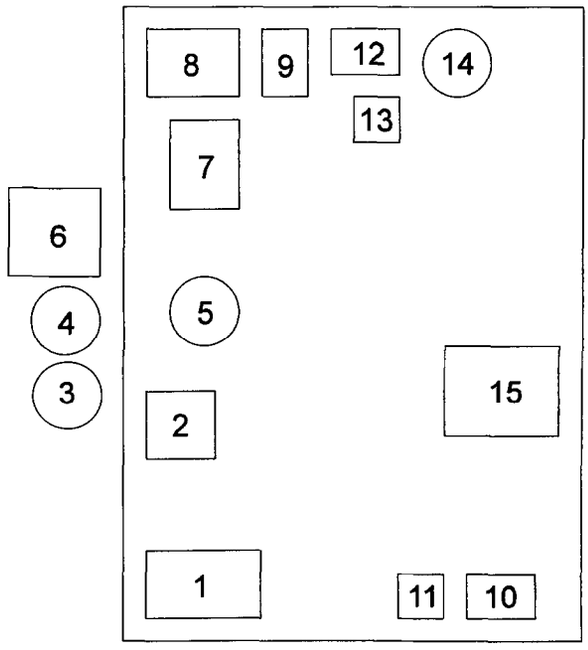


**Fig. 3.** Lay out of robotic system for $B_2$ vitamers. Table bottom level. **1.** P5300 printer (NEC); **2.** pump for homogenizer wash solution (MasterFlex 7553-50, speed 7; Cole-Parmer); **3.** homogenizer wash solution (distilled water); **4.** Polytron homogenizer waste; **5.** Dilute and Dissolve waste; **6.** Disposal waste receptacle; **7.** Master Laboratory Station II; **8.** Master Laboratory Station II; **9.** Z830 Power and Event Controller; **10.** Z830 Power and Event Controller; **11.** 10 A Power Controller for Polytron homogenizer; **12.** Instrument Interface for HPLC system (series 4 Pump, Star Workstation); **13.** compressed air controller; **14.** LC Sipping Injection waste; **15.** RTE-100 refrigerated bath/circulator (NESLAB Instruments) for refrigerated rack.

**Table 5. Partitioning of the degrees of freedom (df) in the analysis of variance (ANOVA) table**

| Source | df | |
|---|---|---|
| Among replicates | 3 | |
| Among samples/replicates | 8 | |
| Between days/samples/replicates | 12 | |
| Age | | 1 |
| Method | | 1 |
| Residual | | 10 |
| Aliquots | 24 | |
| Total | 47 | |

## DISCUSSION

The HPLC method makes use of polymer-based columns that are sensitive to dissolved organic impurities in purified water. Distillation and treatment with cartridge-based water purification systems did not reduce the TOC load in the water to the levels needed to remove interfering artifacts. Flat HPLC baselines were obtained by treating glass-distilled water with any one of the four procedures outlined in the Experimental section. The efficiency of the treatments varied with the initial TOC content of the water. In this study, use of the EasyPure UV system (involving cartridge cleanup and UV photolysis) after glass-distillation gave the most consistent results.

The riboflavin in Western diets is primarily supplied by animal products and fortified cereal products. In this study, the manual and robotic methods are compared using samples of unfortified foods that are significant dietary sources of TRF (Block *et al.*, 1985). These included beef liver (organ meat), beef steaks (muscle meat), eggs, and milk. Raw liver is included as an example of a food substrate that contains active phosphatase enzymes, which are capable of degrading the riboflavin coenzymes.

Table 6 presents the mean contents of RF, FMN, FAD, and TRF for each food type, by method of analysis. With the exception of cooked beef liver, the robotic method generally produces slightly higher vitamer concentrations; in most cases the differences are statistically significant at the 5% level. This may indicate that manual extraction causes a slight overall degradation of the riboflavin vitamers relative to the robotic method. In addition, the ratio of coenzymes:RF is higher by the robotic method (except for cooked liver), indicating that there may be a slight hydrolysis of the coenzymes to RF during manual extraction. The reasons for the distinct behaviour of cooked liver, relative to all other food types, are unclear.

The precision of the manual and robotic methods is similar, as shown by the variability ($s$) associated with the means (Table 6). Not surprisingly, variation tended

**Table 6. Mean content of riboflavin vitamers and TRF by food and analytical method**

| Type of food | Method of analysis | $n$[a] | Vitamin content | | | | | | | | Ratio of [Coenzymes] to [RF] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | RF (nmol/g) | | FMN (nmol/g) | | FAD (nmol/g) | | TRF (mg/100 g) | | |
| | | | $\bar{x}$[b] | s[c] | $\bar{x}$ | s | $\bar{x}$ | s | $\bar{x}$ | s | |
| Raw beef liver | Robotic | 24 | 4.73 | 0.297 | 5.98 | 0.474 | 62.8 | 3.47 | 2.77 | 0.144 | 14.5 |
| | Manual | 24 | 4.63 | 0.354 | 6.46 | 0.397 | 62.3 | 3.54 | 2.76 | 0.153 | 11.1 |
| | SEM (df = 10)[d] | | 0.677 | | 0.145 | | 1.49 | | 0.058 | | |
| | Sig. Prob.[e] | | ns | | ** | | ns | | ns | | |
| Cooked beef liver | Robotic | 23 | 19.2 | 0.67 | 36.5 | 1.51 | 72.4 | 8.43 | 4.82 | 0.383 | 5.67 |
| | Manual | 23 | 19.8 | 0.65 | 43.2 | 1.62 | 78.6 | 4.77 | 5.33 | 0.167 | 6.14 |
| | SEM (df = 10) | | 0.37 | | 0.69 | | 3.94 | | 0.150 | | |
| | Sig. Prob. | | ns | | ** | | ns | | ** | | |
| Cooked beef steak | Robotic | 24 | 1.05 | 0.1924 | 0.594 | 0.0282 | 8.42 | 0.327 | 0.379 | 0.0187 | 8.58 |
| | Manual | 24 | 0.862 | 0.2458 | 0.420 | 0.0340 | 6.54 | 0.646 | 0.295 | 0.0184 | 8.09 |
| | SEM (df = 10) | | 0.0558 | | 0.1973 | | 0.241 | | 0.0102 | | |
| | Sig. Prob. | | ** | | ** | | ** | | ** | | |
| Hard cooked egg | Robotic | 22 | 13.2 | 0.56 | 0.106 | 0.0182 | N/D[f] | | 0.503 | 0.0209 | 0.00803 |
| | Manual | 24 | 11.1 | 0.34 | 0.073 | 0.0190 | N/D | | 0.421 | 0.0121 | 0.00658 |
| | SEM (df = 9) | | 0.17 | | 0.0029 | | | | 0.0062 | | |
| | Sig. Prob. | | ** | | ** | | | | ** | | |
| Pasteurized whole milk | Robotic | 24 | 4.09 | 0.183 | 0.350 | 0.0201 | 0.212 | 0.1603 | 0.175 | 0.0115 | 0.137 |
| | Manual | 23 | 3.86 | 0.087 | 0.327 | 0.0167 | 0.153 | 0.0682 | 0.164 | 0.0067 | 0.124 |
| | SEM (df = 10) | | 0.066 | | 0.0041 | | 0.0284 | | 0.0032 | | |
| | Sig. Prob. | | ** | | ** | | ns | | ** | | |

[a] $n$ = number of determinations.
[b] $\bar{x}$ = mean.
[c] s = standard error.
[d] SEM = standard error of the mean; df = degrees of freedom.
[e] Sig. Prob. = probability of significant differences; ns = not significant; ** = significantly different, $p < 0.05$.
[f] Peak not detected.

**Table 7. Range of content for riboflavin vitamers and TRF by food**

| Type of food | Range of vitamin content | | | |
|---|---|---|---|---|
| | RF (nmol/g) | FMN (nmol/g) | FAD (nmol/g) | TRF (mg/100 g) |
| Raw beef liver | 2.30–7.09 | 4.36–9.25 | 52.9–79.9 | 2.32–3.49 |
| Cooked beef liver | 7.07–29.3 | 21.5–65.6 | 32.5–116.7 | 2.84–7.02 |
| Cooked beef steak | 0.310–3.07 | 0.220–0.890 | 3.84–11.42 | 0.180–0.510 |
| Hard cooked egg | 9.35–15.5 | 0.020–0.530 | N/D[a] | 0.360–0.590 |
| Pasteurized whole milk | 3.31–4.59 | 0.280–0.420 | N/D–0.530 | 0.140–0.200 |

[a]Peak not detected.

to increase at low concentration, e.g. FAD in pasteurized whole milk. The ranges of vitamin concentrations (Table 7) reflect the sample-to-sample variability inherent in micronutrient content of unfortified food products. Recoveries from spiked samples are in the same range, 85–115% for both the manual and robotic methods.

The robotic method proved to be equally effective for processed or cooked samples, and for samples known to contain degradative enzymes, such as raw liver. We designed the sampling plan and conducted the analyses to facilitate comparison between the two methods of extraction; values do not represent the food types in general. However, the experimental TRF results were found to be in reasonable agreement with published TRF values for similar foods (United States Department of Agriculture, 1976–1988).

As mentioned earlier, the HPLC method has been used previously to test the same food types used in this study. Reasonable agreement has been demonstrated among TRF values obtained by this method (run manually), those determined by the AOAC International standard fluorometric method, and published nutrient composition data (Russell and Vanderslice, 1992*a*). In this study chromatograms obtained from the manual and robotic methods were nearly identical (Fig. 4). This result is as expected because the HPLC pump, detector, data collection/integration system, and columns were the identical for both methods. Therefore the separation and quantitation portion of the methods can not account for the observed differences in the results.

There are three major differences between the manual and the robotic methods (Table 3). (1) In the manual extraction, the samples were kept cold between the steps of the extraction, and were extracted in batches of four tubes. This meant some delays between steps. In contrast, the robotic method was conducted at room temperature and steps were carried out sequentially from start to finish. (2) The centrifugation step in the manual extraction had a higher relative centrifugal force. (3) The manual method was performed under yellow light but the robotic extraction was run in the dark.

We consider only one of the differences between methods to explain the differences in measured values. Any effects of delays between extraction steps in the manual methods should have been minimized by the use of ice baths, while ambient temperatures in the robotic

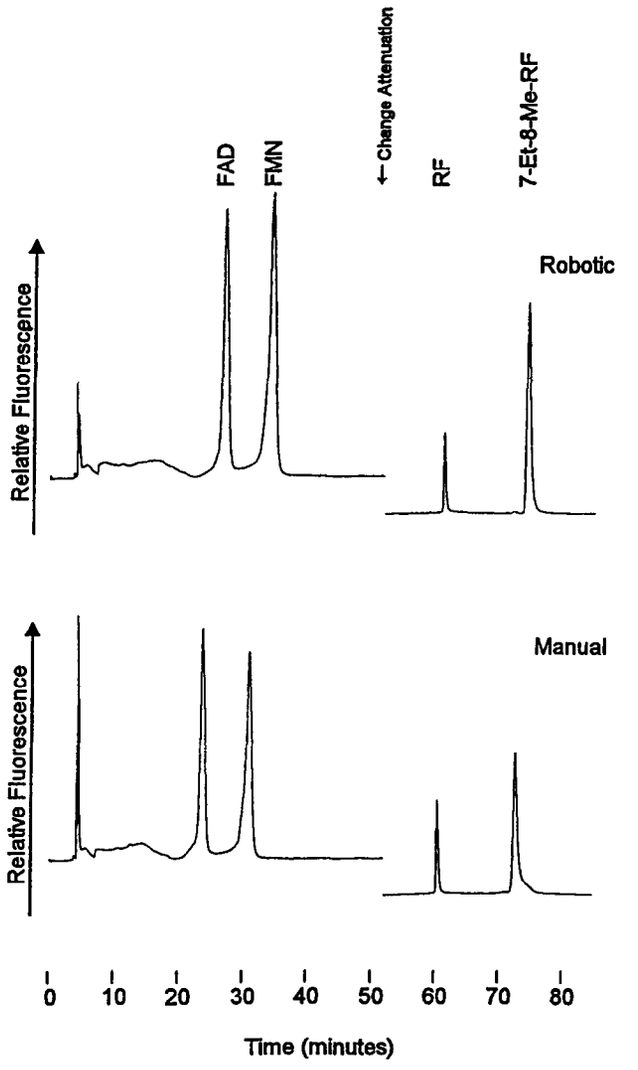


**Fig. 4.** Chromatograms of raw liver samples generated using manual and robotic extraction.

method would have been more likely to degrade the vitamers. Centrifugation in both methods clearly separated the tissue from the aqueous layer and the methylene chloride layer. However, all of the riboflavin vitamers are known to be extremely sensitive to light, which can induce vitamer conversion and degradation (Russell and Vanderslice, 1990). We conclude that running the robotic method in the dark probably exerted a protective effect on the riboflavin vitamers that was not present in the manual procedure.

The robotic method of extraction was as reliable or more reliable than the manual method. In the 9 months it took to complete this study, we experienced one mechanical breakdown of the robotic system. If anything, errors during sample preparation were less likely with the robotic system than the manual procedure, which is time-consuming and requires considerable concentration and technical skill. The robotic method completed the extractions in approximately 75% of the time required to extract the samples manually. It should be noted that the HPLC separation is the overall rate-limiting step in the analysis. Since we found that the riboflavin vitamers were generally more stable in the filtered extracts than in the food samples, the robotic system was programmed to complete all extractions sequentially at the start of the method and wait to inject the prepared extracts as the HPLC reported ready. This is comparable to the manual procedure, in which the extractions are completed in batches and the extracts are placed on the autosampler to await injection.

## CONCLUSIONS

A robotic extraction for the riboflavin vitamers was combined with an existing HPLC separation and tested on a variety of foods. The robotic method compares favourably with that of manual extraction, including determinations on samples known to contain degradative enzymes. It generally produces slightly higher results than the manual method, with higher ratios of the molar concentrations of the coenzymes:RF, which is indicative of less degradation/interconversion of the individual vitamers during extraction. The ability to operate the robotic system in the complete absence of light is most likely responsible. The robotic extraction was faster than its manual counterpart.

The HPLC separation was found to be sensitive to the TOC content of the water, and several alternative methods were successfully applied to clean up 'HPLC grade' water.

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