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The HPLC Analysis of Thiamin and Riboflavin in Potatoes

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ABSTRACT

A simple HPLC method for the analysis of thiamin (as thiochrome) and riboflavin in both raw and cooked potato is described. The potato extract, after a minimum clean up, is injected onto a C_{18} reverse phase column and the vitamins are separated isocratically with water:methanol. The use of fluorescence detection, which is highly specific and sensitive, minimises the number of interfering peaks.

Recoveries of both vitamins, when taken through the method or added to potato samples before extraction, are consistently better than 90%. The results for thiamin in the potato are slightly higher than those obtained by the AOAC (1980) chemical method, whereas the reverse is true for riboflavin. The method may have application to other food matrices and is more rapid than the AOAC (1980) chemical method.

INTRODUCTION

The HPLC analysis of vitamins is not new and has mainly been applied to pharmaceuticals or vitamin premixes (Wittmer & Haney, 1974; Wills *et al.*, 1977; Kamman *et al.*, 1980) where, by virtue of their nature, the level of these vitamins is high and there is little interfering material. Food matrices, however, are difficult to extract for the determination of water-soluble vitamins, which normally occur in trace amounts. Because of extraction problems, the food must be extensively hydrolysed using

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dilute mineral acid (and enzymes in the case of thiamin) at elevated temperatures; for example, refluxing at 100 °C. The resulting extract is therefore highly acidic and contains appreciable amounts of salts, if it has subsequently been neutralised or buffered, in addition to a wide range of potentially interfering substances derived from the hydrolysis of the food matrix. Furthermore, large or sudden pH and salt concentration changes may have undesirable effects on the HPLC columns. Normally, these crude extracts would be extensively cleaned up using a variety of methods including filtration, neutralisation, desalting, chromatography or solvent extraction, before separation by HPLC because of the difficulty of separating the desired component from a wide range of compounds, some of which will have similar retention times.

The difficulties of resolution have led to the use of elution gradients and ion pairing (PIC) reagents.

However, since the measurement of water-soluble vitamins in foods is important for nutrient intake calculations, the use of potentially rapid and sensitive procedures like HPLC needs to be investigated as an alternative to the time-consuming chemical and microbiological assays. The time involved in the classical methods is a major disincentive to analysts, so vitamin analysis in foods is normally only undertaken for small numbers of samples.

The HPLC analysis of riboflavin and thiamin in selected foods has been reported (Ang & Moseley, 1980; Skurray, 1981; Fellman *et al.*, 1982), but no attempts have been made to measure these vitamins in potato by HPLC.

In this paper we describe a robust and rapid method for the extraction and estimation of riboflavin and thiamin in raw and cooked potato using HPLC with a sensitive and highly specific fluorescence method of detection. A comparison with the AOAC (1980) chemical procedure, which involves extraction in butan-2-ol, is presented. The method may have application to other food matrices.

MATERIALS AND METHODS

Sample preparation

A 20-g sample consisting of raw or cooked diced, frozen potato was placed in a 250-ml conical flask containing 65 ml 0·1 M HCl and a magnetic stir bar. The solution was heated with vigorous agitation and refluxed

for *ca.* 30 min. After cooling to below 50 °C, *ca.* 16 ml 1·25% (w/v) Takadiastase (Serva, Heidelberg) solution in 1 M acetate buffer (pH 4·6) was added. The buffering action brings the pH to *ca.* 4·5, which is required for maximum enzyme activity. This crude enzyme preparation contains both phosphatases, to hydrolyse any remaining thiamin phosphate esters (AOAC, 1980) and amylases, which help to complete the hydrolysis of any remaining starch which is essential to ensure good filtration.

The sample was then incubated at 45–50 °C for 2 h in a shaking water-bath. After incubation the solution was left to cool to room temperature, after which it was transferred to a 100-ml volumetric flask, made to volume with distilled water, mixed and filtered through Whatman 541 filter paper into polythene bottles and stored at −20 °C until required for analysis.

HPLC determination of thiamin and riboflavin

Thiamin in the filtrate was converted to its fluorescent derivative, thiochrome, by the addition of 3 ml of 0·03 M potassium ferricyanide solution in 3·75 M aqueous sodium hydroxide to 5 ml filtered extract contained in a 10-ml volumetric flask.

The flask was stoppered and the solution shaken vigorously for *ca.* 2 min and left to stand in the dark for 10 min, after which it was filtered through a Millipore filter (0·45 μm) into vials for HPLC analysis. Five-millilitre aliquots of standard and blank solutions were treated in the same way.

Blanks, standards and filtered sample solutions were analysed for thiamin and riboflavin on separate chromatograms. Ten microlitres were injected and separated on a Waters 25 cm × 4·6 mm μ-Bondapak C_{18} column, isocratically at ambient temperature with 70:30 water:methanol at 2 ml/min using a Waters Associates Modular HPLC System consisting of a Model 660 programmer, a 6000 LC pump and a U6K injector.

The peaks were detected using a Perkin-Elmer LS-5 Luminescence Spectrometer fitted with a 8 μl flow cell. Excitation and emission wavelengths (nm) for thiamin and riboflavin were 365 and 435 and 450 and 510, respectively.

Quantitative data were obtained using fluorescence peak heights of standards from a Model PM8251 single pen recorder (Philips) and calibration curves were constructed by plotting peak heights versus concentration. Detector responses were linear within the range 0·10–1·0 μg/ml

and 0·02–0·10 μg/ml for thiamin and riboflavin, respectively. The detection limits for thiamin and riboflavin were found to be 0·5 ng and 0·1 ng, respectively.

Chemical determination of thiamin and riboflavin

Filtrates prepared for HPLC were analysed by the AOAC (1980) methods.

RESULTS AND DISCUSSION

The extraction procedure outlined in this paper is that of the AOAC (1980), with some slight modifications. The pH adjustment is achieved by the addition of a standard volume of Takadiastase solution in acetate buffer (pH 4·6). This brings the pH to *ca.* 4·5 which is required for maximum enzyme activity. Takadiastase is the enzyme of choice as Clarase was unobtainable.

Although a single extraction method was used, it was not possible to measure both vitamins simultaneously by HPLC because the excitation and emission maxima are different and a much higher sensitivity was needed for riboflavin as the level of this vitamin in potato is low.

The specificity and sensitivity of HPLC with fluorescence detection eliminates the need for the base-exchange silica purification step for thiamin. In addition, the permanganate oxidation step of co-fluorescing compounds and sodium thiosulphite blank determination in the AOAC chemical procedure for riboflavin are not required.

The mobile phase used is much simpler than those used in other studies (Kamman *et al.*, 1980; Skurray, 1981; Toma & Tabekhia, 1979) and does not require the use of PIC reagents or complex elution gradients. Although the use of guard columns was considered to prolong the life of the column with these crude extracts, in practice more than 100 separations each of riboflavin and thiochrome have been carried out on the same column without noticeable loss of resolution or changes in retention time.

Figure (1) (a) to (d) shows the chromatograms for thiamin and riboflavin standards and potato extracts.

It should be noted that the retention times for both vitamins in the sample are slightly less than the standards, due to differences in the matrix extracts.

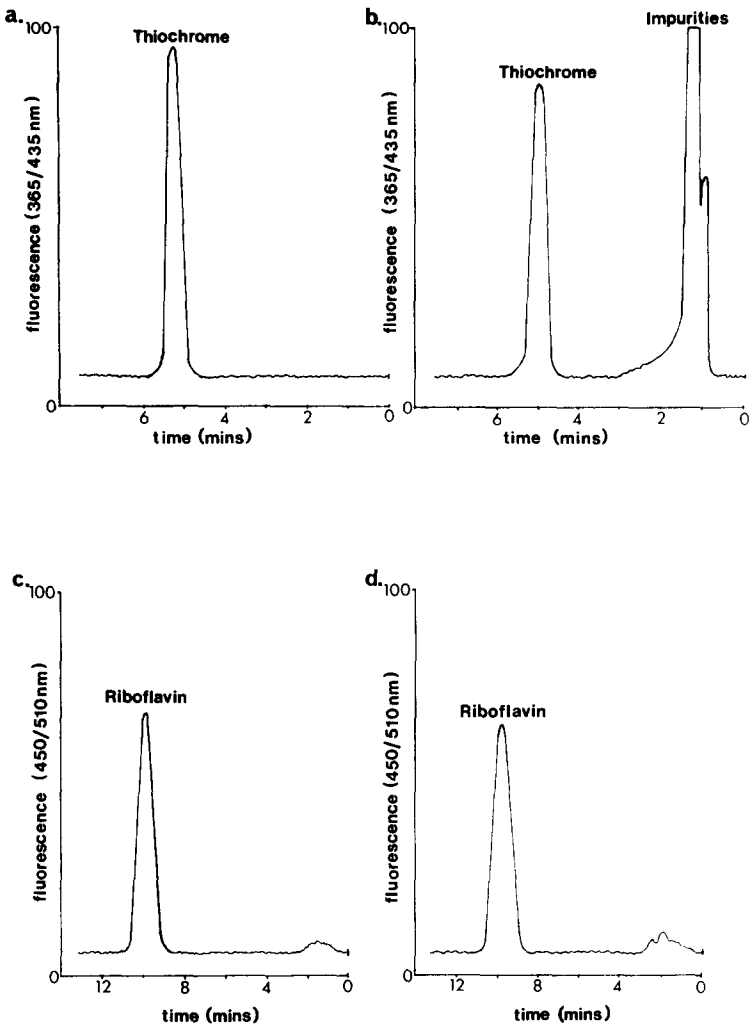

Fig. 1. Chromatograms of thiamin (as thiochrome) and riboflavin standards and potato extracts. (a) 10 μl 0·4 μg/ml thiamine standard. (b) 10 μl potato extract. (c) 10 μl 0·04 μg/ml riboflavin standard. (d) 10 μl potato extract.

Changes in retention time due to matrix effects have not been reported previously and, since retention time is normally one of the criteria used to identify components, it was of some concern. However, peak identity was confirmed by spiking potato samples before and after extraction and by emission scanning of column eluates containing the peaks at the excitation frequencies for thiochrome and riboflavin. The change

TABLE 1
Thiamin and Riboflavin Values in Raw and Cooked Potatoes

Sample	*Method*	*Thiamin*	*Riboflavin*
Raw[a]	HPLC	0·18 (0·15–0·24)	0·02 (0·01–0·02)
	AOAC	0·12 (0·07–0·16)	0·03 (0·02–0·04)
Boiled[b]	HPLC	0·16 (0·14–0·21)	0·02 (0·01–0·02)
	AOAC	0·10 (0·05–0·15)	0·03 (0·02–0·03)
Chips[b]	HPLC	0·18 (0·16–0·26)	0·02 (0·01–0·02)
	AOAC	0·10 (0·05–0·15)	0·03 (0·03–0·04)

[a] Average value and range from ten samples analysed in duplicate.
[b] Average value and range from four samples analysed in duplicate.

in retention time must therefore be as a result of co-elution with a non-fluorescing compound or transient changes on the column due to components in the extract. The most probable causes could be the effects of salt concentrations or pH since it has been demonstrated (Wills *et al.*, 1977) that both salt concentrations and pH have considerable effects on retention time, particularly for thiamin, in the C_{18} reversed phase separation of vitamins.

The HPLC procedure gave slightly higher values for thiamin than did the AOAC method (Table 1). Recovery of vitamins added to the samples before extraction (Tables 2 and 3) was consistently better than 90 % when calculated from a standard curve. The recovery data show that riboflavin is stable during the oxidation of thiamin, as shown by Fellman *et al.* (1982), but the HPLC procedure gave lower values for riboflavin than the AOAC procedure. The reasons for this are not clear, but it may be

TABLE 2
Recovery of Thiamin from Potato (20 g Sample)

μg added	*μg recovered**	*Per cent recovery*
50	47·9	95·8
100	96·5	96·5
200	189·2	94·6
400	375·5	93·9
		Average = 95·2
	Per cent coefficient of variation =	1·2

* Duplicate means.

TABLE 3
Recovery of Riboflavin from Potato (20 g Sample)

μg added	*μg recovered**	*Per cent recovery*
2·5	2·4	96·0
5·0	4·6	92·0
7·5	7·3	97·3
10·0	9·2	92·0
		Average = 94·3
		Per cent coefficient of variation = 2·9

* Duplicate means.

that in the AOAC chemical method there is fluorescent contamination that causes an overestimation of the amount of riboflavin.

The HPLC value for riboflavin in potatoes is in good agreement with that quoted by Burton (1966) of 0·02 mg/100 g for riboflavin, the latter being determined by microbiological assay.

It has been reported (Toma & Tabekhia, 1979; Skurray, 1981) that HPLC values for riboflavin were lower than those found using the AOAC chemical procedure, particularly in those foods that contained small amounts of the vitamin, due to the interference of fluorescent compounds present in foods.

A measure of reproducibility was performed for thiamin and riboflavin by the multiple injection of the same sample ($n = 5$); the data are given in Table 4.

TABLE 4
Reproducibility of Determination of Thiamin and Riboflavin (mg/100 g)

Sample	*Thiamin*	*Riboflavin*
1	0·20	0·020
2	0·19	0·022
3	0·20	0·018
4	0·21	0·019
5	0·23	0·021
	$\bar{x} = 0{\cdot}206$ Per cent coefficient of variation = 7·4	$\bar{x} = 0{\cdot}020$ Per cent coefficient of variation = 7·9

When compared with the manual AOAC procedures, the HPLC system outlined in this paper has the advantage of not requiring such time-consuming steps as the base-exchange silica purification and the solvent extraction for thiamin and the sodium thiosulphite blank determination for riboflavin. It also offers good recoveries (>90%) when compared with standards and good precision for both vitamins. Currently, the method is being evaluated by estimating riboflavin and thiamin in dried milk samples and in 'fast' foods in conjunction with both microbiological assay and alternative HPLC methods to determine applicability to other food matrices.

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