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Note

Simultaneous analysis of thiamin and riboflavin in foods by high-performance liquid chromatography

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Thiamin and riboflavin are essential nutrients that occur in foods and other biological materials either free or bound to other components. While there are a number of methods available for the analysis of thiamin and riboflavin, they are most commonly determined by fluorometric methods. Thiamin is extracted from the food matrix by acid and enzyme treastment, purified by ion-exchange chromatography and then oxidised to thiochrome which is measured fluorometrically at 435 nm[1]. Riboflavin is liberated from the food by acid treatment and after interfering substances are removed by oxidation, it is quantified fluorometrically at 525 nm[1]. There is, however, need for methods that are less time-consuming and less subject to interference and the technique of high-performance liquid chromatography (HPLC) has been of increasing interest, in particular, the development of methods which allow simultaneous determination of both thiamin and riboflavin.

Separation of thiamin and riboflavin by passage through reversed-phase or ion-exchange columns appears to present little problem but the co-elution of interfering substances has resulted in some emphasis being placed on detection systems. Simultaneous determination of thiamin and riboflavin has been reported for high potency pharmaceutical products and vitamin mixtures using UV detection systems[2–4]. While the use of UV detection systems has been reported to resolve thiamin and riboflavin in rice products[5] and in cereal products[6], the relatively low sensitivity of these vitamins to UV[7] and the presence of UV-absorbing interfering substances in many foods[8] make the widespread use of such systems for food analysis seem unlikely without the inclusion of sample concentration and clean-up procedures.

Various workers[7–10] have proposed HPLC methods which determine thiamin as thiochrome and riboflavin as either riboflavin or lumiflavin with fluorescence detection systems. The use of fluorescence increases the sensitivity of the analysis and reduces the range of interfering factors[10]. The thiochrome was formed either before[7,10] or after[9] column separation while lumiflavin was formed pre-column[8]. However, these methods have only been examined on a small number of foods and therefore their general application to food analysis is not known. We have examined the feasibility of simultaneous analysis of thiamin and riboflavin in a range of foods by HPLC using a reversed-phase column, post-column derivatisation of thiamin and fluorescence detectors. The necessity for and development of suitable purification and concentration techniques for specific foods is also reported.

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EXPERIMENTAL

Analyses were carried out on a μBondapak C_{18} (10 μm) Radial-Pak column (Waters Assoc.) installed in a Waters Assoc. liquid chromatograph (Model ALC/GPC 244) equipped with a 41-mPa pump and U6K injector. The mobile phase was methanol–water (40:60, v/v) containing 5 m*M* Pic B_6 (Waters Assoc.) and was filtered and degassed under vacuum before use. The flow-rate was 1.5 ml/min. Column effluents were monitored by various detectors; a UV detector set at 254 nm (Waters Assoc. Model 440) for thiamin and riboflavin, a fluorescence detector with 8-μl flow cell (Waters Assoc. Model M420) and 360 nm excitation and 500 nm emission filters for riboflavin (designated as F_{500}) and a fluorescence detector with 360 nm excitation and 425 nm emission filters for thiochrome (designated as F_{425}). Thiochrome was formed by post-column derivatisation where the column effluent was passed into a stainless-steel T-piece reaction block into which an oxidising solution of 1 m*M* potassium ferricyanide in 0.375 *M* sodium hydroxide was pumped at 1.0 ml/min using a 41-mPa pump. The concentration of sodium hydroxide was reduced to one-tenth of that commonly used[1] to minimise corrosion in the detector system but the solution still produced a quantitative conversion of thiamin to thiochrome. The reaction mixture was passed through a stainless-steel coil (250 cm × 0.5 mm I.D.) which allowed the reaction to proceed to completion before the solution arrived at the detector.

The general method for extraction of thiamin and riboflavin from foods was to digest a homogenised sample (10–25 g) with 0.1 *M* hydrochloric acid followed by enzymic digestion with clarase (Miles Labs., Sydney, Australia) according to AOAC methods[1]. The resulting solution was filtered through paper (Whatman 541) then through a membrane/ultrafilter cell (Diaflo, Amicon). An aliquot of filtrate (50–100 μl) was injected onto the HPLC system.

Concentration and further purification of the filtered extract was carried out for certain foods using a C_{18} Sep-Pak (Waters Assoc.), a short plastic disposable column containing μBondapak C_{18}. A C_{18} Sep-Pak cartridge was placed on the tip of a syringe barrel and the column preconditioned by washing with methanol (10 ml) containing 5 m*M* Pic B_6 followed by double distilled water (10 ml) containing 5 m*M* Pic B_6. A sample (2–10 ml) was placed on the Sep-Pak which was washed with the water–Pic B_6 solution (5 ml). The vitamins were eluted with the methanol–Pic B_6 solution (1 ml) and collected in amber coloured vials. Concentration of vitamins was controlled by varying the volume of solution added to the Sep-Pak.

The specificity of the peak for thiochrome was examined by chromatographing the sample solution before and after treatment with sodium sulphite which destroys thiochrome[11] while the specificity of the peak for riboflavin was similarly determined before and after irradiation with UV light for 90 min[11].

RESULTS AND DISCUSSION

Studies with pure compounds (Roche Products, Sydney, Australia) showed that riboflavin and thiamin were eluted from the column after about 5 min and 6 min respectively as sharp peaks. The sensitivity of the UV detector allowed both riboflavin and thiamin to be detected at 0.005 μg while the limits of detection by fluorescence

were lower, being 0.003 μg for riboflavin on the F_{500} and 0.002 μg for thiamin on the F_{425} detector.

Initial studies with foods examined the chromatographic profiles obtained with UV and fluorescence detectors of extracts from various meats and processed breakfast cereal products that were chromatographed without purification on a Sep-Pak cartridge. The chromatograms for pork, beef and lamb meats showed similar features and Fig. 1 presents those obtained for pork. This shows that the use of the UV detector did not resolve riboflavin and thiamin from interfering compounds but that the F_{500} detector produced a resolved peak corresponding to the retention time for riboflavin and the F_{425} detector produced a resolved peak at the retention time for thiamin. The F_{500} and F_{425} detectors did not record a peak for thiamin and riboflavin respectively.

The chromatograms for cereal products were quite different and Fig. 2, which presents those obtained for muesli, shows that unsatisfactory resolution of thiamin and riboflavin was obtained on all three detectors. However, when the food extracts were purified using a Sep-Pak cartridge, resolved peaks for thiamin and riboflavin were obtained (Fig. 3) with the fluorescence detectors (F_{425} and F_{500} respectively) but unsatisfactory resolution was obtained with the UV detector. Spiking of food sample extracts with thiamin and riboflavin showed a recovery of 98.2 ± 1.2% and 98.0 ± 2.2% on the respective fluorescence detectors. Similar patterns were obtained for all the breakfast cereals examined which covered the range of types available.

No further studies were conducted with the UV detector but a range of foods were analysed using fluorescence detection. All foods examined were found to give satisfactory resolved peaks for thiamin and riboflavin on the F_{425} and F_{500} detectors respectively. Analysis of fresh fruit and vegetables (cauliflower, orange and grape) showed that a sample clean-up procedure was required to obtain resolved chromatograms and quantitation of the low levels of thiamin and riboflavin naturally present was markedly improved by a high degree of concentration of the vitamins in the purification procedure.

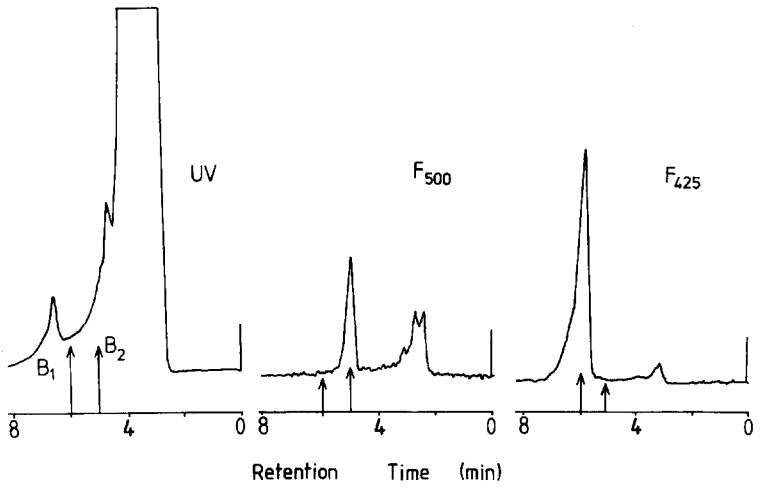

Fig. 1. Chromatograms of a pork extract obtained by UV and fluorescence (F_{500} and F_{425}) detection. $\uparrow B_1$ and $\uparrow B_2$ indicate retention times of thiamin and riboflavin respectively.

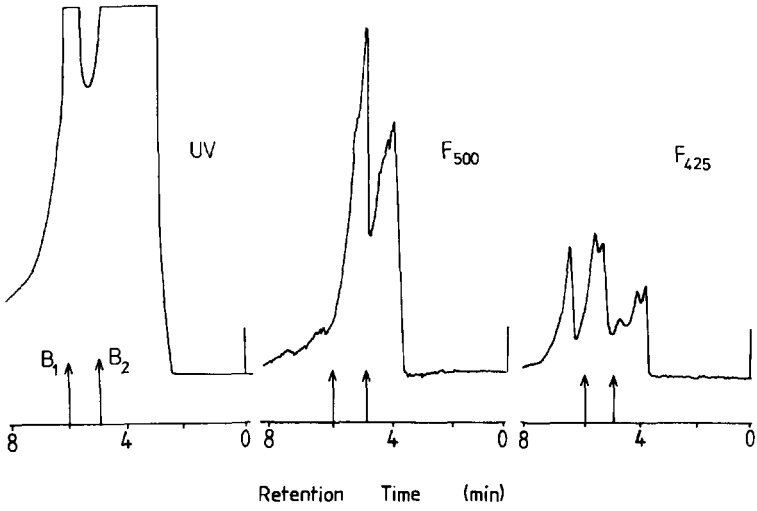

Fig. 2. Chromatograms of a muesli extract before purification obtained by UV and fluorescence (F_{500} and F_{425}) detection. ↑B_1 and ↑B_2 indicate retention times of thiamin and riboflavin respectively.

Extracts of egg, milk and plain yoghurt did not require purification to obtain satisfactory chromatograms but flavoured yoghurt required purification. While extracts of raw meats did not require purification before analysis, it was necessary to purify processed meats such as ham, salami and liverwurst. Whole grain cereals such as wheat and rice contain lower levels of thiamin and riboflavin than the previouslly examined processed cereal products that are fortified with these vitamins and it was found necessary to concentrate the grain extract to obtain a satisfactory peak area.

The levels of thiamin and riboflavin found in all the foods analysed were consistent with expected values and spiked samples of at least one food in each group

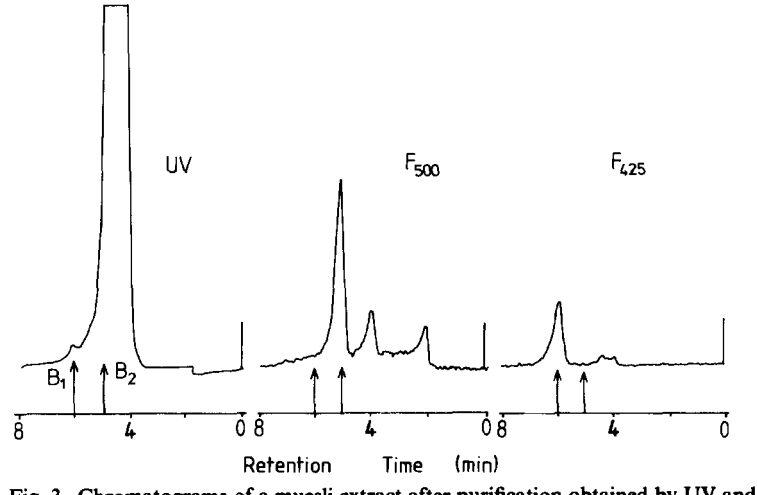

Fig. 3. Chromatograms of a muesli extract after purification obtained by UV and fluorescence (F_{500} and F_{425}) detection. ↑B_1 and ↑B_2 indicate retention times of thiamin and riboflavin respectively.

all gave recoveries greater than 97%. The specificity of the peaks for thiochrome and riboflavin were confirmed for all groups of foods.

Simultaneous analysis of thiamin and riboflavin requires two fluorescence detectors to be connected in series with the sample from the column first passing through the F_{500} detector to detect riboflavin and then through the T-piece reaction block with alkaline ferricyanide addition and finally to the F_{425} detector for thiochrome analysis. The time for analysis of a set of eight samples including the purification/concentration step is about 60 min for each sample. This compares with a total time by standard AOAC methods of 90 min/sample (60 min for thiamin and 30 min/sample for riboflavin).

While optimal operation of the method requires two fluorescence detectors, if only one detector is available, the food extract can be chromatographed twice with a change of detector filters to determine thiamin and riboflavin separately. The ease of operation and time for analysis would still be advantageous when compared to standard methods of analysis.

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