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Application of high-performance liquid chromatography to the analysis of niacin and biotin in Italian almond cultivars

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ABSTRACT

An extraction method involving heat sonication with simultaneous high-performance liquid chromatographic separation of water-soluble vitamins from almonds is described. For the removal of interfering compounds a clean-up procedure on a strong cation-exchange column is suggested. Recovery data after the complete procedure for the six water-soluble vitamins, as well as qualitative and quantitative data for various Italian cultivars of almonds are reported. The method is suitable for the routine evaluation of niacin and biotin.

INTRODUCTION

Almonds are an important crop in Southern Italy, but the numerous cultivars grown vary widely in yield and composition. This is a drawback for the development of Italian almond production. With the aim of screening cultivars for agronomic, technological and nutritional features, to find those most suitable for growth, a research project has been granted funding by the Development Fund for the South of Italy (CASMEZ).

Among the nutritional characteristics to be evaluated, water-soluble vitamin content is of interest, as almonds are considered a good source of B-complex vitamins, mainly niacin (1–10 mg per 100 g), biotin (10–100 μg per 100 g) and thiamin (10–100 μg per 100 g) [1].

Data in the literature are scarce and usually do not specify the cultivar or its origin, or the analytical methods [2]. Therefore we propose that systematic research on water-soluble vitamin content of Italian cultivars should be undertaken. As for the method of choice, the capability for simultaneous multi-vitamin analysis offered by high-performance liquid chromatography (HPLC) caught our attention. This capability, together with its precision, accuracy, specificity and speed, is generally recognized (as also pointed out by Eitenmiller [3]), and is one of the main reasons for the tremendous developments in HPLC methods applied to the analysis of these compounds [4–9]. However, the possibility of using HPLC for the analysis of B-complex vitamins in oil seeds such almonds has not been sufficiently investigated, as the above-quoted reviews show. The preliminary defatting step for almonds was used, as it was

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previously adopted for highly fatty products such as chocolate, almonds and peanuts by Hurst *et al.* [10] for the analysis of thiamin.

EXPERIMENTAL

Apparatus

A Jasco BIP-I liquid chromatograph was used, equipped with a Rheodyne 7125 variable-loop injection valve fitted on a stainless-steel column (33 × 4.6 mm I.D.) prepacked with 3-μm Supelcosil LC-8-DB (Supelco Cat. No. 5-8976) plus a guard column (20 × 4.6 mm I.D.) packed with the same stationary phase, placed in a Dani HPLC oven and connected to a Jasco 850 UV variable-wavelength detector coupled with a Shimadzu Chromatopac C-R1B data processor.

Materials

Standard solutions (0.02, 0.05 and 0.1%) of thiamin, riboflavin, folic acid, pyridoxine, biotin and niacin were prepared dissolving standard compounds (Serva) in 0.1 *M* HCl. The 5 m*M* hexanesulphonic acid sodium salt–0.1% triethylamine solution was brought to pH 2.8 with 75% phosphoric acid and filtered through a Millipore HA 0.45-μm membrane prior to the addition of methanol [11]. The mobile phase was degassed by sonication under vacuum. Before HPLC analysis the standard solutions were filtered through a Millex GV 0.22-μm unit (Millipore).

All the other reagents were of analytical grade.

Methods

Sample preparation. All the samples were previously de-fatted with *n*-hexane by extraction of the ground almonds in a Soxhlet apparatus for 6 h; 30 g of de-fatted almonds, ground to a 0.2-mm powder in a hammer mill, were placed into a 300-ml flask; after having moistened the powder with 10 ml of distilled water, 70 ml of 0.1 *M* HCl were added. The mixture was then sonicated at 75°C for 15 min and, after shifting the pH to 4.6 by adding 0.1 *M* NaOH, transferred into a 100-ml volumetric flask and brought to the mark with water. This extract was filtered through a Gooch filter with Whatman No. 4 paper.

Then, 50 ml of the extract were filtered on a Millipore HA 0.45-μm membrane filter; the vitamins were adsorbed on an aromatic sulphonic acid extraction column (strong cation exchange) (Baker-10-SPE), previously conditioned by passing 6 ml of methanol and 6 ml of water.

After washing (6 ml of water, 6 ml of methanol, 6 ml of water), the retained compounds were eluted from the column with 4.5 ml of 2 *M* KCl–methanol (60:40) solution previously warmed to 70–75°C.

The eluate was collected into a 5-ml volumetric flask and after cooling, brought to volume with the mobile phase.

Prior to injection, the solution was filtered through a Millipore GV 0.22-μm membrane filter.

The whole sample preparation was performed under half-light.

HPLC analysis. Separation was achieved at 35°C according to the following parameters: Stationary phase: Supelcosil LC-8-DB 3 μm; mobile phase: 5 m*M* hexanesulphonic acid sodium salt containing 0.1% triethylamine (pH 2.8)–methanol

(85:15); flow-rate: 1.0 ml/min; detection: 200 nm at 0.08 a.u.f.s. range; sample volume: 10 μl; average retention times: niacin 1.18 min, pyridoxine 1.74 min, thiamin 3.08 min, biotin 4.09 min, riboflavin 5.39 min, folic acid 7.09 min.

RESULTS AND DISCUSSION

For the HPLC separation of the six water-soluble vitamins (thiamin, riboflavin, folic acid, pyridoxine, niacin and biotin) which are reported to be present in almonds [12,13], we applied the conditions suggested by Supelco [11] for the chosen stationary phase (LC-DB-8), specifically made for the fast routine analysis of water soluble vitamins in pharmaceutical products such as multi-vitamin tablets. This column is a deactivated phase which, according to the manufacturer, should provide a best peak shape and a better separation of the vitamins than the standard RP-8 column. The same column was used by Bonomi *et al.* [14] for feed premixes.

Fig. 1 shows a typical separation of a standard mixture containing equal amounts (0.05%) of each vitamin. As almond seeds contain high amounts of lipids (50–65% or more according to the cultivar), prior to the water-soluble vitamin extraction step it was necessary to de-fat the samples by means of a Soxhlet extraction with *n*-hexane. According to Hurst *et al.* [10] the de-fatting treatment should not affect the recovery of B-complex vitamins.

Optimal conditions for acid digestion by means of heat sonication was tested both for the temperature (from 60 to 90°C) and for the time (from 5 to 60 min) required to achieve the highest extraction yield for the least time of digestion. The most suitable conditions were found to be 75°C for 15 min. Increases in temperature and time beyond these points did not noticeably affect the extraction yield.

Since no enzymatic hydrolysis treatments were performed in order to simplify the method, the interfering components overlapping the peaks of the vitamins to be

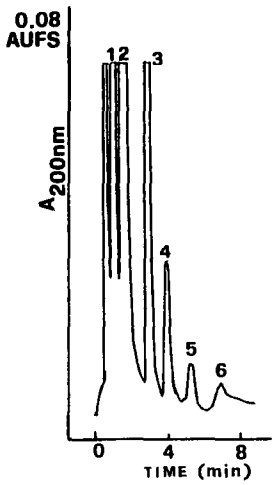

Fig. 1. HPLC separation of 0.05% standard solution containing six water-soluble vitamins. Peaks: 1 = niacin; 2 = pyridoxine; 3 = thiamin; 4 = biotin; 5 = riboflavin; 6 = folic acid. For HPLC conditions see Experimental.

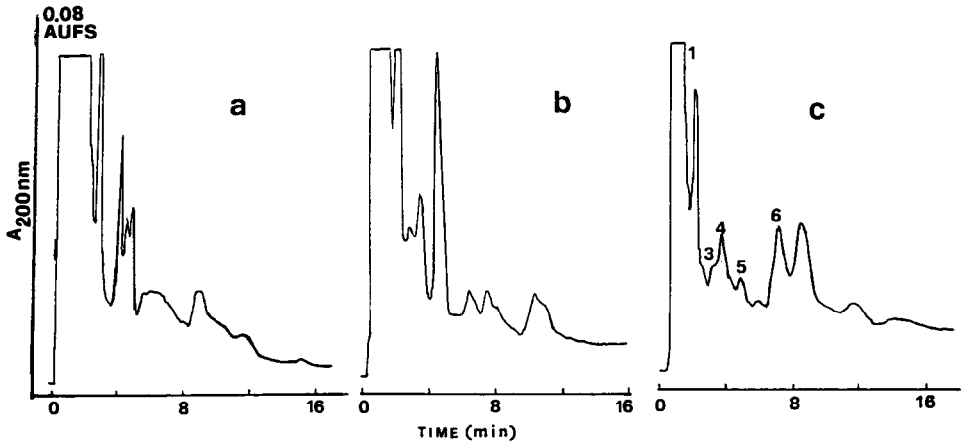

Fig. 2. Effect of clean-up procedure on the HPLC analysis of a *Filippo ceo* c.v. almond extract. (a) Crude extract; (b) removed interfering substances; (c) cleaned-up vitamins. Peaks: 1 = niacin; 3 = thiamin; 4 = biotin; 5 = riboflavin; 6 = folic acid.

quantified (Fig. 2a) were removed by clean-up on a strong cation-exchange phase. We used the method reported by Ayi and co-workers [15,16] for infant formula, with slight modifications as described in the Experimental section. It appears possible to achieve the removal of almost all interference (Fig. 2b) from the water-soluble vitamins (Fig. 2c).

However, only for niacin and biotin did we obtain resolution of the peak which was sufficient to make their quantification possible; for the other vitamins (*i.e.* riboflavin, thiamin, folic acid and pyridoxine) it was only possible to give an estimation of their presence as relative abundance in the extract. In the cleaned-up extract the identification of each vitamin was performed by injecting the same extract spiked with 0.2% standard slution.

The calibration equations for niacin and biotin, over the range 2–10 μg of vitamin, were: $y = 35.06\,x + 2.649$ for niacin, and $y = 24.46\,x + 4.893$ for biotin.

As for the recovery of each vitamin after complete sample preparation (Table I), tests made on 0.1% standard solutions gave good results for niacin, riboflavin and thiamin, while the other three vitamins showed lower recoveries ranging from 79.2%

TABLE I

RECOVERY DATA FROM 0.1% STANDARD SOLUTION SUBMITTED TO THE COMPLETE SAMPLE PREPARATION PROCEDURE (AVERAGE OF FOUR TESTS)

Vitamin	Recovery (%)
Biotin	79.8 ± 8.92
Niacin	97.6 ± 1.01
Riboflavin	92.8 ± 2.05
Thiamin	96.5 ± 1.06
Pyridoxine	85.7 ± 6.42
Folic acid	79.2 ± 8.75

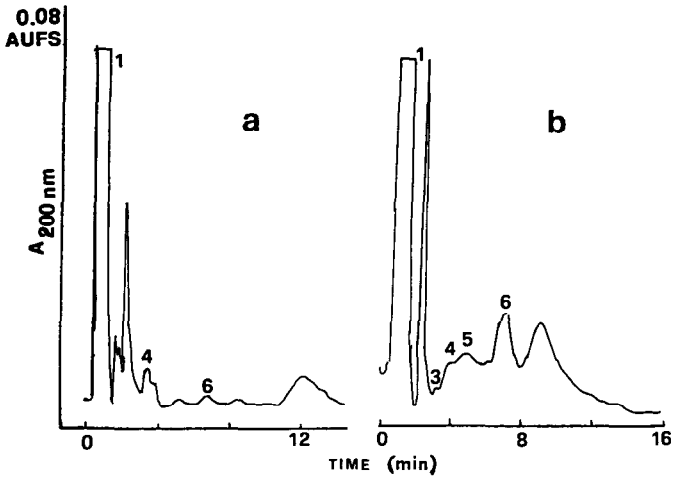

Fig. 3. Examples of HPLC analysis of almond cultivars: (a) *tuono* c.v.; (b) *ferraduel* c.v.

for folic acid to 85.7% for pyridoxine. Furthermore, lower recoveries were associated with a larger variability of data.

Fig. 3 reports examples of analysis after purification of the digest from other two almond cultivars.

As to quantitative data, niacin varied from 1.47 mg per 100 g (*tuono* c.v.) to 3.41 mg per 100 g (*bottara* c.v.) and this range of concentrations agrees with reference data. As for biotin, the range was from 0.12 mg per 100 g (*tuono* c.v. and *scummissa* c.v.) to 0.9 mg per 100 g (*texas* c.v.) and was slightly higher than data in the literature.

Folic acid was present in all the cultivars analyzed, only five cultivars out of nine contained riboflavin (*Fillipo ceo*, *San contino*, *ferraduel*, *scummissa* and *catanese*), and two out of nine contained thiamine (*F. ceo* and *ferraduel*).

Furthermore, it was not possible to detect pyridoxine because of the presence of an interfering peak which was not eliminated with this purification procedure, but this does not mean that it is absent.

For thiamine it should be necessary to enhance detection sensitivity using derivatization to thiochrome and fluorescence detection, as UV detection is not sufficient for the quantitation of this vitamin in almonds.

For folic acid and riboflavin, the resolution is so low as to impair valid integration of the peaks.

Further studies both on the clean-up and concentration steps in sample preparation, and on the chromatographic separation adopting a slightly different mobile phase (*i.e.* a gradient elution) could improve this analysis. For routine purpose therefore this method can be helpful in the analysis of biotin and niacin in almonds.

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