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SIMULTANEOUS DETERMINATION OF ASCORBIC ACID AND DEHYDROASCORBIC ACID BY REVERSED-PHASE ION-PAIR HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH PRE-COLUMN DERIVATISATION

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SUMMARY

A method is described for the separation and quantitation of ascorbic acid and dehydroascorbic acid by reversed-phase ion-pair high-performance liquid chromatography, following pre-column derivatisation of dehydroascorbic acid with 1,2-phenylenediamine. Use of tridecylammonium formate or hexadecyltrimethylammonium bromide as ion-pairing reagents in the mobile phase produced good retention for ascorbic acid and excellent resolution between ascorbic acid and the derivatised dehydroascorbic acid. The UV absorptivity of dehydroascorbic acid was greatly enhanced by derivatisation, permitting its simple determination in foodstuffs. A change of detector wavelength from 348 nm (for the dehydroascorbic acid derivative) to 290 nm (for ascorbic acid) was employed for the analysis of solutions containing a five-fold excess of ascorbic acid over dehydroascorbic acid, using the same detector sensitivity (0.1 a.u.f.s.) for each. The method has been applied to the analysis of orange juice.

INTRODUCTION

Ascorbic acid has been determined by high-performance liquid chromatography (HPLC) using a wide range of column types and mobile phases. When columns containing a bonded-hydrocarbon functionality (*e.g.* C_2, C_8 or C_{18}) were used, adequate retention of the ascorbic acid was achieved only when ion-pair reagents such as tetrabutylammonium phosphate[1–3], tridecylammonium formate[4] or hexadecyltrimethylammonium bromide[1] were added to the mobile phase. Ascorbic acid has also been successfully determined using ion-exchange[5–7] and bonded amino functionality columns[1,8], however, it has been reported that ion-exchange columns easily became irreversibly poisoned[9] and that the mobile phases used with the amino column seriously reduced the lifetime of the column[10].

Dehydroascorbic acid is produced when ascorbic acid is oxidised by even mild oxidising agents. It has the same antiscorbutic activity as ascorbic acid so its de-

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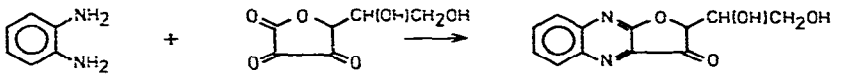
termination in foodstuffs is of some importance: indeed, the "total vitamin C" content of a sample comprises the sum of ascorbic acid and dehydroascorbic acid present. The determination of dehydroascorbic acid by HPLC is complicated by its extremely low UV absorptivity, and in only one case has quantitative separation and determination of ascorbic acid and dehydroascorbic acid been achieved[2]. In this work, up to three C_{18} columns in series were necessary to separate the two species and since UV detection was used, only relatively large amounts of dehydroascorbic acid could be detected, even at 210 nm. The deficiencies of this method are that the low wavelength used introduces problems of solvent purity and also that only partial resolution of ascorbic acid and dehydroascorbic acid was achieved using two columns in series.

The most widely accepted instrumental method for the determination of ascorbic acid involves oxidation to dehydroascorbic acid with iodine, Norit or N-bromosuccinimide, followed by reaction with 1,2-phenylenediamine to produce the fluorophor 3-(1,2-dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one (DFQ), the amount of which is determined by spectrofluorometry[11]. This reaction is shown in Fig. 1. Omission of the oxidation step allows determination of dehydroascorbic acid in the presence of ascorbic acid. We have adapted this method to permit the simultaneous determination of ascorbic acid and dehydroascorbic acid by reacting the latter with 1,2-phenylenediamine and separating the resultant quinoxaline from ascorbic acid using reversed-phase ion-pair HPLC with UV detection. This method allows determination of dehydroascorbic acid in the presence of large excesses of ascorbic acid, a situation which commonly occurs in foodstuffs.



I II III

Fig. 1. Reaction of 1,2-phenylenediamine (I) with dehydroascorbic acid (II) to produce 3-(1,2-dihydroxyethyl)furo[3,4-*b*]quinoxaline-1-one (DFQ) (III).

EXPERIMENTAL

Instrumentation and reagents

The liquid chromatograph used consisted of a Waters Assoc. (Milford, MA, U.S.A.) Model 6000 solvent pump, Model U6K injector, Model 450 variable-wavelength detector and an Omniscribe Model B5217-1 recorder.

For the studies on fluorescence detection, a Waters Assoc. Model 420G fluorescence detector was connected in tandem with the ascorbance detector. A Waters Assoc. μBondapak C_{18} column (30 cm × 3.9 mm O.D.) was used.

Analytical grade methanol was doubly distilled from all glass apparatus and water was distilled using Millipore Milli-Q water purification system. Solvents were filtered and degassed prior to use.

The following reagents were used: L(+)-ascorbic acid (Merck, Darmstadt, G.F.R.), dehydroascorbic acid (Pfaltz and Bauer, Stamford, CT, U.S.A.), 1,2-phenylenediaminedihydrochloride, hexadecyltrimethylammonium bromide and tridecylamine (all from Fluka, Buchs, Switzerland).

Chromatographic procedure

Ascorbic acid (0–30 mg), dehydroascorbic acid (0–6 mg) and 1,2-phenylenediaminedihydrochloride (180 mg) were added to a 100-ml volumetric flask containing 50 ml of methanol and the flask shaken to dissolve the solids. The flask was then diluted to the mark with methanol and allowed to stand in the dark for 80 min. A 10-μl aliquot of this solution was injected onto the column using methanol–water (30:70) as mobile phase at a flow-rate of 1 ml/min and with a detector sensitivity of 0.1 a.u.f.s. The wavelength of the detector was initially set at 290 nm, however after elution of the ascorbic acid, the wavelength was manually changed to 348 nm to permit detection of DFQ.

An alternative procedure was adopted when an ion-pair reagent was used. In this case, the dehydroascorbic acid was derivatised as above, however the mobile phase used consisted of methanol–water (60:40) containing $2.5 \cdot 10^{-4}$ *M* hexadecyltrimethylammonium bromide or methanol–water (40:60) containing $4 \cdot 10^{-2}$ *M* tridecylammonium formate. The detector wavelength was initially set at 348 nm until DFQ had eluted, after which it was changed to 290 nm for the detection of ascorbic acid.

A sample of reconstituted orange juice was analysed by adding 200 mg of 1,2-phenylenediaminedihydrochloride to 70 ml of juice in a 100-ml volumetric flask, after which the flask was shaken and diluted to the mark with methanol. The solution was then centrifuged for 5 min, filtered through Whatman 31 filter paper and allowed to stand for 1 h. A small portion of the sample was filtered through a 0.45-μm filter and a 10-μl aliquot was injected onto the column using a mobile phase of methanol–water (60:40) containing $2.5 \cdot 10^{-4}$ *M* hexadecyltrimethylammonium bromide. The detector wavelength was manipulated as described above.

RESULTS AND DISCUSSION

Stability of DFQ

It is well documented[11] that DFQ is unstable in aqueous solution in the presence of light. When DFQ was prepared in aqueous solution and subsequently chromatographed, five separate peaks were observed. DFQ formed in methanolic solution proved to be much more stable, with as little as 30% methanol in the solution producing a product which was stable for up to 3 h; greater concentrations of methanol further enhanced the stability of DFQ. The main disadvantage of using methanolic solutions was that the time required for complete formation of DFQ was increased in these solvents. When 100% methanol was used, approximately 70 min was required for DFQ formation, compared to 35–40 min in aqueous solutions[11]. Despite this, methanolic solvents were used because of the greater stability of DFQ in these solvents.

Separation of ascorbic acid and DFQ

Initial attempts were made to separate ascorbic acid and DFQ without the use of ion-pairing reagents. Optimum separation was achieved using methanol–water (30:70) as mobile phase, as shown in Fig. 2, where a different detector wavelength was used for each component. Fig. 2 shows that when ascorbic acid was present in a five-fold excess over DFQ, peaks due to both compounds could be simultaneously dis-

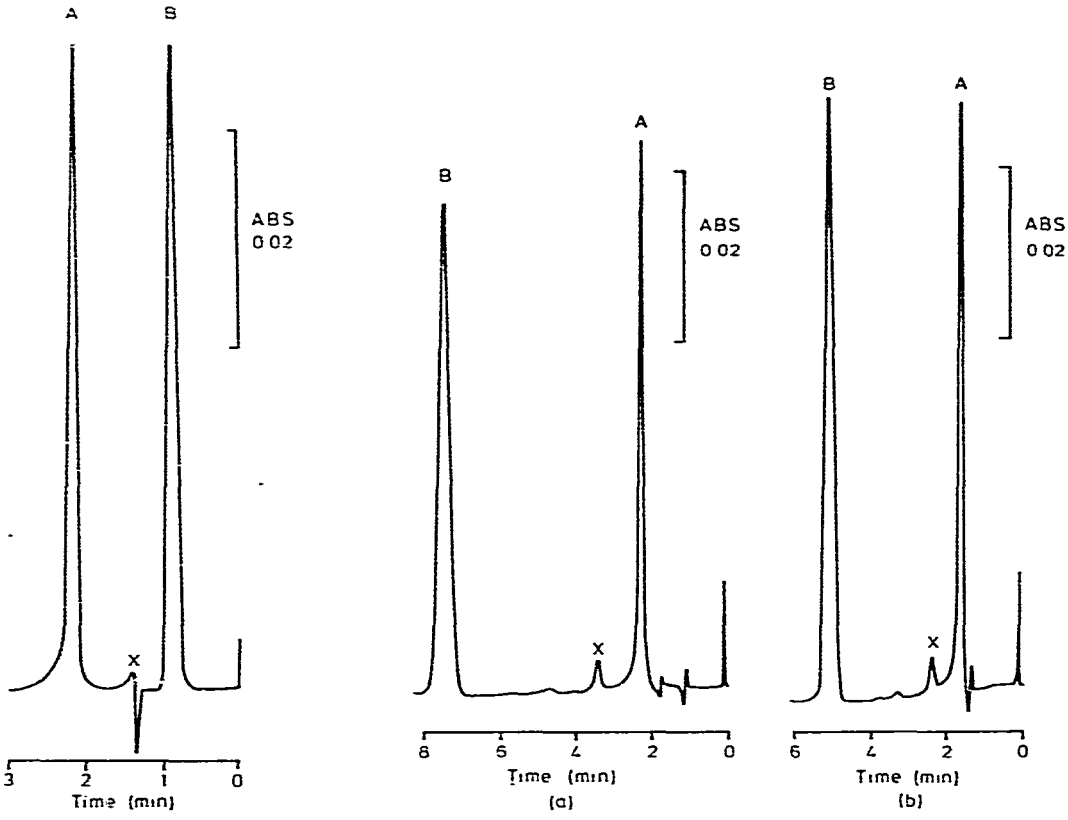


Fig. 2. Separation of DFQ (A) and ascorbic acid (B) without the use of ion-pairing reagents. Conditions: mobile phase, methanol–water (30:70); flow-rate, 2.0 ml/min; detector sensitivity, 0.1 a.u.f.s.; wavelength, 290 nm initially and changed to 348 nm at point X; sample, 10-μl injection of a solution containing 25 mg ascorbic acid 100 ml and 5 mg dehydroascorbic acid, 100 ml, derivatised with 1,2-phenylenediamine.

Fig. 3. Separation of DFQ (A) and ascorbic acid (B) using ion-pairing reagents. Conditions: mobile phases, (a) methanol–water (40:60) containing $4 \cdot 10^{-3}$ *M* tridecylammonium formate, (b) methanol–water (60:40) containing $2.5 \cdot 10^{-4}$ *M* hexadecyltrimethylammonium bromide; flow-rate, 2.0 ml/min; detector sensitivity, 0.1 a.u.f.s.; wavelength, 348 nm initially and changed to 290 nm at point X; sample, as for Fig. 2.

played using the same detector sensitivity (0.1 a.u.f.s.) if the most sensitive wavelength for DFQ (348 nm) was used for its detection, whilst a less sensitive wavelength (290 nm) was used for ascorbic acid. If the wavelength of maximum absorbance of ascorbic acid (265 nm) was used to detect this substance, then the detector sensitivity also required adjustment when the wavelength was changed. The relatively high concentration of ascorbic acid present in many foodstuffs permits use of a less sensitive wavelength, however other wavelengths would be more appropriate for detection of small amounts of ascorbic acid.

The separation shown in Fig. 2 was considered unacceptable because ascorbic acid eluted at the solvent front where it would be subject to interference from non-retained species in real samples. The retention of ascorbic acid was not markedly

altered by decreasing the percentage of methanol in the mobile phase. For these reasons, the use of ion-pairing reagents to increase retention of ascorbic acid was studied.

Previous studies[1,3] have shown that adequate retention of ascorbic acid on reversed-phase columns was achieved only with very lipophilic ion-pairing reagents. We investigated the use of tridecylammonium formate and hexadecyltrimethylammonium bromide as ion-pairing reagents. Both of these reagents produced satisfactory results (Fig. 3) with the retention of ascorbic acid being increased markedly, whilst that of DFQ was unchanged. Hexadecyltrimethylammonium bromide was considered to be the superior ion-pairing reagent because it was obtainable as a pure, crystalline solid and was effective at low concentration ($2 \cdot 10^{-4}$ *M*), whereas tridecylammonium formate had to be prepared from tridecylamine and was effective in increasing ascorbic acid retention only when present in the mobile phase at a concentration greater than $4 \cdot 10^{-2}$ *M*. In addition, prolonged use of tridecylammonium formate (more than 5 h), led to the appearance of multiple peaks for ascorbic acid. This situation could be rectified by flushing the column with methanol and then re-equilibrating the column with the mobile phase containing tridecylammonium formate. When hexadecyltrimethylammonium bromide was used in the mobile phase, at least 3 h of continuous pumping was necessary for the retention time of ascorbic acid to stabilise. However once the column was conditioned in this manner, the retention time of ascorbic acid was constant, even after the column was flushed with water and stored overnight in methanol–water (50:50). On completion of the ion-pairing studies, the strongly adsorbed ion-pairing reagent was removed by flushing the column with methanol.

Calibration plots

Using the conditions described in Fig. 3, linear calibration curves were obtained for dehydroascorbic acid in the range 0–6 mg/100 ml and for ascorbic acid in the range 0–30 mg/100 ml, with both ion-pairing reagents. The upper level in the ranges stated above refers to the maximum amount of each substance measurable with a detector sensitivity of 0.1 a.u.f.s. and does not imply non-linearity of the calibration plots above these levels. The ranges shown above were selected because they were applicable to the determination of ascorbic acid and dehydroascorbic acid in fruit juices. A detailed evaluation of the application of the proposed method to the analysis of a variety of foodstuffs will be discussed in a future communication; however, Fig. 4 illustrates the analysis of orange juice.

Accuracy and precision

The precision of the method was evaluated by carrying six replicate solutions containing 5 mg dehydroascorbic acid and 30 mg ascorbic acid/100 ml through the entire analysis. The relative standard deviations for the ascorbic acid and DFQ peak heights were 1.5% and 3.9%, respectively; these values were comparable to those achieved using repeated injection of the same sample solution, indicating that the precision of the derivatisation procedure was high. No improvement in precision of the ascorbic acid peak height was obtained using the wavelength of maximum absorbance (265 nm), together with a change in densitivity (1.0 a.u.f.s.). This latter result shows that manual setting of the wavelength to 290 nm was quite reproducible, since

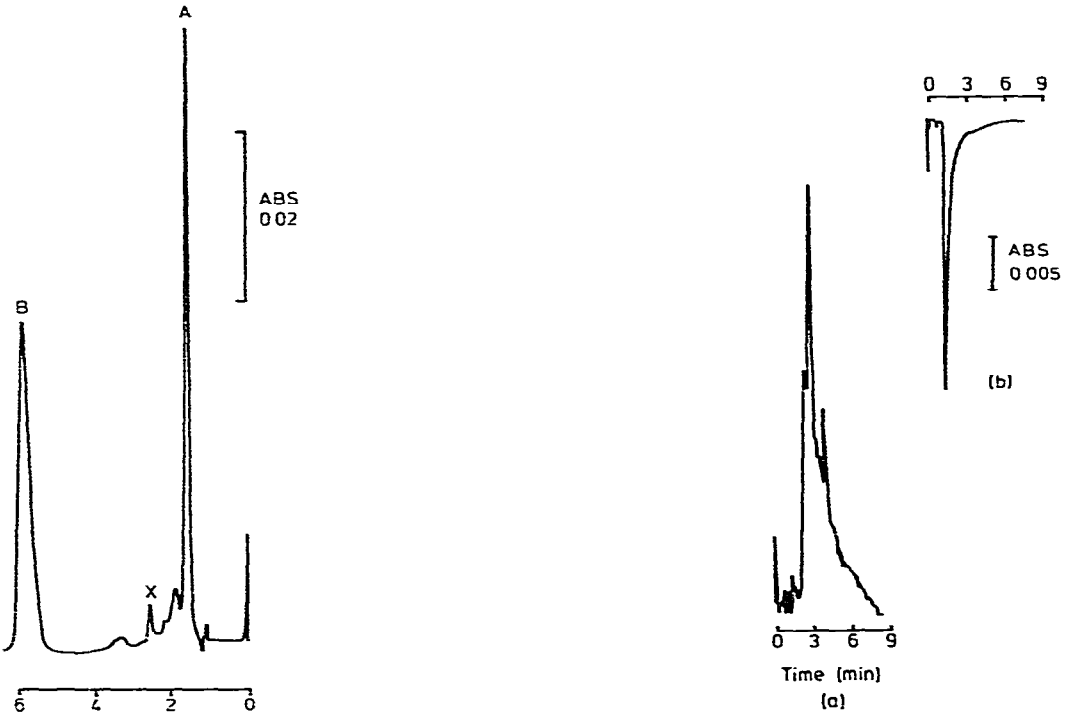


Fig. 4. Determination of DFQ (A) and ascorbic acid (B) in a reconstituted orange juice concentrate. Conditions: Mobile phase, methanol–water (60:40) containing $2.5 \cdot 10^{-4}$ *M* hexadecyltrimethylammonium bromide; flow-rate, detector sensitivity and wavelength as for Fig. 3; sample, 10-μl injection of orange juice. Sample prepared as described in the Experimental section.

Fig. 5. Comparison of fluorescence (a) and absorbance (b) detection of DFQ. Conditions: Mobile phase and flow-rate as for Fig. 3b; detector sensitivity for (b) was 0.05 a.u.f.s.; wavelengths, (a) excitation 340 nm, emission 525 nm; (b) 348 nm; sample, 3-μl injection of a solution containing 1.5 mg dehydroascorbic acid/100 ml, derivatised with 1,2-phenylenediamine. The fluorescence detector gain control was set at a value of 16.

any slight variation in wavelength would cause a considerable change in peak height because the wavelength of 290 nm falls on a steep portion of the absorbance spectrum for ascorbic acid.

Recovery studies were performed using additions of standards throughout the working range of the method. Average recoveries of added ascorbic acid and dehydroascorbic acid were 101.6% (standard deviation 2.4%) and 98.7% (standard deviation 4.1%), respectively. These results indicate that the method has satisfactory accuracy.

Fluorescence detection

DFQ is highly fluorescent and forms the basis of the fluorometric procedure for ascorbic acid and dehydroascorbic acid[11]. In view of this, the possibility of fluorescence detection of DFQ was studied using a Waters Assoc. Model 420G fluorescence detector connected in tandem with the absorbance detector. The results (Fig. 5)

show that a number of fluorescent impurities eluted close to DFQ, rendering its quantitation difficult. These impurities were not detected by UV absorbance at 348 nm. It seems therefore that the excitation filter used in the fluorescence detector had a sufficiently wide spectral bandpass to permit excitation and hence fluorescence of these species. The results suggested that the increased sensitivity of fluorescence detection was largely offset by increased background due to fluorescent impurities.

CONCLUSIONS

The simultaneous determination of ascorbic acid and dehydroascorbic acid may be achieved by reversed phase ion-pair HPLC, following pre-column reaction of dehydroascorbic acid with 1,2-phenylenediamine. Under the chromatographic conditions described, excellent resolution was obtained between the two species and the derivatised dehydroascorbic acid showed good UV absorptivity, thereby facilitating its simple determination at the low concentrations found in foodstuffs.

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