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Analytical Methods Section

Study of natural ascorbigen and related compounds by HPLC

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The effect of processing of vegetables on ascorbigen formation, the ultimate fate of its transformation, their pharmacological evaluation are areas requiring detailed study using analytical methods capable of measuring various individual components. A reversed-phase chromatographic system for separation of ascorbigen, which is an indole containing a derivative of L-ascorbic acid, and its synthetic analogs with different substituents in the indole nucleus was developed. The isocratic chromatographic system was also developed for separation of ascorbigen and its transformation products in acidic media. Ascorbigen B which was previously described as the 2-epimer of natural ascorbigen was shown by HPLC to be a mixture of compounds, where natural ascorbigen 'dimer' was the major component, and natural ascorbigen and its 'trimer' were the minor components. To determine the content of ascorbigen and its transformation products in extracts of fresh or sour cabbage a reversed-phase chromatographic system with gradient elution was developed, with 1'-methylascorbigen as an internal standard. Extracts of fresh or sour cabbage contained the ascorbigen (2·4–5·5 mg per 100 g fresh weight), as the major component and ascorbigen 'dimer' (0·1–0·3 mg per 100 g fresh weight), ascorbigen 'trimer' (0·1–0·3 mg per 100 g fresh weight), 3-hydroxymethylindole (0·2–0·3 mg per 100 g fresh weight) and (indole-3-yl)acetonitrile (0·1–0·5 mg per 100 g fresh weight) as the minor components.

INTRODUCTION

Ascorbigen—an indole containing derivative of L-ascorbic acid 2-C-[(indol-3-yl)methyl]-α-L-threo-L-glycero-3-hexulofuranosonic acid 1,4-lactone was isolated from fresh juice of Savoy cabbage in 1957 (Prochazka *et al.*, 1957). It is formed in damaged or in aging plant tissues from the mustard oil glucoside glucobrassicin, (indol-3-yl)acetothio-*S*-(β-D-glucopyranosyl) hydroximyl-*O*-sulfate (1), which produces under the action of mirosinase (indo-3-yl)methylisothiocyanate (2). The latter is transformed into 3-hydroxymethylindole (3), which reacts non-enzymatically with L-ascorbic acid (AA) to yield ascorbigen (4a) (**Scheme 1**) (Gmelin & Virtanen, 1961; Kutacek *et al.*, 1969). The other alkaloids of glucobrassicin group, e.g. neoglucobrassicin (1-methoxyglucobrassicin) (Gmelin & Virtanen, 1962) or 4-methoxyglucobrassicin (Truscott *et al.*, 1982), which have been identified in several plant tissues, serve for sources of substituted ascorbigens in plant extracts. Hydrolysis of 1 may lead also to (indol-3-yl)acetonitrile (5) and to di(indol-3-yl)methane (6). The concentration of ascorbigen is especially high in different species of cabbage (e.g. Kutacek *et al.*, 1957; McDannell *et al.*, 1987).

By the interaction of 3 and AA, synthetic ascorbigen (4a), identical to natural ascorbigen, was formed. Additionally to 4a, small amounts of so-called Ascorbigen B (7) were isolated, which was supposed to be the 2-epimer of Ascorbigen A, i.e. 2-C-[(indol-3-yl)-methyl]-α-L-threo-D-glycero-3-hexulofuranosonic acid 1,4-lactone (7) (**Scheme 2**) (Kiss & Neukom, 1966). Series of ascorbigens substituted in the indole ring were prepared by the interaction of substituted 3-hydroxymethylindoles with AA (Mukhanov *et al.*, 1984; Bukin *et al.*, 1987; Plikhtyak *et al.*, 1988, 1989, 1991). Minor ascorbigen-like compounds formed as by-products in these reactions were supposed to be Ascorbigen B analogs.

The transformation of ascorbigen in water at pH < 7 proceeds by a mechanism involving the release of AA and addition of (indol-3-yl)methyl cation to another molecule of 4a to yield 2'-[(indol-3"-yl)-methyl] ascorbigen (8a) and 2'-(2"-[(indol-3'"-yl)-methyl]-

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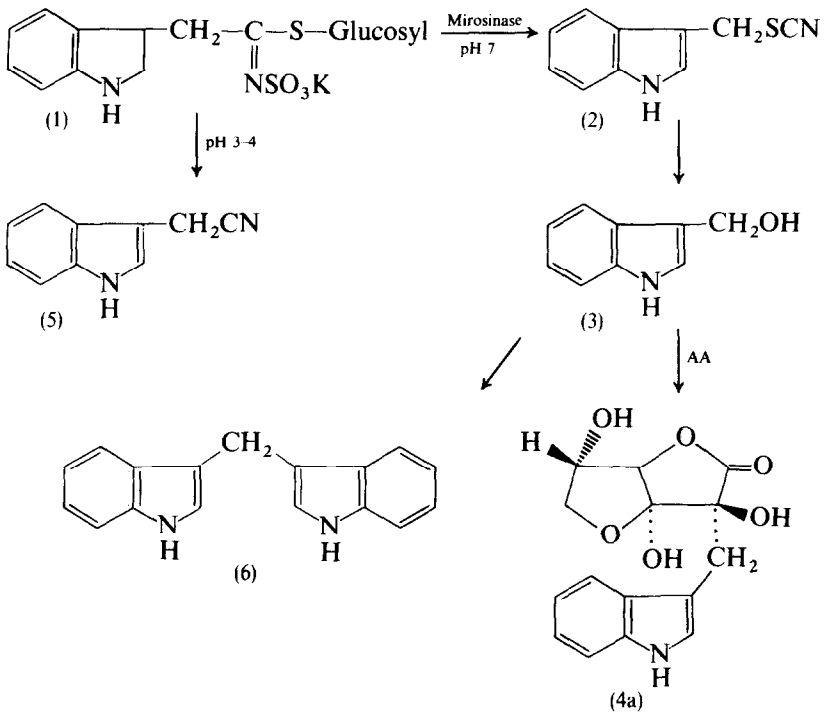
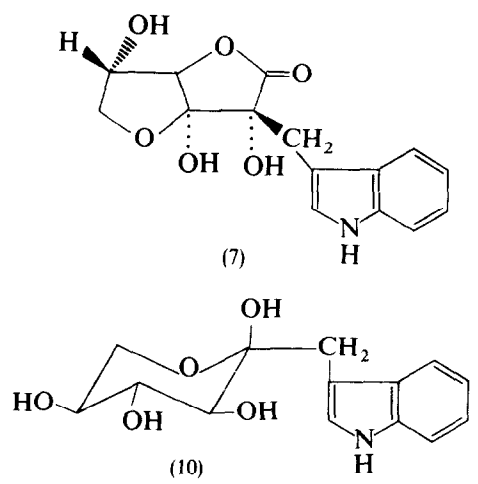


Scheme 1. Enzymatic breakdown of glucobrassicin: (1) glucobrassicin; (2) (indol-3-yl)methylisothiocyanate; (3) 3-hydroxymethylindole; (4a) ascorbigen; (5) (indol-3-yl)acetonitrile; (6) 3,3'-diindolylmethane.

indol-3"-yl)methylascorbigen (9a) (**Scheme 3**) (Korolev *et al.*, 1991). It can be supposed that the compounds 8a and 9a can occur under biological conditions, for example in the stomach.

In mild alkaline media, a reducing sugar 1-deoxy-1-(indol-3-yl)-L-sorbopyranose (10) is formed from 4a via alkaline decarboxylation and isomerization (**Scheme 2**) (Plikhtyak *et al.*, 1989). The present authors' preliminary data show that this compound is present in blood after administration of 4a to animals.

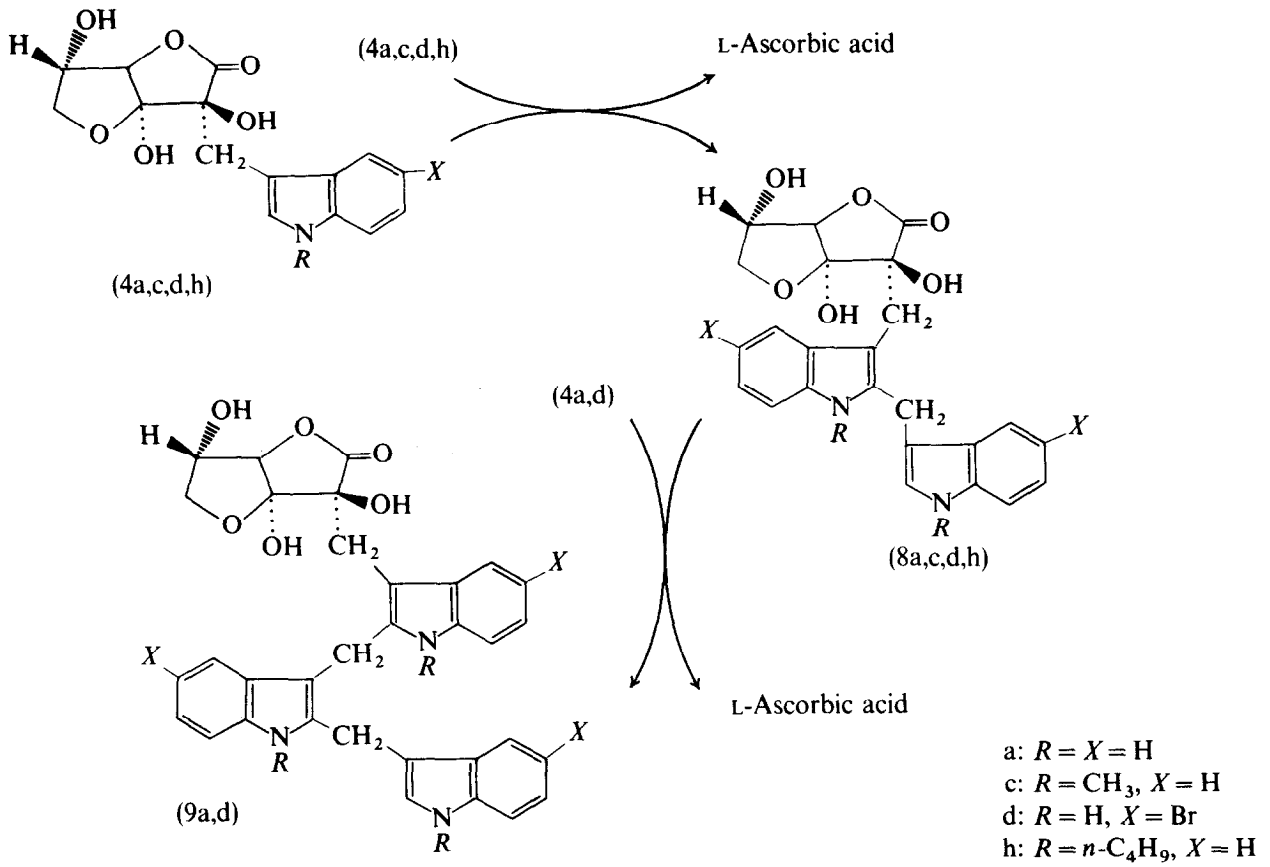
Some authors consider 4a not to be the natural compound, but regard it as an artifact, as it is produced during vegetable processing (e.g. Parry, 1972). However, as humans and animals obtain noticeable amounts



Scheme 2. Structure of so-called Ascorbigen B (7) and 1-deoxy-1-(indol-3-yl)-L-sorbopyranose (10).

of ascorbigen from food, it is reasonable to assume that this compound shows biological effects when vegetables are consumed in the diet. In most papers ascorbigen is considered as a depot form of L-ascorbic acid, as it liberates AA after heating. This has led to the dubious conclusion that biological effects of ascorbigen depend only on amounts of released AA and 3-hydroxymethylindole. This has led in turn to conclusion that 3-hydroxymethylindole (indol-3-carbinole) (3) is present in cruciferous vegetables in high amounts. A series of investigations were undertaken to determine any carcinogenic or anticarcinogenic effects of 3 fed to animals (e.g. Boone *et al.*, 1990; Fong *et al.*, 1990). Meanwhile, 3-hydroxymethylindole is an unstable compound, sensitive to acids, which at pH < 7 easily releases formaldehyde and forms di- and polyindolylmethanes and products of its transformation (Spande, 1979). In vegetables, which are rich with AA, 3-hydroxymethylindole easily interacts with AA during food processing and ascorbigen represents the main indole containing product of glucobrassicin transformation. In recent years, interest in ascorbigen has declined, but new data show that its biological properties do not depend only on amounts of 3 and AA released (Efimov, 1989; Preobrazhenskaya *et al.*, 1991).

Preliminary data show that ascorbigen and some of its congeners (e.g. 1'-methylascorbigen) have immunomodulating activity: when given to animals they enhance activity of macrophages, neutrophils, T-killers and NK cells, influence metabolism of arachidonic acid and prostaglandins, and enhance production of tumor necrosis factor (Efimov, 1989; Preobrazhenskaya *et al.*,



Scheme 3. Transformation of ascorbigen (4a) , 1'-methylascorbigen (4c), 5'-bromoascorbigen (4d) or 1'-butylascorbigen (4h) in acidic media.

1991). When considering biological activity of vegetable diets rich in cruciferous species not only biological effects of 3-hydroxymethylindole must be taken into account, but also the complex biological effect of ascorbigen and its transformation products. As cruciferous vegetables make a significant contribution to the diet in many countries, the study of ascorbigen is important for understanding the biological roles of these food components and their quality estimation.

Until now no investigations have been published devoted to the study of ascorbigen derived compounds in vegetable food. The data on these compounds found in animals fed with ascorbigen containing food are out-of-date (e.g. Sanda *et al.*, 1962).

The effect of vegetables processing on ascorbigen formation, the ultimate fate of its transformation products, and their pharmacological evaluation are areas requiring detailed study using analytical methods capable of measuring various individual components.

The separation and quantification of glucosinolates in vegetables has received much attention in recent years. Glucosinolates have been separated using reversed-phase high-performance liquid chromatography (HPLC) with a solvent system, containing an ion-pairing agent (Helboe *et al.*, 1980) or gradient HPLC with mobile phase, containing high percentages of water (Minchinton *et al.*, 1982; Spinks *et al.*, 1984).

Procedures for the estimation of glucosinolate enzymatic hydrolysis products are sparse in the literature (Mullin, 1978; Josefsson *et al.*, 1979). Recently, HPLC methods have been described for the separation and quantification of glucobrassicin hydrolysis products: 3-hydroxymethylindole (3), (indol-3-yl)acetonitrile (5), di(indol-3-yl)methane (6) and ascorbigen (4a), using acetonitrile and water or buffer solution in gradient mode (McDannell *et al.*, 1987; Fong *et al.*, 1990).

This paper presents: (a) a chromatographic investigation of retention of ascorbigen, its synthetic analogs and some of their transformation products; (b) a study of chromatographic properties of Ascorbigen B; and (c) a reversed-phase gradient liquid chromatography method for the quantitative analysis of natural ascorbigen and its transformation products in extracts of fresh and sour cabbage.

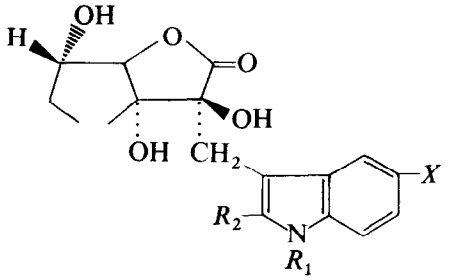
EXPERIMENTAL

Preparation of samples

Compounds investigated

Table 1 lists the structure of ascorbigen and its congeners, which were obtained synthetically by methods described elsewhere. Ascorbigen B, supposed to be the

Table 1. Structure and retention times of ascorbigen and its analogs



Compound	Retention time (min) in System 1	R_1	R_2	X	References
Ascorbigen (4a)	4·1	H	H	H	Kiss & Neukom (1966)
2'-Methylascorbigen (4b)	4·4	H	CH_3	H	Plikhtyak *et al.* (1991)
1'-Methylascorbigen (4c)	5·5	CH_3	H	H	Mukhanov *et al.* (1984)
5'-Bromoascorbigen (4d)	6·5	H	H	Br	Mukhanov *et al.* (1984)
1'-Ethylascorbigen (4e)	7·2	C_2H_5	H	H	Bukin *et al.* (1987)
1'-Allylascorbigen (4f)	8·7	CH_2CHCH_2	H	H	Plikhtyak *et al.* (1989)
1'-Propylascorbigen (4g)	10·7	*n*-C_3H_7	H	H	Plikhtyak *et al.* (1989)
1'-Butylascorbigen (4h)	17·8	*n*-C_4H_9	H	H	Plikhtyak *et al.* (1989)

2-epimer of ascorbigen was isolated by extraction of reaction mixture with ether (Kiss & Neukom, 1966) and was studied by HPLC without additional purification. (Indol-3-yl)acetonitrile was purchased from Aldrich (USA). 3-Hydroxymethylindole was synthesized by the described method (Plikhtyak *et al.*, 1991).

Chemicals

HPLC grade acetonitrile and ammonium acetate were purchased from Merck (Darmstadt, FRG). Deionized water was obtained by the use of the Millipore Q system.

Plant materials

The heads of white fresh and sour cabbage of unknown cultivar, which were stored during the winter, were obtained commercially. White fresh cabbage samples were treated as follows: the outer leaves of the cabbage were discarded, the cabbages were quartered and the individual leaves were collected. One hundred grams of the leaves or 100 g of chopped sour cabbage were cut and homogenized, then NaCl (10 g) was added. The suspension was treated with acetone (100 ml) and a 1% solution of 1'-methylascorbigen (100 μl) as internal standard was added. The suspension was stirred at room temperature for 1 h. After filtration, the solution was evaporated to about 90–100 ml and extracted with ethylacetate (2 × 50 ml). The extracts were combined, dried over Na_2SO_4, filtrated and transferred to a 100-ml volumetric flask. Each solution was evaporated *in vacuo*, to afford ascorbigen and its transformation products. The residues were dissolved in acetonitrile (2 ml) and adjusted to 10 ml with the mobile phase. These solutions were filtered and used for HPLC analysis.

Standard preparation for HPLC calibration curve

Aliquots of standard 1% ascorbigen solution (10; 50; 100; 500 μl) and 100 μl of 1% 1'-methylascorbigen solution were added to 100 ml of water; each of these solutions was extracted with ethylacetate (2 × 50 ml). Extracts were dried over Na_2SO_4, filtered, transferred to a 100-ml volumetric flask and each extract was evaporated *in vacuo*. The residues were dissolved in acetonitrile (2 ml), adjusted to 10 ml with the mobile phase, filtered and used for HPLC calibration. Peak areas were measured by the integrator and used for calculation of the ascorbigen peak-area ratios. Five standard samples covered the expected concentration range of 0·01–0·5 mg ml^{-1}. Peak areas for ascorbigen and internal standard were recorded in integrator units and expressed as a ratio of ascorbigen to internal standard. In the range of concentration from 0·01 to 0·5 mg ml^{-1} the standard curve was linear and yielded the following equation: $y = 0{\cdot}084 + 0{\cdot}007x$ ($r = 0{\cdot}999\ 571\ 6$). While analyzing the cabbage extracts, samples were taken so that the amount of ascorbigen was in the linear concentration range of the standard curve. The quantitative analysis of the minor components was made using the external method. Response factors were determined using standard preparations of ascorbigen 'dimer' (8a), ascorbigen 'trimer' (9a), 3-hydroxy-methylindole (3) and (indol-3-yl)acetonitrile (5), and each analysis as conducted in duplicate.

The detection limits for these compounds were: for ascorbigen 'dimer' (8a)—2 μg ml^{-1}; for ascorbigen 'trimer' (9a)—1 μg ml^{-1}; for 3-hydroxymethylindole (3) and (indol-3-yl)acetonitrile (5)—0·5 μg ml^{-1}.

HPLC of ascorbigens and their transformation products

A DuPont binary gradient instrument, fitted with Reodyne 7125 injector was coupled to the variable-wavelength ultraviolet detector. A wavelength setting at 280 nm was used for HPLC separations. Chromatograms were recorded and peak areas and retention times were measured by means of SP-4000 chromatography data system. The chromatograph was operated in the constant flow mode at 1·0 ml min^{-1}, with an oven temperature of 35°C. A 250 mm × 4·6 mm Zorbax ODS colum was purchased from DuPont.

Isocratic chromatographic system (System 1). The mobile phase was 0·1 M ammonium acetate buffer (pH 5·7) modified with 30% of acetonitrile. Gradient chromatographic system (System 2). The mobile phase was: (A) 20% acetonitrile in 0·1 M ammonium acetate buffer (pH 5·7), and (B) 60% acetonitrile in 0·1 M ammonium acetate buffer (pH 5·7). The program consisted of a linear gradient of 100% of (A) to 100% (B) over 25 min during which all compounds were separated; the program returned to 100% (A) by linear gradient over 1 min followed by 10 min equilibration. HPLC profiles were obtained by injection of 20 μl at 0·16 AUFS.

RESULTS AND DISCUSSION

HPLC of natural ascorbigen and its synthetic analogs

This first part of the article discusses the chromatographic investigation of ascorbigen and its synthetic derivatives: ascorbigen (4a); 2'-methylascorbigen (4b); 1'methylascorbigen (4c); 5'-bromoascorbigen (4d); 1'-ethylascorbigen (4e); 1'-allylascorbigen (4f); 1'-propylascorbigen (4g) and 1'-butylascorbigen (4h) (Table 1).

The separation was performed on an *n*-octadecyl-bonded stationary phase. Mobile phases consisting of methanol, acetonitrile, water, ammonium acetate buffer at different concentrations were tested for separation of these compounds. Since ascorbigen and its derivatives are unstable in acidic (pH < 5) and basic solutions, water and ammonium acetate (pH 5·7) modified with

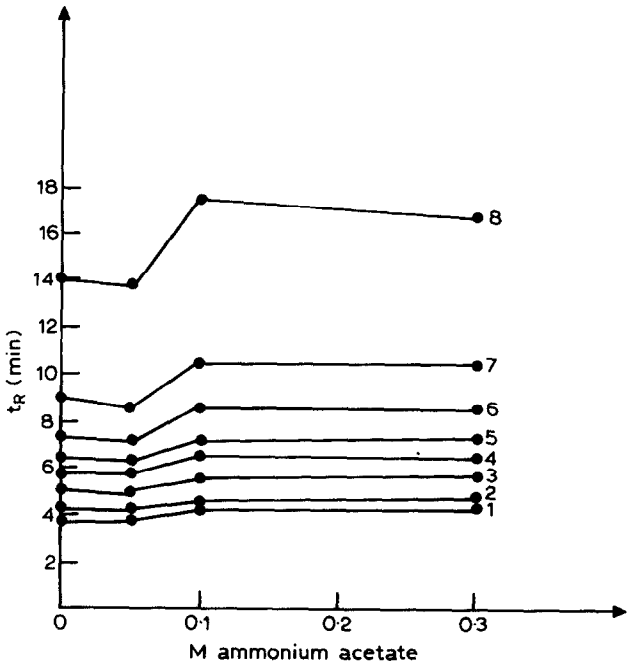


Fig. 1. Effect of buffer concentration on retention times of ascorbigen and its analogs. Column Zorbax ODS 250 × 4·6 mm i.d.; mobile phase CH_3CN/NH_4OAc pH 5·7 (30 : 70 v/v), flow rate 1 ml min^{-1}. Components 1, 4a; 2, 4b; 3, 4c; 4, 4d; 5, 4e; 6, 4f; 7, 4g; 8, 4h.

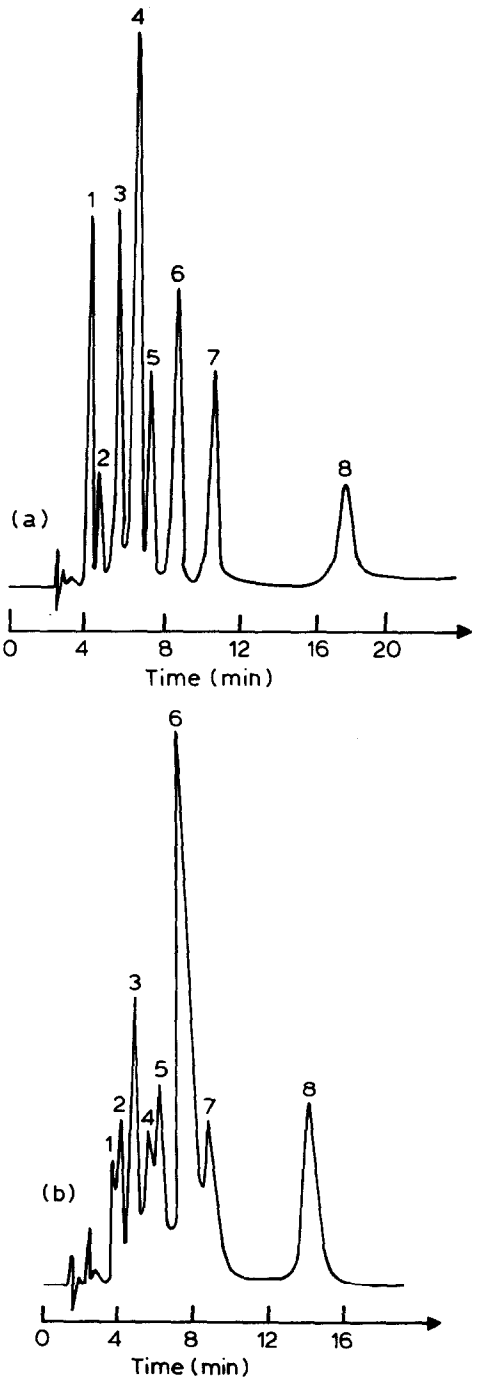


Fig. 2. Chromatogram of ascorbigen and its analogs. Mobile phase; (a) CH_3CN/0·1 M NH_4OAc pH 5·7 (30 : 70 v/v); (b), CH_3CN/water (30 : 70 v/v). Peaks: 1, 4a; 2, 4b; 3, 4c; 4, 4d; 5, 4e; 6, 4f; 7, 4g; 8, 4h.

acetonitrile and methanol were used as a mobile phase. Further investigations showed that methanol as an organic modifier was not suitable, while the use of acetonitrile in mobile phase resulted in good separation and selectivity.

Figure 1 shows the influence of ionic strength on retention of ascorbigen and its derivatives 4a–4h at pH 5·7 by varying the buffer concentration in eluent. The increase in the buffer concentration from 0 to 0·1 M resulted in an increase in the retention times of all compounds. The further increase of buffer concentration from 0·1 to 0·3 M did not change the retention times of these derivatives, except for the 1'-butylascorbigen, for which the retention time was reduced.

As a result of this study, the mobile phase consisting of 0·1 M ammonium acetate (pH 5·7) modified with acetonitrile was selected for further investigations. Figure 2 shows the chromatograms of artificial mixture of ascorbigen (4a) and 2'-methylascorbigen (4b); 1'-methylascorbigen (4c); 5'-bromoascorbigen (4d); 1'-ethylascorbigen (4e); 1'-allylascorbigen (4f); 1'-propylascorbigen (4g) and 1'-butylascorbigen (4h), using two different mobile phases: (a) 0·1 M ammonium acetate buffer/acetonitrile (70 : 30, v/v); and (b) water/

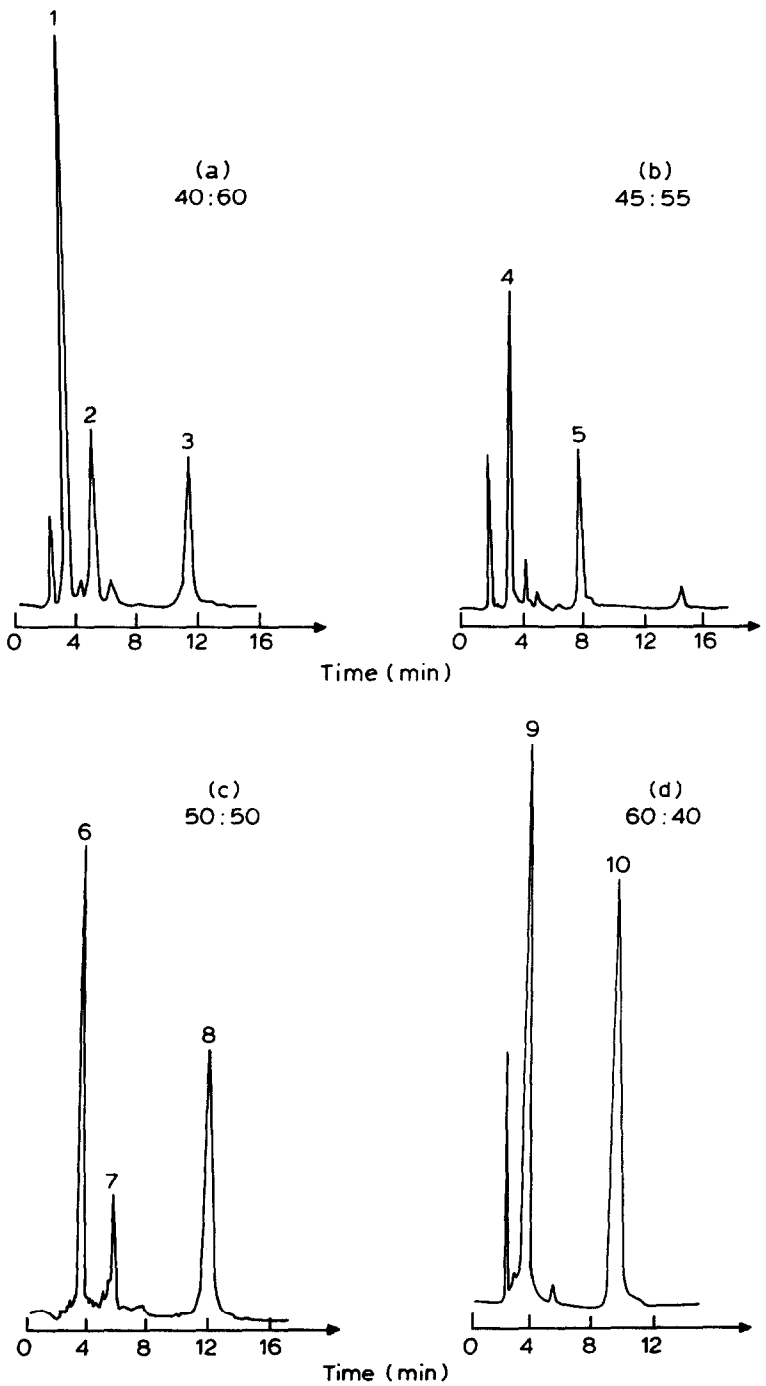


Fig. 3. Chromatograms of artificial mixtures of ascorbigen or its analogs and the products of their acidic transformation. Mobile phase: CH_3CN/0·1 M NH_4OAc pH 5·7 in various proportions. Peaks: 1, 4a; 2, 8a; 3, 9a; 4, 4c; 5, 8c; 6, 4d; 7, 8d; 8, 9d; 9, 4h, 10, 8h.

acetonitrile (70 : 30, v/v). Water containing mobile phases resulted in wide and asymmetrical peaks with poor separation, while the use of eluents, consisting of ammonium acetate buffer, resulted in increased efficiency and good separation of the above-mentioned compounds. On the basis of these data, 1'-methylascorbigen, which had been found to be similar in chemical properties and extraction behavior to natural ascorbigen, was chosen as internal standard in the authors' further investigations.

HPLC of ascorbigen and its transformation products

As mentioned above, ascorbigen (4a) is unstable in acidic or alkaline media. After heating the aqueous solution of 4a at 37°C and pH 5·0 the ascorbigen 'dimer' 8a and ascorbigen 'trimer' 9a are formed. It has also been shown that 1'-methylascorbigen, 1'-butylascorbigen and 5'-bromoascorbigen in acidic media give the analogous transformation products: from 1'-methylascorbigen (4c) or 1'-butylascorbigen (4h) 'dimers' 8c and 8h were obtained; 5'-bromoascorbigen (4d) yielded 'dimer' 8d and 'trimer' 9d (**Scheme 3**). The structures of these compounds were confirmed by NMR (Korolev *et al.*, 1991).

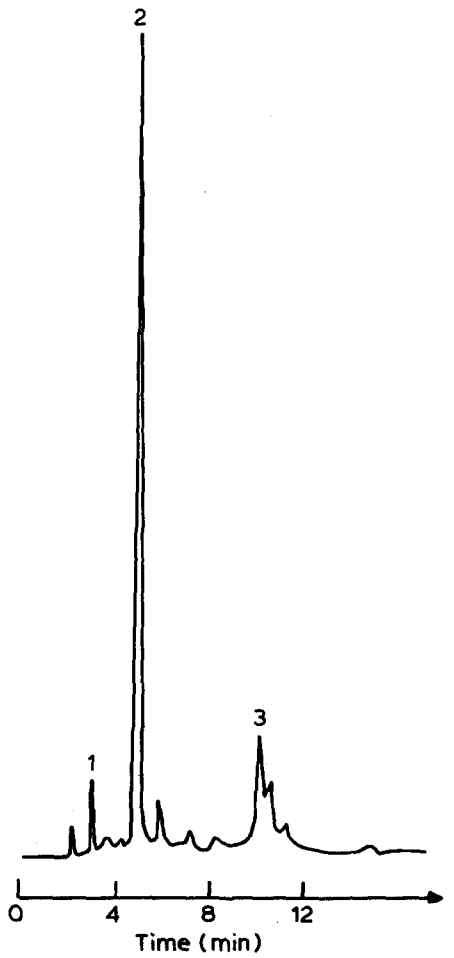


Fig. 4. Chromatogram of Ascorbigen B. Mobile phase: CH_3CN/ 0·1 M NH_4OAc pH 5·7 (40 : 60, v/v). Peaks: 1, 4a; 2, 8a; 3, 9a.

HPLC of 4a, c, d or h and their corresponding transformation products 'dimers' 8a, c, d or h and 'trimers' 9a or d were performed using a mobile phase consisting of 0·1 M buffer solution and acetonitrile in different proportions. Figure 3 shows chromatograms of synthetic mixtures of these compounds. The more lipophylic the substituents in the indole nucleus the higher the concentration of acetonitrile in mobile phase needed for elution. There was a good separation of related compounds: (a) 4a, 8a, 9a; (b) 4c, 8c; (c) 4d, 8d, 9d; (d) 4h, 8h. The elution order was as follows: first, 'monomer' 4a, c, d or h; secondly, 'dimer' 8a, c, d or h, lastly 'trimer' 9a or d.

Figure 4 demonstrates the chromatogram of Ascorbigen B. It is clearly seen that it is not an individual compound, but a mixture of components, the major of which is the 'dimer' 8a and the minor components are the parent ascorbigen 4a and the 'trimer' 9a. The compounds 8a and 9a were isolated from the so-called Ascorbigen B and it was shown that they were identical to the ascorbigen 'dimer' 8a and ascorbigen 'trimer' 9a, obtained after heating of the aqueous solutions of ascorbigen 4a, respectively. Analysis of CD spectra of this mixture and of the each component demonstrated that the chirality of C_2 atom in all these compounds is similar (Korolev *et al.*, 1991). It means that the structure of this compound presented in monographs and papers (e.g. Parry, 1972; Budavari, 1991) is not correct.

HPLC assay of natural ascorbigen and its transformation products in cabbage extracts

Facile formation of ascorbigen transformation products led to the conclusion that compounds 8a and 9a may accompany ascorbigen in natural extracts. Here, the authors present the determination of the content of these compounds as well as 3-hydroxymethylindole (3) and (indol-3-yl)acetonitrile (5) in fresh and sour cabbage.

Ethyl acetate extracts of both fresh and sour cabbage were investigated. Chopped and then homogenized fresh or sour cabbage leaves and not frozen-dried cabbage leaves were used because freezing has been shown to eliminate the possibility of enzyme-induced glucosinolate breakdown and formation of ascorbigen. This factor is important, because frozen-dried cabbage is not usually consumed. The extracts contained a great number of polar compounds in addition to the compounds of interest. In isocratic HPLC system, developed for separation of ascorbigen and its analogs, the peaks of these unidentified components overlapped the peaks of interest, complicating the assay of ascorbigen. To avoid this, a chromatographic system with gradient elution was developed. Figure 5 shows a chromatogram of artificial mixture of ascorbigen (4a), compounds 8a, 9a, 5, 3 and 1'-methylascorbigen (4c) as the internal

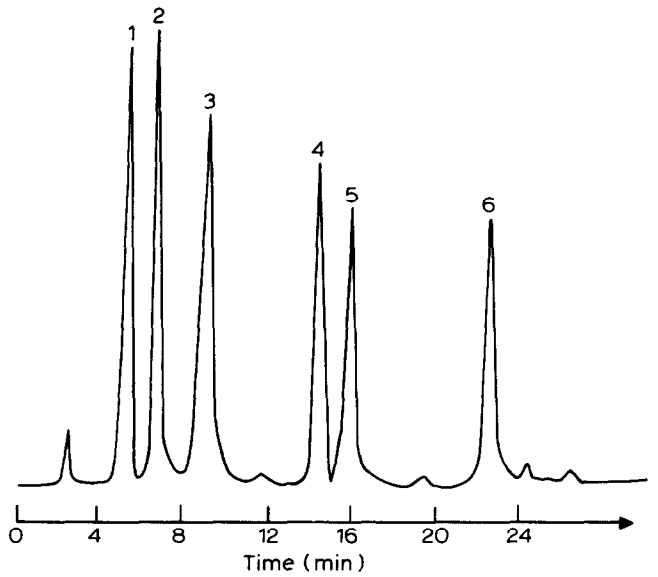


Fig. 5. Chromatogram of artificial mixture of ascorbigen and its transformation products in the gradient mode (System 2). Peaks: 1, 4a; 2, 3; 3, 4c; 4, 5; 5, 8a; 6, 9a.

standard. Using this gradient solvent program, good separation of components was achieved and all the peaks were completely resolved. The retention times of these components listed in Table 2 remained constant during the course of the studies described here. The identification of the extract components was made on the basis of retention times. Figure 6 demonstrates chromatograms of the fresh and sour cabbage extracts. It is clearly seen that the fresh cabbage extract contains ascorbigen (4a) and small amounts of ascorbigen 'dimer' (8a). The sour cabbage extract contains in addition to these components the small amounts of 3-hydroxymethylindole (3), (indol-3-yl)acetonitrile (5) and ascorbigen 'trimer' (9a).

To check the reproducibility of the method, a sample of white cabbage was analyzed on five occasions. Each analysis was performed in triplicate and the mean results (content of ascorbigen) were 2·3, 2·5, 2·4, 2·4 and

Table 2. Content of natural ascorbigen and related compounds in the extracts of white cabbage

Compound	Retention time (min) in System 2	Content mg per 100 g fresh weight					
		Fresh cabbage			Sour cabbage		
		1	2[a]	3	4	5	6
4a	5·5	5·5	2·4	3·4	3·1	5·2	3·3
3	6·7	—	—	0·3	0·3	0·2	0·3
4c	9·5	Internal standard					
5	14·4	—	—	—	0·1	0·5	—
8a	16·4	0·1	0·1	—	0·1	0·1	0·3
9a	22·6	—	—	—	—	0·1	0·3

[a] Fresh cabbage of this year's harvest.

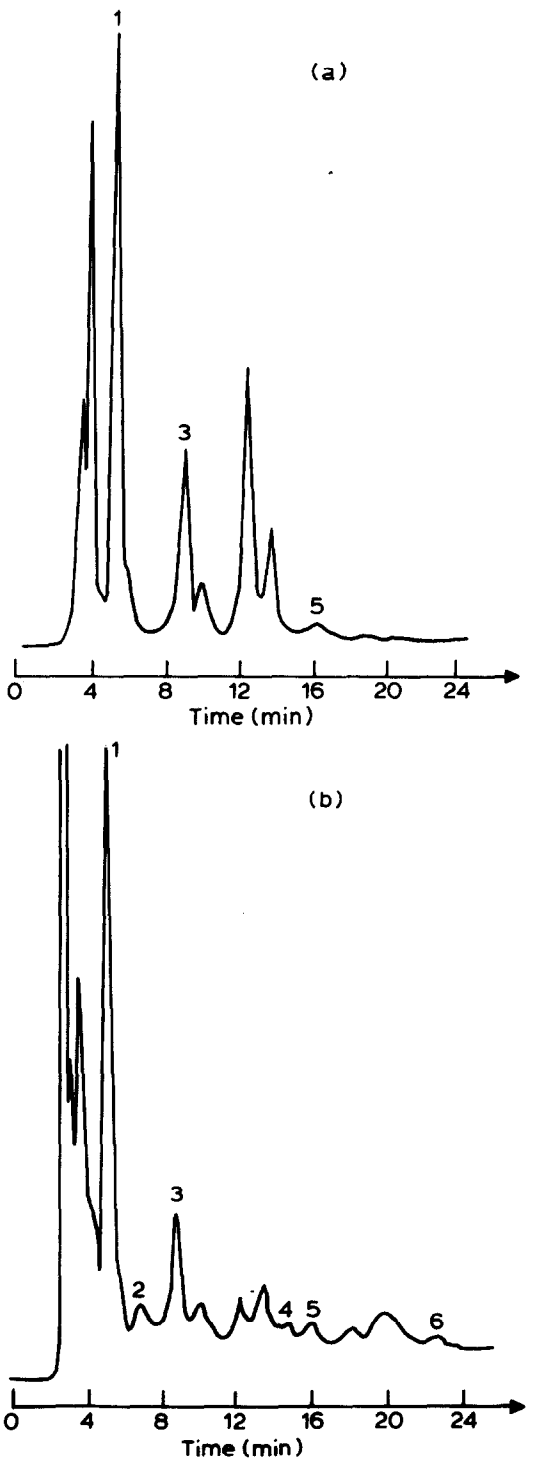


Fig. 6. Chromatograms of cabbage extract samples with added internal standard (1'-methylascorbigen) in the gradient mode (System 2). (a) Fresh cabbage; (b) sour cabbage. Peaks: 1, 4a; 2, 3; 3, 4c; 4, 5; 5, 8a; 6, 9a. Note: all unlabeled peaks are unknown compounds.

2·3 mg per 100 g fresh weight, with an over-all mean of 2·38 mg per 100 g fresh weight. The standard deviation was 0·075 mg per 100 g fresh weight and coefficient of variation was 3·14%.

Six samples of fresh and sour cabbage were analyzed using the proposed procedure and the results were listed in Table 2. The fresh cabbage extracts contained

ascorbigen as the main component (about 2·4–5·5 mg per 100 g fresh weight); and ascorbigen 'dimer' 8a as a minor component (about 0·1 mg per 100 g fresh weight). The content of ascorbigen 'trimer' 9a was below the detection limit. Sour cabbage extracts contained the same amounts of ascorbigen and minor ascorbigen 'dimer' 8a (0·1–0·5 mg per 100 g fresh weight); and additionally minor ascorbigen 'trimer' 9a (0·1–0·3 mg per 100 g fresh weight); 3-hydroxymethylindole (3) (about 0·2–0·3 mg per 100 g fresh weight) and (indol-3-yl)acetonitrile (5) (about 0·1–0·5 mg per 100 g fresh weight).

Cabbage extracts contain not only ascorbigen but also its transformation products. Investigation of their biological effects, including immunomodulating properties, quantification of ascorbigen and its transformation products in vegetable diet of different origin, after various processing, as well as the study of release of AA from ascorbigen and its derivatives in various conditions and on pathways of ascorbigen biotransformation, will be the subject of further investigations.

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