Determination of Ascorbigens in Autolysates of Various *Brassica* Species Using Supercritical Fluid Chromatography

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A new method of analysis based on normal phase supercritical fluid chromatography (SFC) has been developed for investigation of ascorbigens [2-C-(indol-3-ylmethyl)-β-L-xylo-3-hexulofuranosonic acid γ-lactone derivatives]. This method has been adapted to preparative isolation and quantitative determinations of individual ascorbigens comprising ascorbigen, neoascorbigen, and 4-methoxyascorbigen. The structures of these compounds have been revealed from 1D (^{1}H, ^{13}C) and 2D (COSY, HMQC, HMBC) NMR experiments. The developed SFC method had an acceptable linearity for the ascorbigens with correlation coefficients (R^2) > 0.9995 ($n = 10$) in the range of 0.13−4.9 nmol injected, detection limits were below 13 pmol, retention time stabilities were excellent, and relative response factors have been determined. The SFC method has been used for determination of ascorbigens produced during autolysis of indol-3-ylmethylglucosinolates in various *Brassica* vegetables and rapeseed seedlings. Generally, 30−60% of the indol-3-ylmethylglucosinolates in the plants were transformed into ascorbigens, with the concentration in autolysates varying from 0.51 ± 0.002 to 3.72 ± 0.21 μmol/g of dry weight (DW) for ascorbigen, from 0.05 ± 0.01 to 2.42 ± 0.23 μmol/g of DW for neoascorbigen, and from 0.03 ± 0.002 to 0.84 ± 0.07 μmol/g of DW for 4-methoxyascorbigen.

Keywords: *Brassicaceae; Brassica; glucosinolate; ascorbigens; supercritical fluid chromatography*

INTRODUCTION

Glucosinolates form a group of plant-produced allelochemicals with well-defined structures consisting of a β-thioglucopyranoside group and a side chain (R group) attached to the carbon atom number 0 in the (*Z*)-*N*-hydroximine-O-sulfonate group (Kjær, 1960; Ettlinger and Kjær, 1968; Sørensen, 1990). This structure has been found for all glucosinolates identified up to now, which encompass more than 120 different compounds (Kjær and Larsen, 1976, 1977, 1980; McDanell et al., 1988; Sørensen, 1990; Rosa et al., 1997), and it has been revealed from chemical and X-ray crystallographic investigations (Ettlinger and Lundeen, 1956, 1957) as well as from NMR data (Olsen and Sørensen, 1981; Sørensen, 1990). The structural variations are caused by differences in the R group and by acylsubstituents on the thioglucose group (Sørensen, 1990). These variations are reflected in the names of the glucosinolates, which are composed by the chemical name of the side chain (R group) used as prefix to the term glucosinolate (Ettlinger and Kjær, 1968; Olsen and Sørensen, 1981; Sørensen, 1990).

The enzyme myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1) co-occurs with glucosinolates that are present in all plants of the order *Capparales* (Rodman, 1978) and in a few of other plants (Ettlinger and Kjær, 1968; Bjerg and Sørensen, 1987). In seeds of oilseed rape and other *Brassica* species, the total pool of glucosinolates is most often quantitatively dominated by 4-hydroxyindol-3-ylmethylglucosinolate (4-hydroxyglucobrassicin) and methionine-derived glucosinolates (Bjerg et al., 1987). In contrast, vegetative parts of *Brassica* species, including various types of vegetables, have a relatively high content of the indol-3-ylmethylglucosinolates: glucobrassicin, 4-methoxyglucobrassicin, and 1-methoxyglucobrassicin (neoglucobrassicin) (Hansen et al., 1995 and 1997; Doughty et al., 1996; Rosa et al., 1997; Ménard et al., 1999).

Autolysis is the term used for degradation of endogenous glucosinolates in aqueous suspensions of disrupted plant material due to myrosinase-catalyzed hydrolysis of glucosinolates, which lead to many different products (Olsen and Sørensen, 1981; Palmieri et al., 1998). The types of products formed depend especially on the glucosinolate structure in addition to effects from types of myrosinases, other plant constituents, and reaction conditions (Sørensen, 1990; Michaelsen et al., 1991; Bjergegaard et al., 1994; Agerbirk et al., 1996). The indol-3-ylmethylglucosinolates as well as some other arylmethylglucosinolates give unstable isothiocyanates, with release of the thiocyanate ion and a reactive carbonium ion, which will give a complex mixture of reaction products depending on the nucleophiles available and the reaction conditions (Bjergegaard et al., 1994; Agerbirk et al., 1998; Buskov et al., 2000a, 2000b). Although a great number of research projects have been focused on these reactions, much still needs to be done with respect to the relative abundance of the individual compounds in autolysates and the various more or less complex matrix systems in food, feed, and digests. The developed methods for analyses of products resulting from degradation of aliphatic glucosinolates (Sørensen, 1990; Bjergegaard et al., 1999) as well as from aromatic glucosinolates (Buskov et al., 2000a, 2000b) seem now to provide opportunities for

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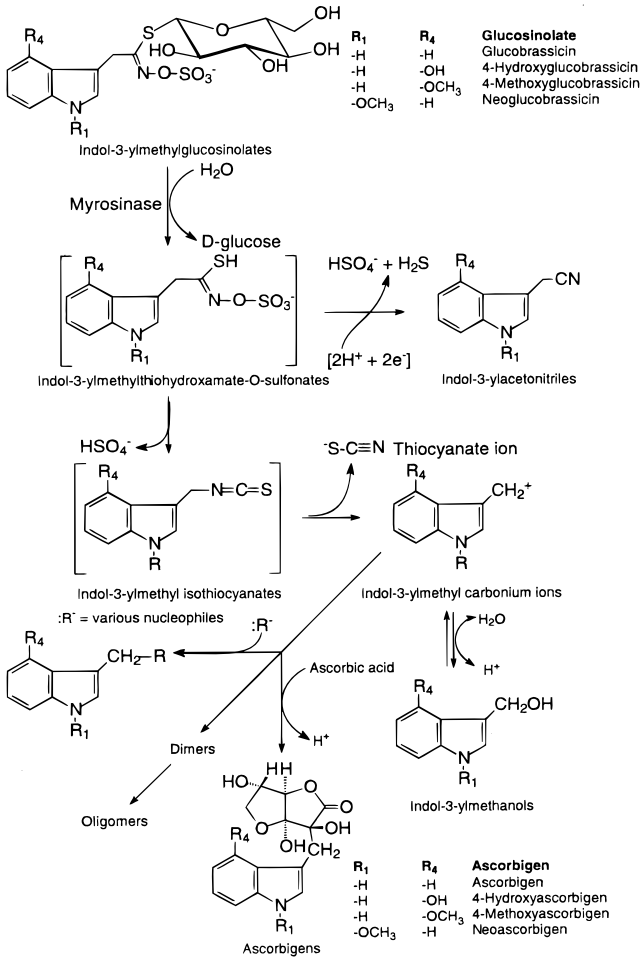

Figure 1. Myrosinase-catalyzed degradation of indol-3-ylmethylglucosinolates: glucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin to the respective ascorbigens, nitriles, alcohols, and oligomers.

increased information on individual glucosinolate degradation products. This is especially important with respect to reliable evaluations of the various physiological effects of arylmethylglucosinolates or products thereof (Jensen et al., 1991; Michaelsen et al., 1994; Adesida et al., 1996; Bonnesen et al., 1999). These effects comprise anticarcinogenic properties of glucosinolate products produced from *Brassica* vegetables (Stoewsand et al., 1978; Boyd et al., 1982; Wattenberg, 1983; Wattenberg, 1990).

The degradation products produced in myrosinase-catalyzed hydrolysis of arylmethylglucosinolates comprise reactive isothiocyanates or carbonium ions, which immediately react with water to give carbinols in acidic solutions (pH ≤ ca. 6.5). The resulting indol-3-ylmethanols easily form indol-3-ylmethyl carbonium ions with subsequent oligomerization or reactions with nucleophilic reagents, e.g., formation of ascorbigens and 5,11-dihydroindolo[3,2-*b*]carbazole (ICZ) (Kiss and Neukom, 1966; De Kruif et al., 1991; Grose and Bjeldanes, 1992; Agerbirk et al., 1996; Buskov et al., 2000a, 2000b) (Figure 1). ICZ is a well-known potent inducer, and the modulation of xenobiotic metabolizing enzymes as cytochrome P-450 by ascorbigen may be related to a possible transformation of ascorbigen to ICZ in acidic media (Preobrazhenskaya et al., 1993b; Stephensen et al., 1999). Only limited experiments have been dedicated to studies of the effects of the structurally related neoglucobrassicin and products thereof (Bradfield and Bjeldanes, 1987; Aleksandrova et al., 1992; Preobrazhenskaya et al., 1993a; Mukhanov et al., 1994; Preobrazhenskaya et al., 1999), and no experiments have focused on the effects of 4-methoxyglucobrassicin and its degradation products, although potential physiological effects of these degradation products have been suggested (McDanell et al., 1987; Loft et al., 1992; Rosa et al., 1997; Ménard et al., 1999).

The present work has been focused on the above-mentioned lacking information on degradation of the indol-3-ylmethylglucosinolates in vivo or in autolysates of vegetative plant parts comprising determinations of structurally different ascorbigens. It is a continuation of our previously performed and ongoing work with new developments in methods of analyses based on supercritical fluid chromatography (SFC), both for analytical separations and preparative purification of a wide range of glucosinolate degradation products, especially those formed during degradation of sinalbin and the indol-3-ylmethylglucosinolates (Buskov et al., 2000a, 2000b). The SFC techniques thus developed have now been modified and used both for analytical determinations and preparative SFC prior to structural determinations based on 1D and 2D NMR techniques. Thereby SFC is found to be an efficient tool for determinations of ascorbigens formed from different indol-3-ylmethylglucosinolates during autolysis of a broad range of *Brassica* species.

MATERIALS AND METHODS

Plant Material. Broccoli (*Brassica oleracea* L. var. *italica*), white cabbage (*B. oleracea* L. var. *capitata*), red cabbage (*B. oleracea* L. var. *capitata*), cauliflower (*B. oleracea* L. var. *botrytis*), and brussels sprouts (*B. oleracea* L. var. *gemmifera*) were obtained from the local market in Denmark. Portuguese cabbages, Troncha de Mirandela (*B. oleracea* L. var. *tronchuda*), Couve-nabiça (*B. napus* L. var. *napobrassica*), and Savoy cabbage (*B. oleracea* L. var. *sabauda*) were obtained from a local market in Vila Real, Portugal. All samples were weighed, freeze-dried, and stored at −20 °C until use. Seeds of single-low and double-low rapeseed (*B. napus* L.) were germinated at room temperature for two weeks, followed by freeze-drying without previous freezing, and stored at −20 °C until use.

Chemicals and Reference Compounds. Carbon dioxide (99.995%) used for SFC was obtained from Hede Nielsen (Taastrup, Denmark). HPLC-grade methanol was obtained from Fisher Scientific (Leicestershire, UK), and trifluoroacetic acid (TFA) was obtained from Fluka (Buchs, Switzerland). Indole and acetone were purchased from Merck (Darmstadt, Germany) and 3-hydroxybenzyl alcohol, indol-3-ylmethanol, methane sulfonic acid (MSA), 4-methoxyindol, diethyl ether, and ascorbic acid were from Sigma-Aldrich (Steinheim, Germany). Anhydrous Na_2SO_4 and ethyl acetate were delivered by Riedel de Häen (Seelze, Germany). Other chemicals were of analysis grade and obtained from various manufacturers. Myrosinase was purified from *Sinapis alba* L. as described previously (Michaelsen et al., 1991). A mixture containing glucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin was purified from 15-day-old *B. napus* L. seedlings using methods as described elsewhere (Bjerg and Sørensen, 1987).

Synthesis of Ascorbigen. Ascorbigen (2-C-(indol-3-ylmethyl)-β-L-xylo-3-hexulofuranosonic acid γ-lactone) was prepared from ascorbic acid and indol-3-ylmethanol at pH 4 as described in Kiss and Neukom (1966). Oligomeric compounds also formed were removed by extraction with diethyl ether. The ascorbigen was then extracted from the aqueous phase with ethyl acetate, the extract was filtrated through anhydrous Na_2SO_4, evaporated under reduced pressure, and the residue was freeze-dried.

Synthesis of 1-Methoxyindol-3-ylmethanol. 1-Methoxyindol-3-ylmethanol was synthesized from 2-nitrotoluene through a series of reactions as described elsewhere (Acheson et al., 1974; Somei et al., 1986; Hanley et al., 1990). The product, a yellow viscous liquid, was pure according to SFC and ^{1}H NMR.

Synthesis of Neoascorbigen. Neoascorbigen [2-C-(1-methoxyindol-3-ylmethyl)-β-L-xylo-3-hexulofuranosonic acid γ-lactone] was prepared as described for the ascorbigen using 1-methoxyindol-3-ylmethanol as starting material instead of indol-3-ylmethanol.

Preparation of Ascorbigens from Glucosinolates. A mixture containing ascorbigen, neoascorbigen, and 4-methoxyascorbigen [2-C-(4-methoxyindol-3-ylmethyl)-β-L-xylo-3-hexulofuranosonic acid γ-lactone] was prepared in vitro from a mixture of the three indol-3-ylmethylglucosinolates [glucobrassicin (15%), neoglucobrassicin (24%) and 4-methoxyglucobrassicin (61%)]. The indol-3-ylmethylglucosinolate mixture (71 mg) was degraded by myrosinase in 100 mM citrate buffer (20 mL, pH 6.0) with the presence of ascorbic acid in excess (100 mg). The mixture was left overnight and extracted with diethyl ether followed by ethyl acetate (2 × 50 mL). The ethyl acetate extract was filtered through anhydrous Na_2SO_4 and evaporated to dryness. The residue was dissolved in ethyl acetate prior to preparative SFC purification (see below).

Sample Preparation. To initiate glucosinolate degradation, 500 mg of freeze-dried plant material was homogenized in water (10 mL) and left for approximately 1 h. Internal standard (3-hydroxybenzyl alcohol, ca. 1 μmol/sample) was added, and the homogenate was extracted with ethyl acetate (3 × 10 mL). Samples were centrifuged to separate the organic phase from the aqueous phase and the plant material. The ethyl acetate phases were combined and filtered through anhydrous Na_2SO_4. The organic phase was evaporated, and the residue was dissolved in 0.5 mL of ethyl acetate prior to SFC analysis. All extractions were done in triplicate.

SFC Analysis. The SFC equipment used for the determination of the indol-3-ylmethylascorbigens was a Gilson SF3 supercritical fluid chromatography system (Gilson, Middleton, WI) consisting of a Gilson 306 pump with chilled head for pumping carbon dioxide. A Gilson 306 pump was used to deliver the methanol modifier (containing additive) followed by mixing in a Gilson 811C (1.5 mL mixing chamber). Sample injection (5 μL) was controlled by the Gilson 233XL autosampler (20-μL loop) and a Gilson 402 syringe pump. The column temperature was maintained in a Gilson 831 column oven followed by detection in a Gilson 119 UV−vis detector equipped with a high-pressure flow cell. The column back-pressure was maintained by a Gilson 821 pressure regulator and a Nupro pressure relief valve. Unipoint system software version 1.91 was used to control the equipment.

Separation of the ascorbigens was done on a HP Hypersil bare silica column (200 × 4.6 mm, 5-μm particles) at 2 mL/min. The column back-pressure was held isobaric at 17 MPa, and the column temperature was held at 35 °C ($\rho_{\text{mobile phase}} \sim$ 0.84 g/mL). The modifier was methanol containing 1 vol % MSA. Initially, the mobile phase was 4 vol % methanol/MSA (99:1) in carbon dioxide for 3 min, followed by a linear gradient to 12 vol % modifier in 4 min. This concentration was held for 6 min run and then returned to initial conditions. Detection was performed at 217 and 280 nm after 5 μL of sample was injected.

The mobile phase density was estimated from the Bender virial equation of state (Bender, 1970). The calculations were done for pure carbon dioxide using the assumption that small amounts of methanol in the carbon dioxide would not greatly influence the density calculations.

Preparative SFC Purification of Ascorbigens. Purification of ascorbigens for identification was done on the Gilson SF3 system as described above but slightly modified to run in preparative mode. A Waters Millipore model 510 HPLC pump (Milford, MA) was connected to the system through a T-connector between the detector and the pressure regulator and set to deliver approximately 1.3 mL/min acetone to prevent precipitation of solutes after the pressure relief valve. The Nupro pressure relief valve was replaced by a low-volume Gilson 7037 pressure relief valve to reduce the dead volume of the system.

Purification of the three ascorbigens was done on a HP Hypersil bare silica column (200 × 4.6 mm, 5-μm particles). The pressure was held at 20 MPa, the temperature was held at 40 °C, and the flow rate was set at 2 mL/min. The mobile phase consisted of carbon dioxide with 10 vol % methanol. Detection was performed at 217 and 280 nm after injection of 10−15 mL of ascorbigen mixture (approximately 1−2 mg/injection).

Relative Response factors (RRF) of Ascorbigens. Relative response factors (RRF, relative to the internal standard, 3-hydroxybenzyl alcohol) were used to determine the amount of the individual ascorbigens in the samples. $RRF_{\text{ascorbigen}}$ and $RRF_{\text{neoascorbigen}}$ were determined from the slope (α) of calibration curves for the ascorbigens and the internal standard ($RRF_x = \alpha_{3-\text{hydroxybenzyl alcohol}}/\alpha_x$). $RRF_{4-\text{methoxyascorbigen}}$ was calculated from $RRF_{\text{ascorbigen}}$ and the relative response between indol and 4-methoxyindol, due to the lack of sufficient amounts of purified standard. It is assumed that the absorbency ratio between the chromophore system in indol and ascorbigen is equal to the ratio between 4-methoxyindol and 4-methoxyascorbigen. Thereby, it was possible to calculate the RRF from differences in absorptions between indol and 4-methoxyindol as determined by injections of different concentrations of these compounds in the SFC system. The RRFs were calculated at both 217 and 280 nm.

Determination of Individual Glucosinolates. The glucosinolates present in the various samples were extracted and purified according to our standard procedures as described elsewhere (Bjerg and Sørensen, 1987; Sørensen et al., 1999). The isolated glucosinolates were transformed into their corresponding desulfoglucosinolates by sulfatase and analyzed by micellar electrokinetic capillary chromatography (MECC) on a HP^{3D} CE capillary electrophoresis instrument (Hewlett-Packard, Waldbronn, Germany) or an ABI 270A-HT capillary electrophoresis system (Applied Biosystems, Foster City, CA) (Bjergegaard et al., 1995; Sørensen et al., 1999).

NMR Spectroscopy. 1D (^{1}H and ^{13}C) and 2D experiments (COSY, HMQC, and HMBC) were recorded on a Bruker Avance 400 NMR spectrometer. Spectra were recorded at room temperature in methanol-d_4 using TMS as reference.

RESULTS

Purification and Identification of Ascorbigens. The indol-3-ylmethylglucosinolates are transformed in myrosinase-catalyzed reactions through thiohydroxamate-O-sulfonates to their highly unstable isothiocyanates, which releases the stable thiocyanate ion and the resonance-stabilized indol-3-ylmethyl carbonium ions (Figure 1). These carbonium ions, or their isothiocyanate precursors, react easily with nucleophilic reagents such as water or ascorbic acid. It is found that ascorbic acid, at weakly acidic to neutral conditions, is a much better nucleophile than water. Ascorbigens (Figure 1) are thus the quantitatively dominating products formed (vide infra) when the indol-3-ylmethyl isothiocyanates are released in a buffer containing an excess of ascorbic acid. Purification of the three ascorbigens formed during degradation of a mixture of glucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin in the presence of excess ascorbic acid (Figure 2) was easily achieved on the HP Hypersil silica column in about 10 min/run. The absence of additive in the mobile phase increased the tailing of all three ascorbigens, but this tailing was not found to have an influence on the purity of the isolated ascorbigens. All of the ascorbigens were thus nearly pure as determined by rechromatography on SFC and as revealed from the NMR experiments discussed below.

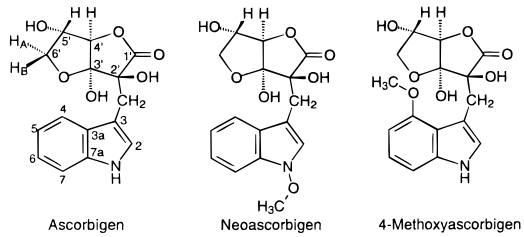

Figure 2. Structure of ascorbigen, neoascorbigen, and 4-methoxyascorbigen as found in cabbage autolysates. Numbers refer to the system used during assignment of protons and carbons in NMR experiments.

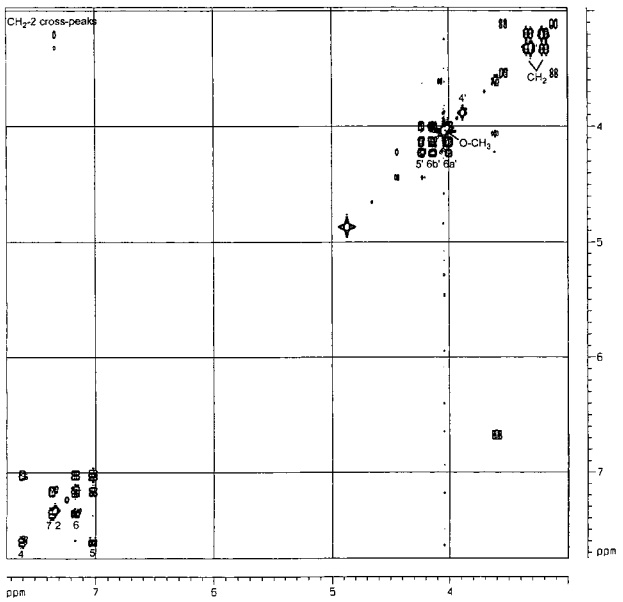

Figure 3. COSY spectrum of neoascorbigen, showing couplings between protons.

The structures of ascorbigen, neoascorbigen, and 4-methoxyascorbigen were confirmed from various 1D and 2D NMR experiments (^{1}H, ^{13}C, COSY, HMQC, and HMBC). This is shown for neoascorbigen in Figure 3. Proton and carbon chemical shifts were easily assigned to the respective atoms (Table 1). In 4-methoxyascorbigen, the presence of a methoxy group at carbon C-4 was confirmed from COSY and HMBC experiments. The COSY spectrum confirmed that there were three adjacent protons on the heteroaromatic ring. The position of the methoxy group at C-4 was finally confirmed from an HMBC experiment, in which only the proton at C-2 showed coupling to carbon C-3. In ascorbigen and neoascorbigen, the proton at C-4 also showed coupling to C-3. The ^{1}H spectrum for neoascorbigen showed a similar pattern as found for ascorbigen itself (Table 1).

SFC Method Development. A normal phase silica column was chosen for the separation of ascorbigens. This selection was based on previously obtained results from our SFC research that have shown efficient separations and reproducible retention times for compounds structurally related to those of interest in this study. The carbon dioxide used as supercritical fluid is a nonpolar fluid and is, therefore, unable to elute polar compounds from a polar column. Therefore, methanol was chosen as a modifier to increase the eluting strength of the mobile phase. The carbon dioxide−methanol mobile phase was also too weak to fully overcome nonideal interactions, which occur between the analytes and the stationary phase. These interactions are at least in part caused by hydrogen bonding and resulted in severe tailing of the ascorbigens, which initially were suppressed by addition of small amounts of the strong acid, trifluoroacetic acid (TFA), i.e., 1 vol %, to the modifier. Additional improvement of the SFC method was obtained by the use of a gradient, which was necessary to elute the polar ascorbigens, especially the ascorbigens with a free heteroaromatic N-H group. However, the presence of increasing amounts of TFA in the mobile phase increased the background absorption at low wavelengths (217 nm) during the run. This effect was eliminated by substituting the TFA with MSA, which gave only limited UV background absorption, but still decreased the unwanted interaction between the stationary phase and the ascorbigens at the same mobile phase concentration as TFA (1 vol % MSA in methanol).

The effect of temperature on the separation of some minor compounds from the ascorbigen peaks was studied at a constant density as calculated by the Bender equation of state (Bender, 1970) assuming that the amount of methanol in the mobile phase did not largely influence the density. A test mixture produced by myrosinase-catalyzed degradation of a mixture of indol-3-ylmethylglucosinolates in the presence of ascorbic acid was used during the optimization. The temperature had an appreciable effect on the resolution between the ascorbigens and some minor unknown components present in the test mixture. The resolution between the ascorbigens largely did not change in the tested temperature interval (30−50 °C), and as the best separation was found at approximately 35 °C and 17 MPa, this was chosen as the optimal conditions.

Linearity, Reproducibility, and Detection Limits. 3-Hydroxybenzyl alcohol was chosen as an internal standard and was found not to coelute with other ethyl acetate extractable compounds from autolysates of the various *Brassica* samples investigated. Both ascorbigen and neoascorbigen were directly used in the linearity test for calculation of their RRF, whereas 4-methoxyascorbigen only was available in more limited amounts. Therefore, the RRF for 4-methoxyascorbigen was calculated from the difference in absorption between indol and 4-methoxyindol as determined by injecting various amounts of these solutes. The RRF of 4-methoxyindol with indol as reference, was found to be 0.75 at 217 nm and 0.97 at 280 nm. The RRFs for ascorbigen, neoascorbigen, and 4-methoxyascorbigen were afterward determined using 3-hydroxybenzyl alcohol as reference compound (Table 2).

The linearity of the developed method was good in the range tested (Table 2), as seen from the correlation coefficients, $R^2 > 0.9995$ ($n = 10$). The detection limit at 217 nm was below 13 pmol for both ascorbigen and neoascorbigen, and this seems to be the same for 4-methoxyascorbigen, although not finally determined for this compound. At 280 nm, the detection limits were slightly higher with approximately 30 pmol for the ascorbigens. The retention time reproducibility within days was excellent with the following means ± standard deviations: 6.91 ± 0.03 min for 3-hydroxybenzyl alcohol, 9.14 ± 0.05 min for neoascorbigen, 12.33 ± 0.09 min for ascorbigen, and 13.10 ± 0.08 min for 4-methoxyascorbigen ($n = 5$). Small changes in retention times were observed between days, but these changes were not found to give any problems with respect to identification based on the peak retention times (Figure 4).

Table 1. Comparison of Measured ^1H and ^{13}C NMR Chemical Shifts (ppm) for Ascorbigen, Neoascorbigen, and 4-Methoxyascorbigen Dissolved in Methanol-d_4 with TMS as Reference[a]

	ascorbigen			neoascorbigen			4-methoxyascorbigen		
atom	$\delta_{H,\,ppm}$	^{H-H}J, Hz	$\delta_{C,\,ppm}$	$\delta_{H,\,ppm}$	^{H-H}J, Hz	$\delta_{C,\,ppm}$	$\delta_{H,\,ppm}$	^{H-H}J, Hz	$\delta_{C,\,ppm}$
2	7.19		126.6	7.33		125.1	7.06		125.4
3			107.7			104.9			107.9
3a			129.2			125.8			119.0
4	7.61	$J_{4,5}=7.9$	120.1	7.61	$J_{4,5}=8.1$	120.7			155.6
		$J_{4,6}=1.0$			$J_{4,6}=1.0$				
5	6.96	$J_{5,6}=7.0$	119.8	7.02	$J_{5,6}=7.0$	120.7	6.43	$J_{5,6}=7.2$	99.9
		$J_{5,7}=0.9$			$J_{5,7}=0.9$			$J_{5,7}=1.3$	
6	7.05	$J_{6,7}=8.0$	122.3	7.17	$J_{6,7}=8.2$	123.4	6.97	$J_{6,7}=8.3$	122.9
7	7.31		112.1	7.36		109.0	6.92		106.0
7a			137.7			133.3			139.3
O-CH_3				4.04		66.3	3.88		55.3
-CH_2-	3.24	$J=14.2$	31.9	3.20	$J=14.3$	31.6	3.32	$J=14.8$	32.6
	3.40	$J_{H,2}=0.9$		3.33	$J=0.8$		3.57	$J_{H,2}=0.7$	
1′			178.8			178.3			177.4
2′			81.0			80.7			80.1
3′			108.8			108.7			109.2
4′	3.77	$J_{4',5'}=0.7$	88.4	3.89	$J_{4',5'}=0.7$	88.6	4.39	$J_{4',5'}=0.7$	88.2
5′	4.19	$J_{5',6b'}=5.7$	75.6	4.23	$J_{5',6b'}=5.7$	75.7	4.37	$J_{5',6b'}=5.9$	75.6
		$J_{5',6a'}=3.4$			$J_{5',6a'}=3.4$			$J_{5',6a'}=3.5$	
6a′	3.98	$J_{6a',6b'}=9.7$	75.6	4.00	$J_{6a',6b'}=9.7$	75.1	4.07	$J_{6a',6b'}=9.6$	76.4
6b′	4.12		75.6	4.14		75.7	4.22		76.4

[a] Proton coupling constants are given in hertz. See Figure 2 for current numbering of protons and carbons.

Table 2. Relative Response Factors (RRF) of Ascorbigens, as Determined from Linearity Tests

compound	$RRF_{217\ nm}$	$RRF_{280\ nm}$	range tested[a]
3-hydroxybenzyl alcohol	1.00	1.00	0.034−12.4
ascorbigen	0.24	0.35	0.013−4.90
neoascorbigen	0.26	0.40	0.013−4.87
4-methoxyascorbigen	0.18	0.34	*b*

[a] Nanomoles of solute injected. [b] Compound not available.

Determination of Ascorbigens in *Brassica* spp. A range of *Brassica* vegetables often used for human consumption were used as sources for SFC determination of ascorbigens after autolysis of the glucosinolates. The internal standard, 3-hydroxybenzyl alcohol, seemed to be usable as an internal standard in these sources as no other compounds coeluted with it (Figure 5A−D). It gave a good reproducibility of the extractions after autolysis. This may be caused by the complex matrix systems in homogenates of *Brassica* vegetables, which often formed an emulsion of the aqueous and organic phases. These phases were, however, easily separated by use of centrifugations in a small table centrifuge.

Results from SFC determinations of ascorbigens formed in the majority of cabbage and seedling autolysates showed that ascorbigen was the quantitatively dominating compound, where the concentrations ranged from 0.43 μmol/g (140 mg/kg of dry weight) to 3.72 μmol/g (1.1 g/kg of dry weight). Neoascorbigen occurred most often in lower quantities in the autolysates as compared to that of the ascorbigen content, with concentrations from 0.05 μmol/g (17 mg/kg of dry weight) to 1.33 μmol/g (445 mg/kg of dry weight). In most cabbage autolysates, 4-methoxyascorbigen was a minor indol-3-ylmethylascorbigen, with concentrations ranging from 0.03 μmol/g (10 mg/kg of dry weight) in cauliflower to 0.31 μmol/g (104 mg/kg of dry weight) in the Portuguese broccoli, Couve-nabiça (Table 3). To test the applicability of the developed SFC method on autolysates of *Brassica* seedlings, seeds of oilseed rape (*B. napus* L.), both from a variety with high glucosinolate content and from a double low variety were germinated for two weeks. The ascorbigen profiles in autolysates from these two varieties were different from those of the cabbage autolysates, as neoascorbigen was the quantitatively dominating compound in the seedling autolysates followed by 4-methoxyascorbigen and then ascorbigen (Table 3 and Figure 5D).

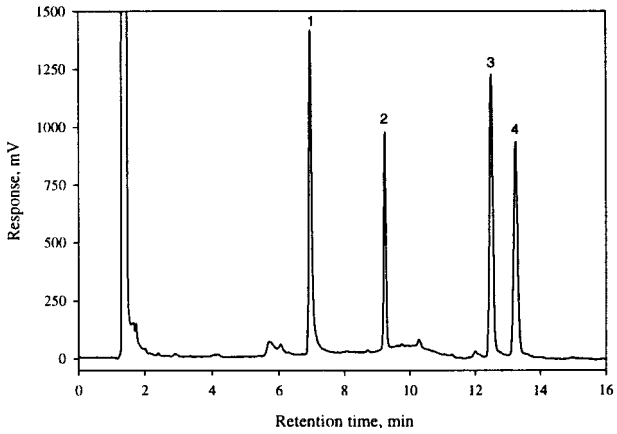

Figure 4. SFC chromatogram of a standard mixture of ascorbigens. (1) 3-Hydroxybenzyl alcohol (internal standard); (2) neoascorbigen; (3) ascorbigen; and (4) 4-methoxyascorbigen.

Generally, the amount of ascorbigens formed in the autolysates were approximately half of the theoretical amounts calculated from the concentrations of intact indol-3-ylmethylglucosinolates, determined by MECC (Table 3). For some cabbages, the higher amounts of ascorbigens were nearly equal to the amounts originally determined as intact glucosinolates. However, only very small amounts of ascorbigens were detected in extracts using boiling 70% methanol (Figure 6), which indicated that the ascorbigens are formed during the autolysis as a result of the reaction between ascorbic acid and degradation products of indol-3-ylmethylglucosinolates produced in the myrosinase-catalyzed degradation.

DISCUSSION

Ascorbigens: Structures and Occurrence. Ascorbigens are a group of compounds formed as results of reactions between C-2 of ascorbic acid (carbanion prop-

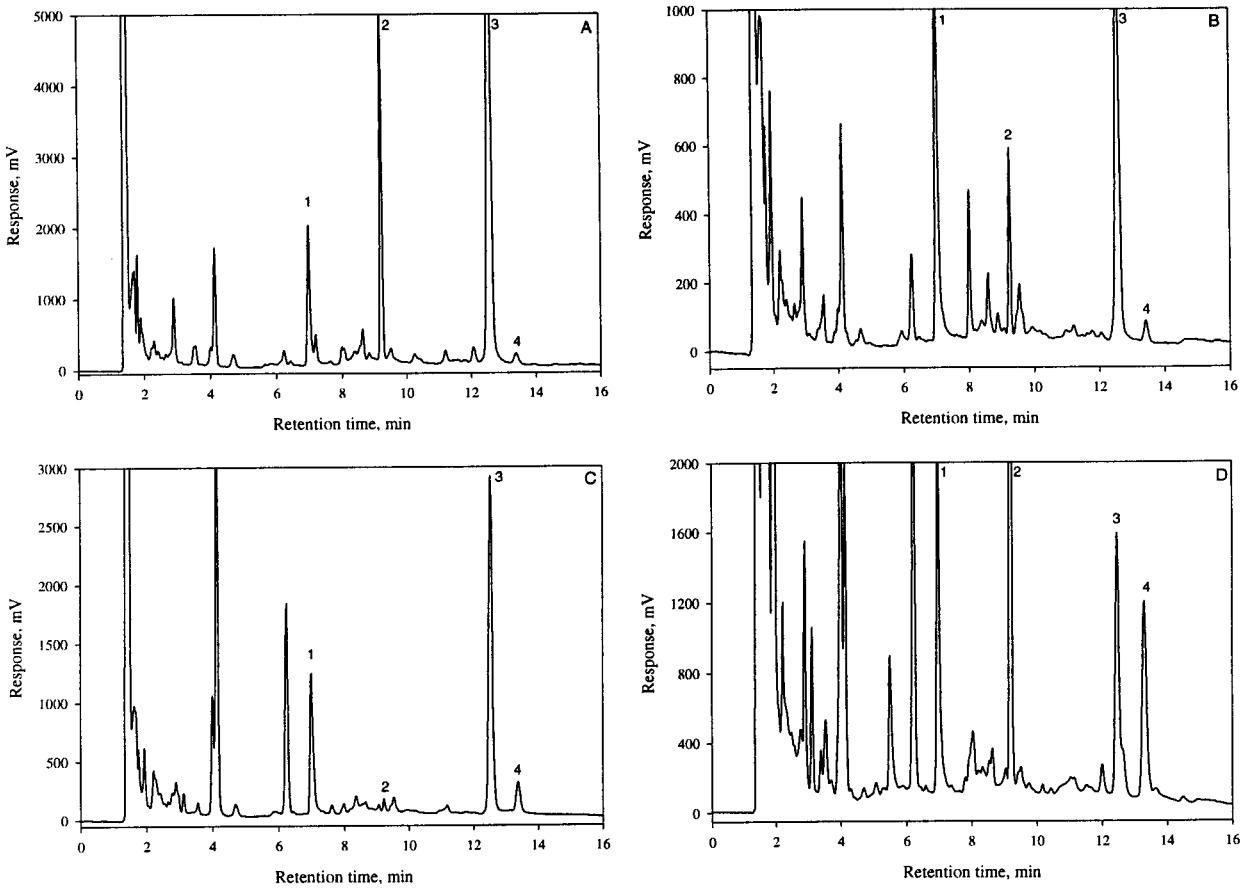

Figure 5. SFC chromatograms of autolysates produced from (A) Troncha de Mirandela; (B) cauliflower; (C) brussels sprouts; and (D) two-week-old rapeseed seedlings (high glucosinolate rapeseed). Separation conditions were as described in Materials and Methods. Labels as in Figure 4.

Table 3. Contents of Indol-3-ylmethylglucosinolates in Intact Plant Material Determined by MECC[b] and Content of Ascorbigens Produced in *Brassica* Autolysates Determined by the SFC Method Now Developed

Brassica species	ascorbigen (glucobrassicin) (μmol/g)[a]	neoascorbigen (neoglucobrassici) (μmol/g)[a]	4-methoxyascorbigen (4-methoxyglucobrassicin) (μmol/g)[a]
white cabbage	1.33 ± 0.04 (3.31)[b]	0.12 ± 0.02 (0.11)[b]	0.15 ± 0.01 (0.81)[b]
red cabbage	0.43 ± 0.01 (1.83)	0.05 ± 0.04 (0.03)	0.16 ± 0.01 (0.98)
brussels sprouts	1.74 ± 0.04 (4.94)	0.05 ± 0.01 (0.04)	0.23 ± 0.04 (0.95)
cauliflower	0.71 ± 0.04 (2.61)	0.20 ± 0.01 (0.21)	0.03 ± 0.002 (0.32)
broccoli	1.37 ± 0.03 (3.77)	0.89 ± 0.04 (1.32)	0.08 ± 0.02 (0.64)
savoy cabbage	2.17 ± 0.06 (7.36)	0.16 ± 0.04 (0.10)	0.13 ± 0.02 (1.49)
Troncha de Mirandela[c]	3.72 ± 0.21 (6.05)	1.33 ± 0.04 (0.82)	0.08 ± 0.01 (0.74)
Couve-nabiça[c]	0.46 ± 0.06 (0.77)	0.49 ± 0.01 (0.38)	0.31 ± 0.01 (0.89)
rapeseed seedlings[d]	0.81 ± 0.01 (2.48)	2.42 ± 0.23 (2.74)	0.84 ± 0.07 (3.77)
rapeseed seedlings[d]	0.51 ± 0.002 (0.97)	1.51 ± 0.12 (1.00)	0.69 ± 0.01 (2.11)

[a] Determined as μmol/g of dry weight. [b] Glucosinolate content determined before autolysis by standard methods based on micellar electrokinetic capillary chromatography (MECC) as described in detail elsewhere (Sørensen et al., 1999; Bjergegaard et al., 1995). [c] Portuguese cabbage. [d] Two-week-old seedlings.

erties) and compounds with the ability to release carbocations as, for example, arylmethylcarbonium ions including indol-3-ylmethylcarbonium ions (Bjergegaard er al., 1994; Buskov et al., 2000a, 2000b) (Figure 1). Ascorbigen is the original identified compound (Kutácek et al., 1960; Gmelin and Virtanen, 1961, 1962; Kiss and Neukom, 1966), which has given the basis for names of compounds in the group of ascorbigens. The structures of ascorbigen, neoascorbigen, and 4-methoxyascorbigen shown in Figure 2 were unambiguous as revealed from the data now obtained by synthesis, chromatography, spectroscopy, and finally confirmation from 1D and 2D

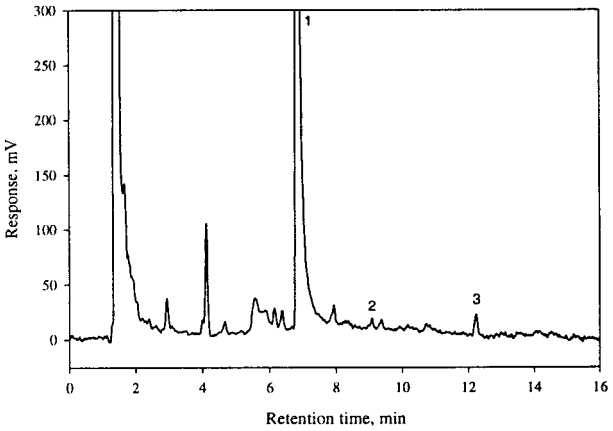

Figure 6. SFC chromatograms of cauliflower extracted in methanol/H_2O (7:3) to deactivate the myrosinase. Separation conditions were as described in Materials and Methods. Labels as in Figure 4.

NMR experiments. These NMR experiments have thus given the basis for fully assigned proton and carbon chemical shifts to all atoms in the compounds (Table 1). The heteroaromatic ^1H NMR pattern for 4-methoxyascorbigen was similar to the data reported by Hanley et al. (1985), with only some minor deviations in assigned chemical shift values. The data for ascorbigen and neoascorbigen are also in agreement with other previously published results (Lazhko et al., 1993; Mukhanov et al., 1994; Agerbirk et al., 1998).

In addition to the above-mentioned ascorbigens, compounds containing oligomeric indol-3-ylmethyl groups are also well-described and characterized (Aleksandova et al., 1992; Buskov et al., 2000b). These products are formed when ascorbic acid is a limiting reactant and when appropriate reaction conditions for oligomerization are present, which is at pH values below 6 (Agerbirk et al., 1996, 1998; Buskov et al., 2000b). However, in reaction media with too low pH, e.g., below 4, the reactivity of ascorbic acid, with a pK_a' of around 4.2, is reduced, and the formation of nitriles in myrosinase-catalyzed reactions is increased at the expense of isothiocyanate formation, especially if electron-releasing compounds as ascorbic acid or ferro ions are present (Bjergegaard et al., 1994; Agerbirk et al., 1998). Recently, it has also been demonstrated that the myrosinase-catalyzed degradation of 4-hydroxybenzylglucosinolate (sinalbin) results in formation of 4-hydroxybenzylascorbigen at appropriate reaction conditions (Buskov et al., 2000a). The myrosinase-catalyzed sinalbin reaction gives an initial 4-hydroxybenzyl isothiocyanate, which easily reacts with nucleophiles as ascorbic acid and water, with formation of 4-hydroxybenzylascorbigen and 4-hydroxybenzyl alcohol. The latter compound can be, as the case is with indol-3-ylmethyl alcohols, the precursor of ascorbigens in nonenzymatic ascorbigen synthesis, but carbinols are not intermediates in the formation of ascorbigens from the corresponding glucosinolates (Bjergegaard et al., 1994; Agerbirk et al., 1996). The type of glucosinolate and the reaction conditions during the autolysis processes will thus define the type of products formed. In Brassicaceae, broccoli, cabbage, and other glucosinolate-containing plants used as food, indol-3-ylmethylglucosinolates as glucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin as well as other arylmethylglucosinolates are the precursors for the nonascorbic acid part of ascorbigens. This is considered to be of interest in relation to the quality of cruciferous food constituents, as various types of indolyls are bioactive compounds that can have both negative effects including smell and taste (Hansen et al., 1995, 1997; Bæk et al., 1997) and positive effects as biocides (Ménard et al., 1999) and anticancerogenic compounds (Loft et al., 1992; Bonnesen et al., 1999). Very little is, however, known concerning glucosinolate catabolism in plants, whereas progress in studies of in vitro transformations of indol-3-ylmethylglucosinolates has been obtained (Sørensen et al., 1999; Buskov et al., 2000a, 2000b).

SFC as Method of Analysis for Ascorbigens. SCF has been used for the development of new and efficient methods of analysis for ascorbigens. These methods are based on normal phase SCF using a bare silica-packed column for qualitative and quantitative analytical determination of individual ascorbigens and for preparative isolation of pure ascorbigens. The methods have been used for studies of ascorbigens formed during myrosinase-catalyzed degradation of the indol-3-ylmethylglucosinolates, glucobrassicin, neoglucobrassicin, and 4-methoxyglucobrassicin (Figure 1). RRFs have been determined and used in connection with the SFC method, which allowed fast and reproducible determinations of the ascorbigens produced in autolysis using 3-hydroxybenzyl alcohol as the internal standard. The RRF for 4-methoxyascorbigen was determined individually from standard curves of indol, 4-methoxyindol, and ascorbigen, assuming that the ascorbic acid part of the ascorbigen molecules has the same influence on the molar absorption of both indol and 4-methoxyindol.

Ascorbigens Formed during Autolysis of *Brassica* Plants. Qualitative and quantitative determinations of transformation of indol-3-ylmethylglucosinolates into ascorbigens during autolysis have been performed for several *Brassica* species, both vegetables and seedlings. Intact glucosinolates present in the plant materials before start of the autolysis have been determined by standard procedures including the use of MECC (Bjergegaard et al., 1995; Sørensen et al., 1999). Ascorbigen determinations in autolysates were based on the developed SFC method. The results clearly demonstrate that ascorbigen, neoascorbigen, and 4-methoxyascorbigen might be the main degradation products from their respective indol-3-ylmethylglucosinolates (Table 3). This also seems reasonable as cabbage and other green plant materials often contain high amounts of the efficient nucleophile, ascorbic acid, which easily reacts with the indol-3-ylmethylglucosinolate degradation products with the formation of the respective ascorbigens. As has been noted earlier, it might be questioned whether indol-3-ylmethanol or indol-3-ylacetonitrile is the most important anticancerogenic compounds formed during degradation of the indol-3-ylmethylglucosinolates (McDanell et al., 1987; Stephensen et al., 1999). In agreement with earlier reports, our results showed that the ascorbigens are formed in quite high amounts in the cabbages during autolysis and therefore might be consumed in reasonable amounts with the food (McDanell et al., 1987; Aleksandrova et al., 1992; Preobrazhenskaya et al., 1999). It should be noted that the conditions used in the present autolysis experiment illustrate the ideal autolysis conditions and therefore represent the upper limit of ascorbigens that can be formed. Quite a few cabbages, i.e., broccoli and white cabbage, are, however, used as fresh material in various salads, and therefore they may be exposed to the same

conditions as in the present experiments, e.g., during cutting and chewing.

Generally, approximately 50% of the indol-3-ylmethylglucosinolate degradation products were recovered as the respective ascorbigens. This might be explained from the fact that both the formed indol-3-ylmethyl isothiocyanates and indol-3-ylmethanols, as has been found for the 4-hydroxybenzyl isothiocyanate, easily react also with, for example, thiols and other groups as found in amino acids, peptides, and proteins (Bjergegaard et al., 1999; Buskov et al., 2000a). These reactions between degradation products formed in myrosinase-catalyzed hydrolysis of indol-3-ylmethylglucosinolates and a number of nucleophiles are the subject for ongoing investigations.

Formation of the ascorbigen oligomers in the cabbage autolysates as has been shown by Aleksandrova and coworkers (1992) was not detected. The ascorbigen oligomers can be produced during the reaction of an ascorbigen molecule at the heteroaromatic carbon C-2 with another indol-3-ylmethanol or indol-3-ylmethyl isothiocyanate. The reactivity of the C-2 carbon is, however, generally lower as compared to the reactivity of the CH_2 group/C-3 carbon. In slightly acidic autolysates, where the pH normally is around pH 5.2, this oligomerization reaction at the C-2 carbon may therefore not be observed as it generally proceeds more easily at more acidic conditions, e.g., below pH 4.5−5.0 (Buskov et al., 2000b).

ABBREVIATIONS USED

Ascorbigen, 2-C-(indol-3-ylmethyl)-β-L-xylo-3-hexulofuranosonic acid γ-lactone; ICZ, 5,11-dihydroindolo[3,2-*b*]carbazole; MECC, micellar electrokinetic capillary chromatography; MSA, methanesulfonic acid; 4-methoxyascorbigen, 2-C-(4-methoxyindol-3-ylmethyl)-β-L-xylo-3-hexulofuranosonic acid γ-lactone; neoascorbigen, 2-C-(1-methoxyindol-3-ylmethyl)-β-L-xylo-3-hexulofuranosonic acid γ-lactone; RRF, relative response factor; SFC, supercritical fluid chromatography; TFA, triflouroacetic acid.

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