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Analytical problems in the study of flavonoid compounds in onions

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Flavonoids, although potentially mutagenic, are widely thought to have beneficial effects in the diet resulting from their antioxidant and metal binding properties. Epidemiological evidence correlates diets rich in flavonoid compounds with a low risk of coronary heart disease. In the present work flavonoids (principally flavonols and anthocyanins) are studied in the onion, a major dietary source of flavonoids. This work concentrates on the development of methods to study flavonoids in their natural form in plant foods as conjugates. Two major components quercetin monoglucoside and quercetin diglucoside account for 80% of the total flavonoids in onions. Anthocyanins are only minor components of the flavonoid spectrum in the edible portion of red varieties. A preliminary study of flavonols in onions, which had been finely chopped, suggests that in most varieties there is only a small loss in total flavonol but that there is a progressive loss of the diglucoside component with an accompanying quantitative accumulation of the monoglucoside and the aglycone. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

There is a vast body of epidemiological evidence which correlates high dietary intakes of fruit and vegetables with a low risk of degenerative diseases such as cancer and coronary heart disease (Block *et al.*, 1992). The extent to which non-nutrient plant secondary metabolites contribute to this net protective effect is currently under consideration, and evidence is accumulating which suggests that, among some classes of compounds that were originally thought of as natural toxicants, there are compounds whose effect in the diet may be protective. We are interested in one such group of compounds, the flavonoids which are widely distributed bioactive plant components. From the dietary point of view, four classes of flavonoids are of significance, the 4-oxo-flavonoids (flavones, flavonols, etc.), anthocyanins, isoflavones and the flavan-3-ol derivatives (catechins and tannins). Epidemiological studies in The Netherlands have shown that high intakes of one class of flavonoids (the 4-oxo-flavonoids) are correlated with a low incidence of coronary heart disease (Hertog *et al.*, 1993*a*). These findings have been extended to a study in seven countries which have again shown the correlation between high intakes of flavonols and a low incidence of coronary heart disease (Hertog *et al.*, 1995). However, similar studies have failed to show a correlation between flavonol intake and the incidence of cancer in humans (Hertog *et al.*, 1994; Goldbohm *et al.*, 1995), even though animal model experiments showed that several classes of flavonoids can protect against some chemically-induced cancers (Deschner *et al.*, 1991). Some flavonoids have a strong antioxidant activity (Rice-Evans *et al.*, 1995) and are effective chelating agents for iron and copper, and may mediate against free radical reactions involving these metals. They also inhibit platelet aggregation via an inhibition of cyclo-oxygenase (Laughton *et al.*, 1991) and are good inducers of phase II anticancer enzymes (Zhang & Talalay, 1994).

In plant foods, flavonoids and related phenolic compounds exist in a multiplicity of complex conjugates with sugars and organic acids. Important questions remain on the fate of such conjugates during processing and digestion, on the nature of the nutritionally important form of flavonoids and on the extent of their uptake and metabolism in the gut. An important prerequisite for tackling such problems is the development of convenient high-resolution separation systems to resolve and quantify flavonoid aglycones and conjugates. We will describe the development of suitable sample handling, extraction and diode-array-based HPLC methods to study flavonoids and their conjugates

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and their application to the onion, a major dietary source of flavonoids (Hertog *et al.*, 1993*a*,*b*).

MATERIALS AND METHODS

Reagents

All solvents were of AnalaR grade and HPLC grade where applicable. Methanol was obtained from Fisons, Loughborough, UK; acetonitrile from BDH, Poole, UK; tetrahydrofuran from Aldrich, Wisconsin, USA; trifluoroacetic acid from Sigma Chemical Co., Poole, UK; and water was purified via a Millex Q-plus system, Millipore, Watford, UK.

Standards

Flavonoid standard solutions were prepared by dissolving in methanol and storing at −20°C between analyses.

Samples

Onions were kindly supplied by Dr M. Day from the National Institute of Agricultural Botany, Cambridge, UK.

All the varieties studied were long day types: a white-skinned variety, Albion; a brown variety, Rijnsburger; hybrid Karato, a pink variety; Rose and a red-skinned one, Red Baron. They were stored under normal commercial storage conditions prior to analysis.

Extraction

Five bulbs from each variety were skinned, cut into quarters and immediately frozen in liquid nitrogen. Each sample was lyophilized, ground to a powder in a domestic food processor and stored at −20°C.

For the autolysis experiment, samples (approximately 300 g) were taken from five onion bulbs (from each variety) which had been skinned, chopped and immediately frozen in liquid nitrogen. Each sample was homogenized in a domestic food processor and stored in an aluminium tray at 25°C for 0, 2, 5, 8 and 24 h. Each sample was frozen and lyophilized prior to analysis.

All samples were extracted as follows: 2 g of the dry powder was homogenized three times in 70% methanol (50 ml) at 1200 rpm for 1 min (Pro400 homogenizer, Connecticut, USA) and the mixtures filtered under reduced pressure through filter paper (Whatman No. 1). The combined fractions were evaporated *in vacuo* at 30°C to approximately 20 ml and made up to 25 ml with methanol. An aliquot (1 ml) was diluted with methanol (1 ml) and used for HPLC analysis (20 μl).

HPLC

The analytical HPLC analyses were carried out using a 250×4.6 mm column packed with Dynamax 8 μm reversed-phase (C18) silica with integral guard column (Rainin, Anachem Ltd, Luton, UK). Two solvents, A (water, THF, TFA—98:2:0.1) and B (acetonitrile), were used in gradient sequences of 17% B (2 min), 17–25% B (5 min), 25–35% B (8 min), 35–50% B (5 min), 50–90% B (5 min) and 90% B (5 min) at a flow rate of 1 ml/min. Detection and quantification were performed using a diode-array-detector (HP1050 series) and HPLC3D Chemstation software (Hewlett-Packard, Bracknell, UK).

An external standard of quercitrin (0.175 mg/ml) (Apin Chemicals, Abingdon, UK) was used between every second run and linearity of response for the peak signal areas measured was confirmed for both quercitrin and quercetin (Sigma, Dorset, UK).

Preparative HPLC was carried out using a 250×21.2 mm column packed with the same material as above in order to purify peaks for chemical identification. A gradient system using the same solvents as above with the following sequence: 17% B (5 min), 17–30% B (15 min), 30–50% B (5 min), 50% B (5 min) and 90% B at 5 ml/min was used and the eluate monitored at 270 nm.

Mass spectrometry

Methanolic solutions of the extracts were applied to a drop of glycerol, followed by a drop of 3 M hydrochloric acid on the copper probe tip in the fast atom bombardment attachment to a Kratos MS9/50TC mass spectrometer. The sample was bombarded with a fast atom beam of xenon produced by an Ion-tech NF gun operating at 9 kV (nominal). FAB-mass spectra were recorded in positive mode.

RESULTS

The highest levels of flavonols were found in the red variety, 918 mg/kg fresh weight but this value is only slightly higher (22 and 13%) than that found in the pink and brown varieties, 711 and 803 mg/kg fresh weight, respectively. In contrast to the findings of other workers (Leighton *et al.*, 1992), the white variety contained readily detectable levels of flavonols, even though the level was less than 10% of that found in the other three varieties (50 mg/kg). The level of flavonols in these three varieties is twice as high as that found by Hertog *et al.* (347 ± 63 mg/kg) but within the range reported by Leighton *et al.* (1992) of 57–1096 mg/kg for 25 yellow-skinned varieties and 168–699 mg/kg for two red-skinned varieties.

The anthocyanins in the red cultivars are heavily concentrated in the skin and in the inner edible tissue are restricted to a single layer of cells in the epidermal tissue. Within this edible portion, they are relatively minor components comprising 9.2% of the total flavonoids (estimated by comparison of peak areas at 270 nm). In the pink cultivar the level is even lower at

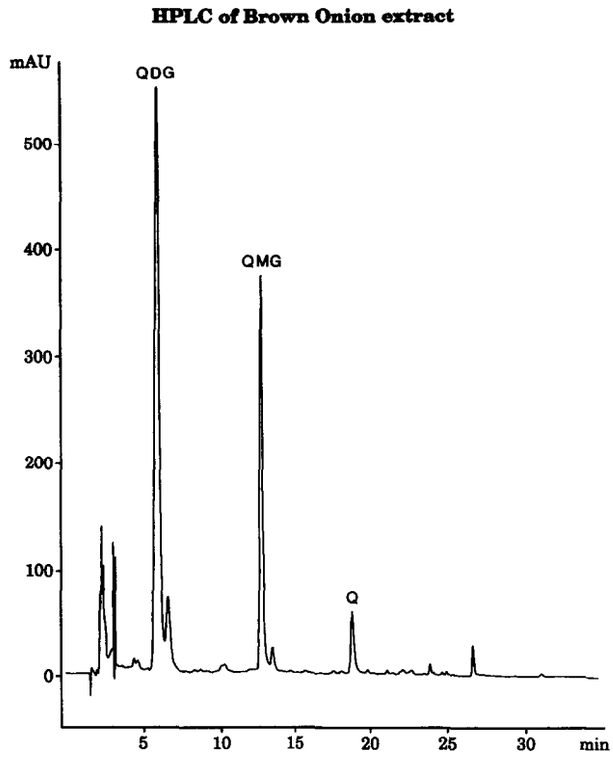

Fig. 1. The HPLC analysis of an extract prepared from brown-skinned onion variety, Rijnsburger. QDG, quercetin diglucosides; QMG, quercetin monoglucosides; Q, quercetin.

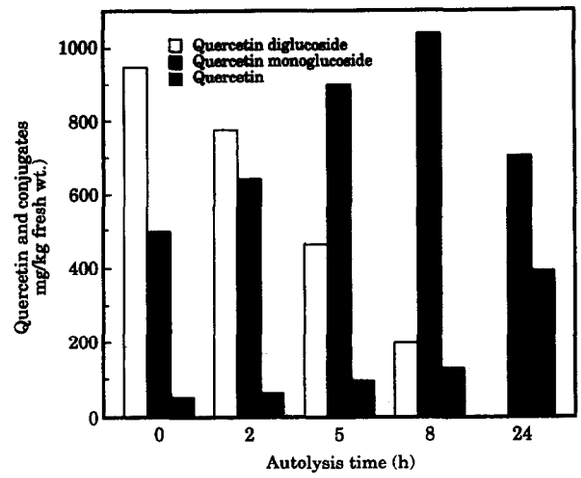

Fig. 2. The effect of autolysis on the composition of flavonol glucosides.

0.7%, and its concentration is less than 10% of that found in the red variety.

Methanolic extracts of the brown, white, red and pink cultivars were analysed by HPLC. Reversed-phase (RP) HPLC analysis of the brown variety, Rijnsburger, is shown in Fig. 1, and when observed at high sensitivity the chromatogram reveals a complex pattern of flavonoid conjugates, the majority of which have UV spectra characteristic of quercetin derivatives. At least two extra peaks in the chromatograms of both red and pink coloured onions have spectra with maxima at 520 nm and are responsible for the red colour of these extracts. A minor peak at a retention time (RT) of 18.7 min was identified as quercetin aglycone on the basis of its retention time, UV spectrum and co-chromatography with an authentic standard. Free quercetin is only a minor component of the total flavonoid spectrum accounting for 4.8, 2.1 and 0.9% of the total for the brown-, pink- and red-skinned varieties, respectively. Although up to 20 minor quercetin derivatives can be detected, when the separation is viewed at lower sensitivity (Fig. 1) it is clear that there are only two major flavonoid components, both of which give spectra consistent with their being quercetin derivatives. The two peaks, RTs of 6.2 and 12.7 min, account for over 80% of the total flavonol content of the brown variety. These two components were isolated by preparative HPLC and each analysed by fast atom bombardment mass spectrometry. The former gave signals consistent with a quercetin diglucoside (MH^+ m/z 627, MH-2h$^+$ m/z 303) and the latter consistent with a quercetin monoglucoside (MH^+ m/z 465, MH-h$^+$ m/z 303). These components have UV spectra which indicate that they are derivatives of quercetin with maxima at 253, 266, 367 nm and 253, 266, 345 nm, respectively, and produce a good fit to the spectra obtained from quercetin 3,4′-diglucoside and quercetin 4′-glucoside, respectively. Co-chromatography of these two standards with an onion extract produced co-eluting peaks at 6.2 and 12.7 min, respectively. However, definitive identification of these compounds must await the purification of sufficient material to allow detailed NMR spectral analysis to be carried out.

These two components were the predominant forms observed in all four varieties tested and this was true for the white variety, even though the total levels of flavonoids were much lower than in the other three types. In the red and pink onion varieties, the two major anthocyanins detected by their absorbance at 520 nm, as well as in the range 260–370 nm with peaks at RTs of 5.1 and 8.2 min, are seen as relatively minor components.

Compositional changes, during autolysis, for the red-, pink- and brown-skinned varieties are shown in Fig. 2. Two of the three cultivars tested showed a slight loss in total 4-oxo-flavonoids over the 24-h period; 18% over 24 h for Red Baron and 11% for Rose, but for Rijnsburger variety no measurable loss could be detected. However, in all three varieties within this period there were significant qualitative changes in flavonoid composition (Fig. 2). For Rijnsburger, within 2 h 19% of quercetin diglucoside was lost with the appearance of an equimolar amount of quercetin monoglucoside, whilst during the first 5 h there was a 50% loss of the diglucosides accompanied by increases in both the monoglucoside and the free aglycone, and by the end of 24 h all of the diglucoside had disappeared. In each time interval the loss of diglucoside could be almost quantitatively

accounted for by the appearance of monoglucoside and aglycone. With the other two varieties studied a similar pattern of changes occurred and the loss of diglucoside could largely, but not completely, be accounted for by the appearance of the monosaccharide derivative and the aglycone.

DISCUSSION

A wide range of 4-oxo-flavonoids has been shown to be present in onion. The majority are glucose derivatives of the flavonol aglycones, quercetin and kaempferol. Quercetin is by far the major agylcone but there are some varieties which have a relatively high content of kaempferol (Bilyk *et al.*, 1984). The 3,4′-, 7,4′- and 3,7-diglucosides, and the 4′- and the 3-monoglucosides of quercetin, have been reported (Herrmann, 1988; Leighton *et al.*, 1992). Small amounts of kaempferol 3- and 4′-glucoside and of isorhamnetin 4′-glucoside have also been reported in onion bulbs (Herrmann, 1988). In other parts of the onion plant more complex derivatives have been reported (Urushibara *et al.*, 1992). In red varieties the main anthocyanins have been reported to be 3-O-monoglucoside and 3-O-diglucosides (3-O-laminariobioside) of cyanidin (Fuleki, 1971). More recent work has shown that in red onion skin these derivatives are extensively malonylated (Terahara *et al.*, 1994).

Our analysis by HPLC has shown that the spectrum of flavonoids is dominated by the quercetin mono- and diglucosides which represent about 80% of the total flavonol fraction. In red varieties, the anthocyanins contribute only a small amount to the flavonoid spectrum and onions are not important sources of anthocyanins unless the skin is eaten.

We have studied the potential for autolytic changes during food processing to influence the flavonol composition and found that flavonols in onions are relatively stable to degradation beyond the aglycone. In the widely used brown-skinned onion, Rijnsburger, overall degradation was not detected, and even in the least stable variety less than 20% was lost even during extended periods of autolysis. However, during this period plant hydrolytic enzymes are active which will break down the quercetin diglucosides almost quantitatively to aglycone via the monoglucose derivative. The significance of this is not clear since although it is often assumed that the aglycones are the biologically active form of flavonols during digestion (Leighton *et al.*, 1992) this is not established and is a matter of some controversy. Clearly the glycosides differ significantly from the aglycones in their hydrophillicity and in their transport properties, and this must affect their activity in the gut during digestive processes. There are clearly outstanding questions on the form in which flavonols occur in plant foods which have been subjected to various preparation techniques. This may influence their biological activity during digestion and their subsequent effects on the biochemistry of gut and liver cells.

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