SESSION 10A

PROSPECTS FOR USING BIOTECHNOLOGY TO PRODUCE HEALTHIER FOOD

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Platform Papers: 10A-1 to 10A-4

An overview of current and potential health benefits from food and agronomic traits derived from first and second generation GM crops

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ABSTRACT

The first generation of GM crops modified through recombinant DNA techniques resulted in food derived from plants engineered to resist pests and herbicides, allowing increased yields, reductions in conventional chemical pesticides and decreased soil erosion. In these first generation crops, targeted pest control and the reduction in insecticide use produced indirect health benefits for Bt- rice producers in China and Bt-cotton small holders in South Africa and lowered mycotoxin levels in the grain of Bt-maize when compared to maize grown using conventional agronomy. Second generation GM crops are under development with significant direct nutritional and health benefits, which could enhance consumer acceptance of foods derived from GM crops. Transgenic rice and mustards have been developed having Pro-vitamin-A pathway targeting Vitamin A deficiency in developing countries. Transgenic soybean, oil seed rape and linseed have been developed with increased levels of omega-3 fatty acids. RNA interference (RNAi) is being used to 'silence' genes encoding for allergenic proteins in food such as hypoallergenic wheat, rice and groundnuts. Second generation crops also involve the use of transgenic plants to produce 'functional foods' where food derived from GM crops is not only a source of sustenance but also of medicine.

INTRODUCTION

Acreages of biotech crops continue to rise. In 2004, 81.0m hectares of transgenic crops were produced in 17 countries by 8.25 million farmers. This accounts for a 20% year on year rise in acreage planted to transgenic crops (Figure 1). In 2005, the billionth cumulative acre of a transgenic crop was planted somewhere in the world. Calculations based on cumulative food production from transgenic crops since first 1997 suggest that more than 800 billion meals containing at least 200 grams of food derived from transgenic crops has been knowingly or unknowingly consumed by a global population approaching 6.5 billion people with no known or confirmed adverse effects. The continued rapid adoption of biotech crops by farmers in developed and developing countries reflects substantial improvements in productivity, the environment, economics, health and social benefits. The global value of commercial transgenic crops, which up until now exhibit almost exclusively first generation agronomic traits, is estimated to be 4.70 billion USD with the value estimated to rise to 5.0 billion USD in 2005. A major step change in value is predicted with the introduction of second generation products exhibiting food quality and other traits which improve nutrition and hence the health of consumers. A global study by Australian economists based on grain, oil seeds, fruit and vegetables from biotech crops projects a global potential gain of 210 billion USD by 2015 (James, 2004).

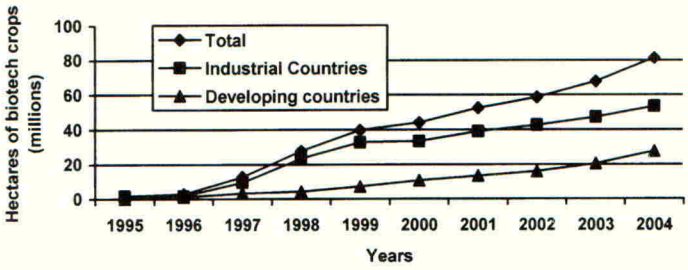

Figure 1. Global area of biotech crops in 1995-2004 (James, 2004)

Healthy diets and physical activity are key to good nutrition and are necessary for a long and healthy life. The predicted new phase in growth in biotech plantings will be fuelled by traits which show tangible health benefits. Many of these benefits will be linked to nutrition traits which will benefit both developed and developing countries and in many cases address the problems of nutrition-related chronic diseases such as obesity, diabetes, cardiovascular diseases, cancer, osteoporosis and dental diseases. According to the WHO, nutrition related chronic diseases account for 59% of the 57million deaths annually and 46% of the global burden of disease (WHO, 2002). In Africa in 2000, 1.7million deaths were attributed to underweight or malnutrition, the second highest cause of death after unsafe sex. Other significant causes of deaths were attributed to the risk factors of vitamin A deficiency, blood pressure, zinc deficiency and iron deficiency (Figure 2). First and second generation biotech products have the potential to address some of the risk factors leading to death through chronic diseases.

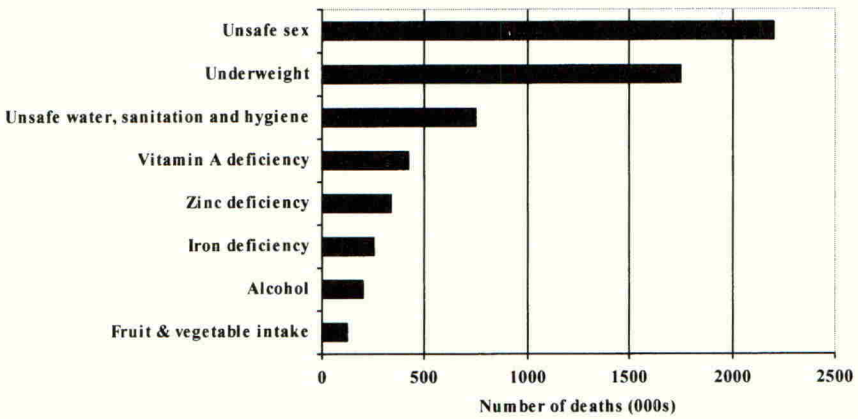

Figure 2. Deaths in Africa in 2000 attributable to selected leading risk factors (WHO, 2002)

This paper seeks to review some of the traits leading to health benefits attributed to first generation products of transgenic crops and discuss some of the second generation biotech products and their potential benefits.

FIRST GENERATION BIOTECH PRODUCTS

Enhanced yield

The first generation of transgenic crops modified through recombinant DNA techniques resulted in food derived from plants engineered principally with agronomic traits. Increased yields and yield stability are the general consequences of growing transgenic crops engineered to resist pests and herbicides and are contributing to addressing the second most important risk factor attributable to deaths in Africa during 2000, viz. underweight (Figure 2). Increased yields are the obvious way to improve food security. The US National Centre for Food and Agricultural Policy reports that transgenic insect-resistant maize raised yields in the United States by 47 million bushels on 1.8 million hectares during 1997, a year of high European Stalk Borer infestation. In 1998, yield gains of 60 million bushels on 6 million hectares were realised (cited by Metcalfe, 2005). Higher yields per unit area have undoubtedly contributed to addressing ever increasing global needs for sustenance and food security. In developing world areas such as Africa, Cassava is a major food source for most Africans, and is the fourth most important crop in the world. Roots yields of Cassava can be devastated by African cassava mosaic virus reducing yields in affected crops by 80%. Transgenic cassava has been developed to withstand the African cassava mosaic virus and hence improve nutrition and food security of affected Africans (Mackey & Montgomery, 2004).

Benefits of increased yield resulting in increased food security and improved living standards are not limited to food crops. The improved yields and resulting income gains from industrial transgenic crops such as insect resistant and herbicide tolerant cotton have had positive effects on the socio-economic wellbeing of cotton growers. Small holder transgenic cotton growers in South Africa (Gregory *et al*, 2002), China (Huang & Pray, 2002) and India (Qaim, *et al*, 2003) in particular, have benefited from yield gains of 30-200% when compared to conventional cotton, devastated by infestations of bollworm (*Helicoverpa, Heliothis, Pectinophora and Erias* spp.).

Resistance to herbicides such as glyphosate and glufosinate leads to reduced weed competition with the crop and the risk of crop damage when compared to some herbicides leading to increased yields. Tolerance to abiotic stressors such as drought and saline soil also result in higher yields and yield stability (Fuchs & Mackey 2003).

Indirect benefits

While yield gains and increased food security were expected outcomes of first generation transgenic crops, other indirect positive effects on health have been recorded, primarily from insect tolerant traits such as stalk borer tolerant Bt-maize which is resistant to European Corn Borer (*Ostrinia nubilalis*) and African stalk borers (*Busseola fusca, Chilo partellus*) and boll worm resistant cotton. A common entry route of maize stalk borers is through the developing cob, causing yield loss and an entry point for secondary infection by pathogens. The advantage of this genetic modification is that the protection of maize borers also drastically reduces the amount of secondary infection caused by the fungus *Fusarium*. The ability to control *Fusarium* results in up to 95% reduction of the level of the mycotoxin fumonisin under some environmental conditions. Fumonisin is known to cause adverse health effects in humans and swine, horses and rodents (Fuchs & Mackey, 2003).

The use of insect resistant cotton substantially reduces or eliminates farmers' use of pesticides for the control of boll worms. Some farmers in China (Pray & Ma 2001) reduced the number of times they sprayed from 30 to 3 times. More often the reduction was from 12 to 3 or 4 sprays.

Small holder farmers in the Makhathini Flats region of South Africa reduced the number of sprays from 7 to 2 on average (Bennett, 2002). Surveys conducted in China provide preliminary evidence that this reduction in insecticide use may have had a positive impact on farmers' health. Farmers were asked if they had headache, nausea, skin pain or digestive problems when they applied pesticides (Table 1). Of the cotton growers that only used transgenic cotton, 4.7% reported poisonings while 22% of farmers who planted conventional cotton reported poisonings.

Table 1. Survey in 1999 of health impacts to Chinese farmers growing Bt or conventional cotton (Pray & Ma, 2001)

Varieties of cotton Cultivated	Number Of Farmers	Pesticide quantity (kg/ha)	Number and Seriousness of Poisonings Reported in 1999 Season					
			Required visit to Hospital	Doctor	Went home to rest	Kept Spraying	Total	Total as % farmers
Only Bt varieties	236	10.3	0	0	2	9	11	4.7
Both Bt and non-Bt varieties	37	29.4	0	0	0	4	4	10.8
Only Non-Bt varieties	9	57.8	0	0	0	2	2	22.2

In an unpublished survey of small holder cotton farmers of the Makhathini Flats region of South Africa, farmers reported fewer incidences of 'cotton sickness' when growing transgenic cotton varieties requiring fewer insecticide sprays than conventional cotton growers.

SECOND GENERATION PRODUCTS

Although most of the initial transgenic crops carried improved agronomic traits, the first plant biotech product was the 'Flavr Savr tomato' which was developed to allow the fruit to ripen longer on the vine and have an increased shelf-life. Second generation transgenic crops are under development with significantly improved quality and nutritional properties (Table 2). These include carbohydrate manipulation to change starch biosynthesis, changes in oil profiles in numerous crops, altering levels of certain amino acids to enhance protein production, increasing the density of vitamins and minerals and the elimination of undesirable food components. Many of these improvements such as vitamin A enrichment of rice and maize will have the greatest impact in the developing countries of Asia and Africa respectively.

Table 2. Selected products under development having improved quality and nutritional properties (updated from Fuchs & Mackey, 2003)

High oleic acid vegetable oils
High-stearate vegetable oils to replace the hydrogenated oils and reduce *trans* fatty acids
Oil seed rape oil high in beta-carotene
Maize and soy with increased levels of lysine, tryptophan and methionine
Maize, rice, wheat and potatoes with improved starch characteristics
Rice high in beta-carotene and iron
Second generation rice even higher in beta-carotene
Sweet potatoes with improved protein quality
Ripening-controlled papayas, tomatoes, cherry tomatoes, bananas, strawberries and pineapples
Tomatoes with increased lycopene
Soybean with increased vitamin E
Isoflavone increases in numerous crops
Maize with increased levels of vitamin E
Cassava with increased levels of iron, zinc, protein and vitamins A and E
Increased protein content in potato using a gene from Amaranth

Improved oil profile

There is a great deal of interest in changing the fatty acid profiles of commodity crops that produce dietary oils which are currently not optimal for most applications. Health, processing and functionality improvements will bring profound benefits to consumers and add significant value for the biotechnology industry. Efforts have focussed largely on altering the fatty acid composition to provide either more oxidatively stable oils or oils with enhanced nutritional characteristics. Soy oil makes up 83% of the total edible consumption of vegetable oils in the United States followed by maize, palm and sunflower oils at 4% each. Improving the soybean oil profile is understandably a target for breeders and the biotech industry. A key goal is to reduce or eliminate saturated fats and increase omega-3-fatty acids with increased levels of oleic acid. Such a product would be more stable and would not require hydrogenation thereby eliminating the production of *trans* fatty acids which have been linked to cardio vascular disease. Fish and marine algae are currently the major dietary sources of the omega-3-fatty acids eicosapentaenoic/docosahexaenoic acids (EPA/DHA). Although ά-linolenic acid (ALA), an omega-3-fatty acid, is produced in the seeds of crops such as oil seed rape and flax, bioconversion to EPA/DHA via stearidonic acid (SDA) is inefficient. Increasing the levels of SDA through genetic modification in selected crops will substantially raise levels of EPA/DHA and could replace the need for marine species as a source of these omega-3-fatty acids.

Vitamins and minerals

Much research is focussed at increasing nutrient density of staple foods with the aim of benefiting those communities of the developing world where the need is greatest. Vitamin A deficiency is most acute in Southeast Asia where 70% of children under the age of 5 years are affected and where rice, which lacks pro-vitamin A in the endosperm of the grain is the staple food. The development of 'Golden Rice' expressing relatively high levels of beta-carotene where 100g of rice provided 0.16mg beta-carotene showed promise. Although laudable, many regarded this as insufficient to adequately address vitamin A deficiency. New research (Paine *et al* 2005)

led to the development of 'Golden Rice 2' with even higher levels (23-fold) of total carotenoids and a preferential accumulation of β-carotene compared to the original 'Golden Rice'. This new development significantly improves the nutritional value of 'Golden Rice' and should be more effective in combating vitamin A deficiency in Southeast Asia. The development of 'Golden Mustard Oil' is underway in India by genetically enhancing mustard oil to over express the enzymes of the beta-carotene synthetic pathway. Like rice, maize is either deficient or low in vitamin A and like rice, maize meal is the leading staple food of many African countries. Using similar approaches projects are underway to develop maize and other crops such as oil seed rape with increased levels of beta-carotene (Mackey & Montgomery, 2004).

Cassava is a major food source for most Africans. Its root is low in protein and several micronutrients. Projects are underway to develop genetically modified roots with higher levels of zinc, iron, protein and vitamins A and E. This project called 'BioCassava Plus' is a 10-institution wide endeavour and is one of the Bill and Melinda Gates Foundation's program grants in its 'Grand Challenges in Global Health' initiative. Besides vitamin A, other projects in a range of crops are aimed at increasing levels of vitamin D, vitamin E, iron, lycopene, folate and other nutrients.

Carbohydrates and protein enhancement

A key aim of the sugar industry is to produce genetically engineered sugarcane varieties with higher sucrose content by re-directing carbohydrates from starch biosynthesis to sucrose production. The introduction of photosynthesis genes from maize into rice led to yield increases of 25% in transgenic rice varieties. Other manipulations in the composition and total amount of starch had led to amylase-free, amylopectin-enriched potatoes which contain less fat when fried.

Developing countries in need of increased intake of dietary protein are key targets of research focussed on increasing amino acid content of the staple foods of these countries. Approaches to increase the amount of lysine in cereals and maize and methionine in legumes are being assessed. A two or three-fold increase in lysine content of maize has been obtained which is positive for human health as well as for improved quality feed for animals (Fuchs & Mackey, 2003).

Removing allergens

While concerns still exist that transgenic crops may produce allergic reactions by introducing novel proteins into the diet, gene modification has the potential of removing known allergens from existing foods.

One new methodology being developed by researchers is called RNA interference (RNAi). Instead of inserting new genes, this approach affects the existing ones. RNAi is being used to 'silence' genes encoding for allergenic proteins in food such as hypoallergenic wheat, rice and groundnuts. Using gene silencing researchers at USDA and Pioneer Hi-Bred Intl.have developed the 'knockout' soybean, which no longer produces the P34 protein which causes the vast majority of allergic reactions to soybean.

Other efforts to reduce or eliminate allergens in foods by modifying their amino acid sequences have also proved successful. Research is underway to suppress allergenic proteins in other crops, too, including wheat, rice and peanuts.

Biopharmaceuticals

Biopharmaceuticals are the result of a breakthrough application of biotechnology to plants to enable them to produce therapeutic proteins that could ultimately be used by the medical community to combat life-threatening illnesses. In this process, plants themselves become 'factories' that manufacture therapeutic proteins. These proteins are then extracted, refined and used in pharmaceutical production. Since most proteins cannot be chemically synthesized, there are very few options for protein production. With nearly 500 biotechnology products approved or in development globally, and with production capacity limited, the need for efficient means of therapeutic protein production is apparent. Using plants to produce pharmaceutical proteins presents several clear advantages. Transgenic plants can now be used to produce pharmacologically active proteins, including mammalian antibodies, blood product substitutes, vaccines, hormones, cytokines and a variety of other therapeutic agents (Goldstein & Thomas, 2004).

CONCLUSION

For the past 10 years transgenic crops have consistently provided a range of benefits to growers who have planted them. There has been a shift in rate of adoption from developed to developing countries driven by increased yield and health benefits for small holder and subsistence farmers. As crop plants are increasingly transformed with traits that improve the nutrition levels of staple foods and the benefits that those bring are recognized, it is likely that acceptance of transgenic crops will grow. It clear that we need no longer to talk only of the potential of crop biotechnology but can point to the mounting evidence that have recorded actual benefits to users of the technology while we can be confident that biotechnology will increasingly have a greater positive impact on human health on the public at large in the future.

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The production of very long chain polyunsaturated fatty acid in transgenic plants as an alternative, sustainable source of health-beneficial fish oils

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ABSTRACT

Very long chain polyunsaturated fatty acids are vital components of the human diet, playing many important roles in optimal health and development. In particular, these fatty acids are vital for neonatal development, and are also protective against adult pathologies such as cardiovascular disease. Current dietary sources of very long chain polyunsaturated fatty acids are predominantly from fish oils, a natural resource in some decline; therefore alternative sustainable sources are required. Recent attempts to engineer transgenic plants with the capacity to synthesise these fatty acids have clearly demonstrated the possibility of such an approach. This represents a major breakthrough in the quest for alternative sources of fish oils, as well as fulfilling the promise of transgenic plant as green factories.

BACKGROUND

One nutritional enhancement output trait that has been the focus of considerable recent interest is the synthesis and accumulation of *n-3* very long chain polyunsaturated fatty acids (*n-3* VLC-PUFAs) (Drexler *et al.* 2003; Domergue *et al.* 2005; Sayanova and Napier, 2004). These fatty acids are found predominantly in fish oils and have historically been associated with health-beneficial properties to humans. Beyond this anecdotal evidence, a large body of clinical, genetic and controlled dietary intervention studies over the last 20 years have confirmed the importance of *n-3* VLC-PUFAs to a range of human stages of growth and illness (Burr *et al.* 1989). For example, the VLC-PUFAs play an important role in neonatal growth and development, in particular brain and eye function: it is for this reason that most replacement formula milks now contain these fatty acids (Graham *et al.* 2004). There is also evidence that *n-3* VLC-PUFAs can play a health-protective role in the prevention of cardiovascular disease and associated symptomatic conditions collectively known as Metabolic Syndrome (Nugent, 2004). Unfortunately, global fish stocks are in considerable decline and since this valuable natural resource represents the current predominant source of VLC-PUFAs, an alternative sustainable source of these fatty acids is urgently required. One rapidly emerging contender for this role is transgenic plants that have been engineered with the genes encoding the primary biosynthetic pathway for VLC-PUFA biosynthesis (Abbadi *et al.* 2001). Considerable progress towards the objective of making "fish oils" in transgenic oilseeds has been made in the last few years, and very recent data has confirmed the earlier promise of using transgenic plants as "green factories" for the synthesis of VLC-PUFAs (Napier *et al.* 2004; Singh *et al.* 2005).

THE PRODUCTION OF VLC-PUFAS IN TRANSGENIC PLANTS.

Several recent studies have successfully demonstrated the demonstrated the feasibility of producing C_{20+} VLC-PUFAs in transgenic plants by the heterologous expression of multiple VLC-PUFA biosynthetic genes from non-plant sources. These studies demonstrated that several different approaches could be used to engineer plants to accumulate C_{20} VLC-PUFAs such as arachidonic acid (20:4, n-6; ARA) and eicosapentaenoic acid (20:5, n-3), albeit at relatively modest levels (Qi *et al.* 2004; Abbadi *et al.* 2004). These studies provided a proof-of-concept for the "reverse engineering" of this pathway into transgenic plants, but also provided insights into potential bottlenecks to be overcome to achieve higher levels of VLC-PUFAs (Napier *et al.* 2004). One key observation related to the substrate requirements of the biosynthetic enzymes: as detailed in Figure 1, aerobic VLC-PUFA biosynthesis is catalyzed by an alternating sequence of desaturation and elongation reactions. In particular, most desaturases from lower organisms (i.e. non-mammalian species) utilise glycerolipid-linked substrates, usually the *sn-2* position of phospholipids. In contrast, fatty acid elongation uses acyl-CoA substrates. Abbadi *et al.* (2004) showed that this "substrate dichotomy" acts a bottleneck in the efficient heterologous reconstitution of VLC-PUFA biosynthesis, resulting in the build up of C_{18} Δ^6-desaturated fatty acids (the product of the first transgene-derived step in the pathway) without any subsequent elongation. Thus, to efficiently reconstitute the synthesis of VLC-PUFAs in transgenic plants, acyl-exchange between phospholipids and the CoA pool is required. This observation holds true for both the Δ^6-desaturase/elongase pathway (the "conventional" pathway) and the so-called Δ^9-elongase/Δ^8-desaturase alternative pathway (Fig. 1). Interestingly, VLC-PUFA biosynthesis in mammals does not suffer from this problem of substrate dichotomy, as the fatty acid desaturases appear to use acyl-CoA substrates, similar to the elongase. This means that the synthesis of C_{22+} VLC-PUFAs occurs exclusively in the acyl-CoA pool. Whilst it might be tempting to consider the utility of producing transgenic plants containing mammalian desaturases to facilitate the efficient synthesis of VLC-PUFAs (via an exclusive acyl-CoA route), considerable regulatory issues arise, as do the likelihood of consumer resistance (Domergue *et al.* 2005). In addition, proof-of-concept experiments using presumptive animal acyl-CoA desaturases have failed to confer any major increase in the synthesis of C_{20} PUFAs in transgenic plants (Robert *et al.* 2005) indicating additional (as yet unknown) constraints.

In spite of this, several major advances have been made in the last year, demonstrating the successful synthesis of EPA and docosahexaenoic acid (22:6, n-3). In particular, significant levels of EPA have been achieved in transgenic soy and Brassicas (Kinney *et al.* 2004). Perhaps rather surprisingly, very similar sets of transgenes were used in these experiments as to those used previously in linseed with very limited success (Abbadi *et al.* 2004). It therefore seems that there are several different hierarchies of constraint on heterologous VLC-PUFA synthesis in higher plants: these can be considered as either generic (such as substrate dichotomy) or species-specific (as seen for linseed or soy). This implies that great care must be taken in the selection of oilseed crop for transgenic engineering, as well as the need for additional activities with which to overcome generic bottlenecks.

CONCLUSIONS AND FUTURE PROSPECTS

The feasibility of producing C_{20} VLC-PUFAs in transgenic plants has now been clearly demonstrated, providing the possibility of an alternative sustainable source of these nutritionally important fatty acids. Given the concerns over the depletion of oceanic fish

stocks and contamination of these by industrial pollutants (such as heavy metals and PCBs), the apparent benefits of such a production platform should be clear (Domergue *et al.* 2005). Moreover, whilst the European consumer has shown very clear resistance to transgenic crops and so-called "GM food", this was primarily to material containing input traits such as herbicide tolerance. Considering the very clear health benefits of a diet enriched in *n-3* VLC-PUFAs, it is possible that transgenic plants which contain the VLC-PUFA output trait might help to demonstrate the value of GM food to a sceptical public. Alternatively, given the dependence of aquaculture on fish oils (for the correct nutrition of farmed fish), it might be possible to use transgene-derived VLC-PUFA-enriched oils, providing an indirect route of enhancing human consumption of these fatty acids without depleting the natural reserves.

ACKNOWLEDGEMENTS.

Rothamsted Research receives grant-aided support from the BBSRC (UK). The authors thank BASF Plant Sciences (Limburgerhof, Germany) for support.

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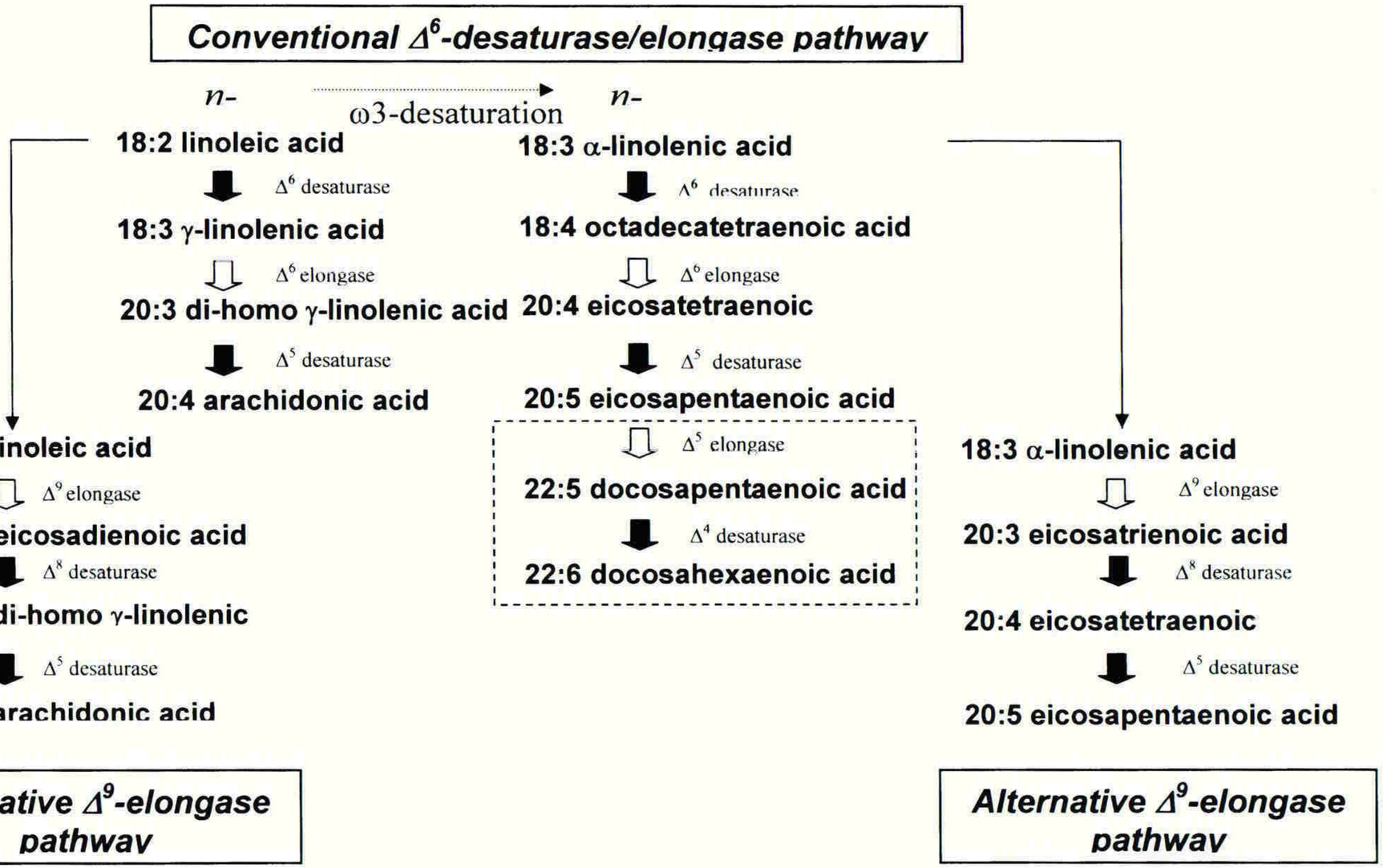
Figure 1 Generalised representation of LC-PUFA biosynthesis.
The conventional Δ^6-desaturase/elongase pathway for the synthesis of arachidonic acid and eicosapentaenoic acid from the essential fatty acids linoleic and α-linolenic acids is shown, as is the alternative Δ^9-elongase route. The Δ^5-elongase/Δ^4-desaturase route for docosahexaenoic acid synthesis is also indicated (boxed), as is the potential role of *ω3*-desaturation in conversion of *n-6* substrates to *n-3* forms. The "substrate dichotomy" of PUFA biosynthesis is represented via solid arrows for glycerolipid-linked reactions and open arrows for acyl-CoA reactions.



Absorption and metabolism of quercetin glucosides after the ingestion of onions by human volunteers

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ABSTRACT

Epidemiological studies have shown an association between increased intakes of dietary flavonols and a reduced incidence of cardiovascular disease. Onions containing flavonols, were fed to human volunteers after which plasma and urine were collected and analysed over a 24 h period. Five flavonol metabolites, quercetin-3'-sulfate, quercetin-3-glucuronide, isorhamnetin-3-glucuronide, a quercetin diglucuronide and a quercetin glucuronide sulfate, were detected in plasma in quantifiable amounts together with trace quantities of six other metabolites. The profile of metabolites excreted in urine was markedly different to that of plasma with many of the major urinary components, including quercetin-3'-glucuronide, two quercetin glucoside sulfates and a methylquercetin diglucuronide, being either absent or present in the bloodstream in only trace quantities. This is indicative of substantial phase II metabolism. Total urinary excretion of quercetin metabolites was 12.9 μmoles which corresponds to 4.7% of intake.

INTRODUCTION

Flavonols are polyphenolic C_6-C_3-C_6 compounds that occur widely in plants and plant-derived foods (Saltmarsh *et al.* 2003) and beverages (Duthie & Crozier 2003). They are secondary metabolites that are synthesized *in planta* via the phenylpropanoid and flavonoid pathways (Crozier 2003). There is much nutritional interest in flavonols, some of which are strong antioxidants Williamson *et al.* 1996). Epidemiological studies have shown an association between increased intakes of dietary flavonols and a reduced incidence of cardiovascular disease (Hertog *et al.* 1993, 1995, Knekt *et al.* 1996). This is in keeping with reports that flavonols inhibit low-density lipoprotein oxidation in vitro (Janisch *et al.* 2004), a process that in vivo leads to atherosclerosis and a narrowing of the blood vessels (DeWalley *et al.* 1990, Vinson *et al.* 1995). Flavonols can also inhibit platelet aggregation (Tzeng *et al.* 1991), induce expression of phase-II detoxification enzymes and have also been shown to inhibit the growth of certain types of human cancer cells Ranelletti *et al.* 1992, Scambia *et al.* 1990, Yoshida *et al.* 1990).

Flavonols, such as quercetin, kaempferol, isorhamnetin and myricetin, are found in fruits, vegetables, teas and red wines as sugar conjugates, typically linked to glucose, rhamnose or rutinose (Saltmarsh *et al.* 2003, Duthie & Crozier 2003). The average intake of flavonols in the Netherlands has been estimated to be 23 mg/d with tea and onions being the major sources at 48% and 29% of total intake, respectively (Hertog *et al.* 1993). The ability of flavonols to provide protective effects against cardiovascular disease and other chronic

conditions requires that they are absorbed into the bloodstream from the gastrointestinal tract and transported to target tissues. A number of studies of flavonol absorption have been carried out with onions which consistently contain high levels of flavonols Crozier *et al.* 1998) in the form of quercetin-3,4'-diglucoside, quercetin-4'-glucoside, and smaller amounts of other conjugates including isorhamnetin-4'-glucoside (Tsushida & Suzuki 1995). Studies typically involve the acute ingestion of an onion supplement after which plasma pharmacokinetics and urinary excretion are determined over a 24 h period.

This publication reports on the use of HPLC with photodiode array (PDA) and MS^2 detection to analyse human plasma and urine collected from six volunteers after the ingestion of red onions which contained high levels of a range of anthocyanins and flavonols. No anthocyanins were detected in either plasma or urine but full scan MS^2 data were obtained for a total of 23 flavonols which comprised a mixture of glucuronide, glucoside, methylated and sulfated metabolites of quercetin. Quantitative pharmacokinetic data on the five main components that accumulated in plasma and the levels 12 metabolites excreted in urine over a 24 h period were also obtained but not presented here. This is the first report of a pharmacokinetic study in which individual glucuronidated, methylated, and sulphated metabolites of quercetin have been identified and quantified. It extends a preliminary qualitative report on the HPLC-MS^2 methodology used to identify flavonol metabolites in plasma and urine after the consumption of onions (Mullen *et al.* 2004).

MATERIALS AND METHODS

Study design

Six volunteers (four males and two females), who were healthy, non-smokers and not on any medication, participated in this study and gave their written consent. They were aged between 23 and 45 years and had a mean body mass index of 23.7 ± 1.2 (range 20.9-27.6). Subjects were required to follow a low flavonoid diet for two days and to fast overnight prior to supplementation. This diet excluded most fruits, vegetables and beverages such as tea, coffee, fruit juices, and wine. On the morning of the study red onions (*Allium cepa*) were skinned, chopped into small slices, and fried for four 4 min in margarine. Aliquots of the fried onions were taken for qualitative and quantitative analysis of their anthocyanin and flavonol content.

All subjects consumed 270 g of fried red onions. Venous blood samples were taken before (0 h) and 0.5, 1, 2, 3, 6 and 24 h post-ingestion. Twelve ml of blood was collected in heparinised tubes at each time point and immediately centrifuged at 4000 *g* for 10 min at 4°C. The plasma was separated from the red blood cells and 500 µl aliquots were acidified to pH 3 with 15 µl of 50% aqueous formic acid and 50 µl of ascorbic acid (10 mM) was added to prevent oxidation. The plasma samples were then stored at -80°C prior to analysis. Urine was collected before and over 0-4, 4-8 and 8-24 h periods after the consumption of the fried onion supplement. The volume of each sample was recorded prior to acidification to pH 3.0 and the storage of aliquots at -80°C. The study protocol was approved by the Glasgow Royal Infirmary Local Research Ethics Committee.

HPLC with diode array and MS^2 detection

Samples were analysed on a Surveyor HPLC system comprising of a HPLC pump, PDA detector, scanning from 250 to 700 nm and an autosampler cooled to 4°C. Separation was carried out using a 250 x 4.6 mm I.D. 4 µm Synergi Max-RP column eluted with a 60 min gradient of 5-40% acetonitrile in 1% formic acid at a flow rate of 1 ml min^{-1} and maintained at 40°C. After passing through the flow cell of the diode array detector the column eluate was split and 0.3 ml min^{-1} was directed to a LCQ DecaXP ion trap mass spectrometer fitted with an electrospray interface. (Thermo Finnigan). Analyses utilised the negative ion mode for flavonols and positive ionisation for anthocyanins as this provided the best limits of detection. Analysis was carried out using full scan, data dependant MS^2 scanning from *m/z* 100 to 1000. Capillary temperature was 350°C, sheath gas and auxiliary gas were 60 and 10 units respectively, and the source voltage was 4 kV for negative ionisation and 1 kV for positive ionisation.

RESULTS

Analysis of fried red onions

Gradient reverse phase HPLC with absorbance detection and full scan data dependent MS^2 was used to identify and quantify the flavonol and anthocyanin content of the fried red onion meals. Absorbance at 365 nm and negative ionisation MS^2 were used for flavonol analysis. The total amount of flavonols in the 270 g onion meal was 275 ± 8.8 µmoles. In keeping with the data of Tsushida and Suzuki (1995), the major components were quercetin-3,4'-diglucoside (107 ± 1.4 µmol), quercetin-4'-glucoside (143 ± 12 µmol) and isorhamnetin-4'-glucoside (11 ± 1.4 µmoles) which accounted for 95% of the 275 ± 8.8 µmoles flavonol intake. The anthocyanins were monitored at 515 nm and positive ion full scan MS^2 data were obtained. In accordance with previously published work seven anthocyanins were detected (Donner et al. 1997) and main components in the 270 g meal were cyanidin-3-glucoside (9.3 ± 0.3 µmoles), cyanidin-3-(6"-malonylglucoside) (48 ± 1.7 µmoles) and cyanidin-3-(6"-malonyllaminaribioside) (14 ± 0.5 µmoles). The total anthocyanin content of the onion meal was estimated to be 75 ± 2 µmoles.

Qualitative analysis of plasma and urine

Plasma and urine samples were analysed by HPLC with PDA and MS^2 detection. No peaks were apparent in the 515 nm traces obtained with either urine or plasma. With the sample sizes analysed and the limit of detection at $A_{515\ nm}$, anthocyanins at levels $\geq$ 0.1 % of the amounts ingested would have been detected. This finding is in keeping with other reports on the fate of dietary anthocyanins following absorption. The picture that has emerged is that a variety of anthocyanins appear in urine after supplementation with berries or berry extracts but at best in extremely low concentrations, typically 0.1% or less of the ingested dose.

In contrast to the anthocyanins, sizable quantities of flavonols were detected in plasma and urine, with a total of 23 quercetin-based compounds being identified. Typical HPLC traces obtained at $A_{365\ nm}$ are illustrated in Figures 1A and B and the identifications based on MS^2 spectra and t_R data are summarised in Table 1.

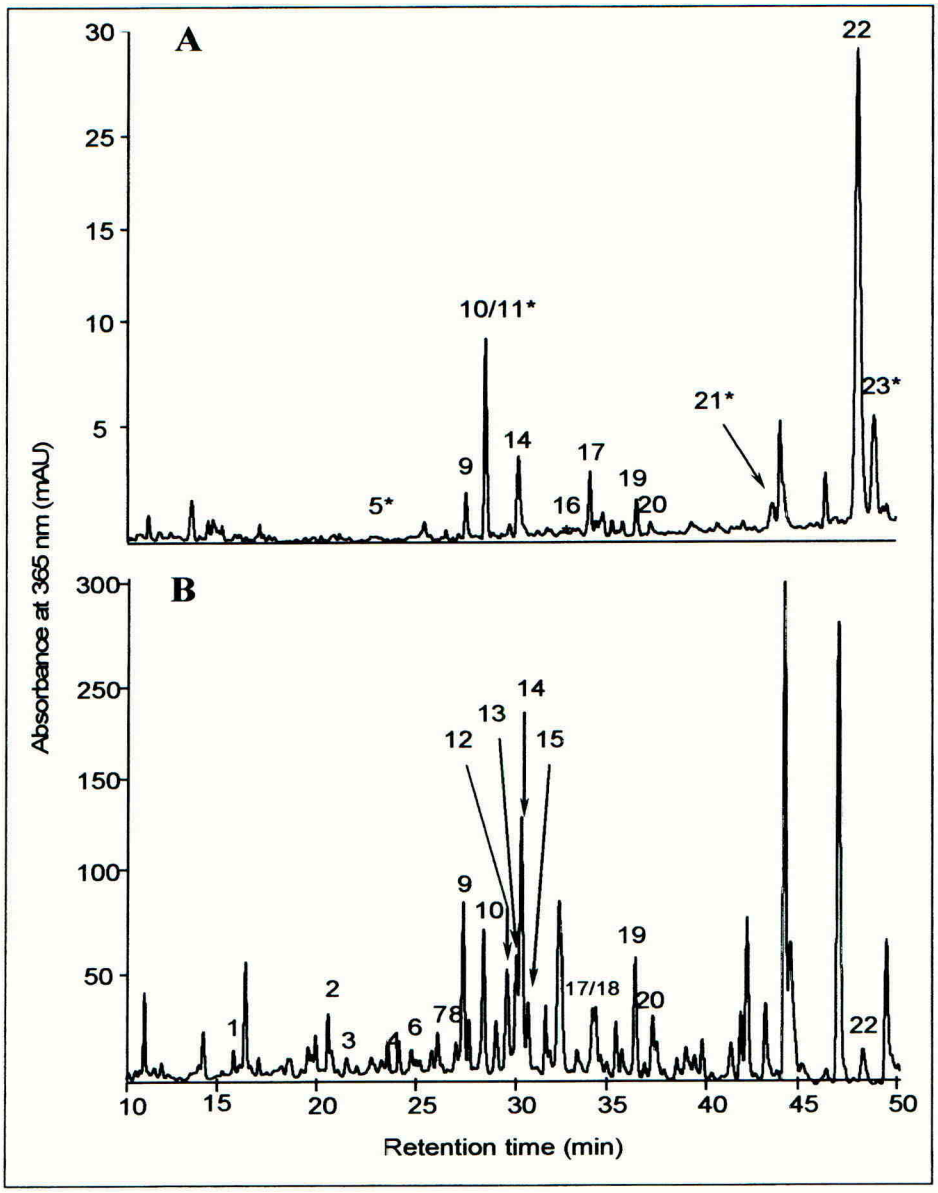

Fig. 1. Gradient reversed-phase HPLC with detection at 365 nm of quercetin metabolites in (A) a plasma extract and (B) urine obtained from a human volunteer after the consumption of fried red onions. Samples analysed on a 250 x 4.6 mm I.D., 4μm Synergi Max-RP column at 40°C and eluted at a flow rate of 1ml min^{-1} with a 60 min gradient of 5-40% acetonitrile in water containing 1% formic acid. Detection was with a diode array detector operating at 365 nm. Peaks 1–23 represent components subsequently analysed by MS^2 with an electrospray interface with negative ionisation – for identity of peaks 1–23, see Table 1. * Indicates peaks detected in samples from only from volunteer 6.

Table 1. HPLC-MS^2 Identification of Quercetin Metabolites Detected in Plasma and Urine After the Consumption of 270 g of Fried Onions by Six Human Volunteers.

Peak	t_R (min)	Compound	$[M-H]^-$ (m/z)	MS^2 fragments ions (m/z)	Location
1	15.6	quercetin diglucuronide	653	477([M-H]$^-$-GlcUA), 301([M-H]$^-$-GlcUA-GlcUA)	urine
2	20.4	methylquercetin diglucuronide	667	491([M-H]$^-$-GlcUA), 315([M-H]$^-$-GlcUA-GlcUA)	urine
3	21.5	quercetin glucoside glucuronide	639	477([M-H]$^-$-Glc), 463([M-H]$^-$-GlcUA), 301([M-H]$^-$-GlcUA-Glc)	urine
4	22.7	methylquercetin diglucuronide	667	491([M-H]$^-$-GlcUA), 315([M-H]$^-$-GlcUA-GlcUA)	urine
5	22.8	quercetin-3,4'-diglucoside*	625	463([M-H]$^-$-Glc), 301([M-H]$^-$-Glc-Glc)	plasma
6	24.8	quercetin diglucuronide	653	477([M-H]$^-$-GlcUA), 301([M-H]$^-$-GlcUA-GlcUA)	urine
7	26.2	quercetin glucoside glucuronide	639	477([M-H]$^-$-Glc), 463([M-H]$^-$-GlcUA), 301([M-H]$^-$-GlcUA-Glc)	urine
8	27.0	quercetin glucoside glucuronide	639	477([M-H]$^-$-Glc), 463([M-H]$^-$-GlcUA), 301([M-H]$^-$-Glc-GlcUA)	urine
9	27.4	quercetin diglucuronide	653	477([M-H]$^-$-GlcUA), 301([M-H]$^-$-GlcUA-GlcUA)	urine, plasma
10	28.4	quercetin-3-glucuronide	477	301 ([M-H]$^-$-GlcUA)	urine, plasma
11	28.4	quercetin-3-glucoside*	463	301 ([M-H]$^-$-Glc)	plasma
12	29.6	quercetin glucoside sulfate	543	463([M-H]$^-$-SO_3), 381([M-H]$^-$-Glc), 301([M-H]$^-$-SO_3-Glc)	urine
13	30.1	quercetin glucuronide sulfate	557	477([M-H]$^-$-SO_3), 381([M-H]$^-$-GlcUA), 301([M-H]$^-$-SO_3-GlcUA)	urine
14	30.3	quercetin glucuronide sulfate	557	477([M-H]$^-$-SO_3), 381([M-H]$^-$-GlcAU), 301([M-H]$^-$-SO_3-GlcUA)	urine, plasma
15	30.6	quercetin glucoside sulfate	543	463([M-H]$^-$-SO_3), 381([M-H]$^-$-Glc), 301([M-H]$^-$-SO_3-Glc)	urine
16	33.2	isorhamnetin-3-glucoside*	477	315 ([M-H]$^-$-Glc)	plasma
17	34.1	isorhamnetin-3-glucuronide	491	315([M-H]$^-$-GlcUA)	urine, plasma
18	34.4	quercetin-4'-glucuronide	477	301([M-H]$^-$-GlcUA)	urine
19	36.3	quercetin-3'-glucuronide	477	301([M-H]$^-$-GlcUA)	urine, plasma
20	37.2	isorhamnetin-4'-glucuronide	491	315([M-H]$^-$-GlcUA)	urine, plasma
21	43.2	quercetin*	301	179, 151	plasma
22	47.9	quercetin-3'-sulfate	381	301([M-H]$^-$-SO_3)	Urine,plasma
23	48.3	quercetin-sulfate*	381	301([M-H]$^-$-SO_3)	plasma

Peak numbers and HPLC retention times refer to HPLC trace in Figs. 2A and 2B. t_R - retention time; $[M-H]^-$ - negatively charged molecular ion; Glc - glucosyl unit; GlcUA – glucuronyl unit; *indicates compounds detected only in the plasma of one of the six volunteers

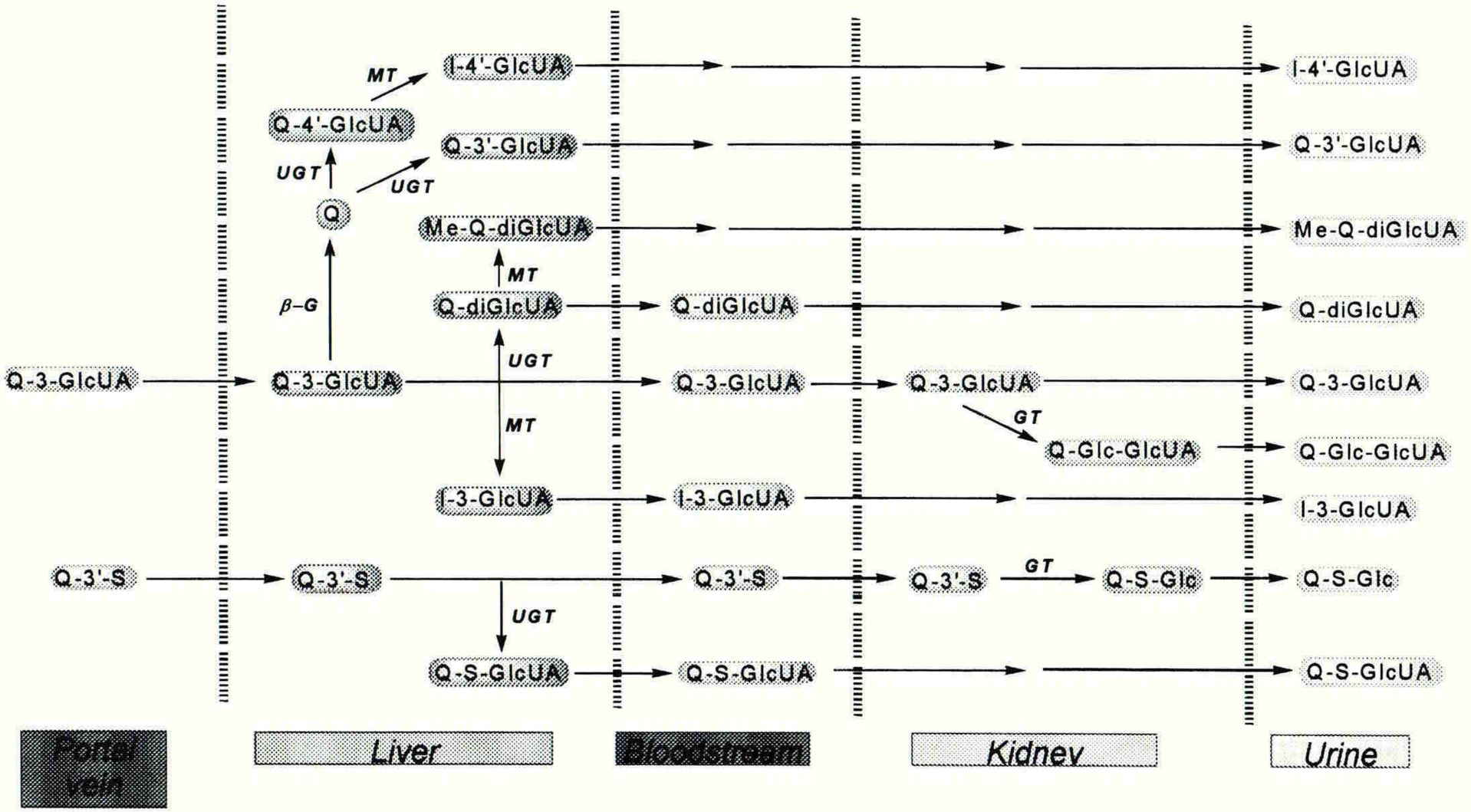

Fig. 2. Schematic of the possible metabolic fate of quercetin-3-glucuronide and quercetin-3'-sulfate as they are transported from the small intestine to the liver where they are further metabolised before returning to the bloodstream and being excreted in urine via the kidneys.. Q – quercetin; I – isorhamnetin; glc – glucoside; glcUA - glucuronide; diglcUA – diglucuronide; S – sulfate; β-G – β-glucosidase; UGT - glucuronyltransferase; MT – methyltransferase; GT – glucosyltransferase.

DISCUSSION

Traditionally, quantitative analysis of plasma and urine after ingestion of either flavonols or flavonol-rich produce by humans or animals has involved enzyme or acid hydrolysis of samples to release aglycones, prior to HPLC (Hollman et al. 1996). The current study with human volunteers in which unhydrolysed extracts were analysed by HPLC with PDA and full scan data dependent MS^2 detection, provided a far more detailed picture of the fate of flavonol glucosides within the body than it was possible to obtain in earlier investigations. In total 23 flavonol derivatives were either identified or partially identified with five being quantified in plasma and 12 in urine. If these samples had been subjected to hydrolysis only quercetin and isorhamnetin would have been detected and quantified. There are several reasons why it was possible to obtain such a detailed insight into the fate of dietary quercetin glucosides following their ingestion. In the case of plasma samples, very clean extracts with high flavonol recoveries were obtained by using the extraction procedures of Day et al. (2001). Secondly, an earlier investigation, in which [2-^{14}C]quercetin-4'-glucoside was ingested by rats and radio labelled metabolites were monitored, alerted us to the possibility that quercetin glucosides may be converted in humans to a much larger number of metabolites than had previously been anticipated (Mullen *et al.* 2002). In addition, recent improvements in the sensitivity of PDA detectors, in terms of flow cell optics with increased path lengths, have lowered limits of detection. Also negative ion mass spectrometry using ion trap MS^n has made it substantially easier to identify metabolite peaks observed in the improved HPLC absorbance traces.

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The improvement of Golden Rice and its implementation for the potential alleviation of vitamin A deficiency

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ABSTRACT

Golden Rice is the name given to genetically modified rice which produces β-carotene (pro-vitamin A) in the grain. In the body, β-carotene is converted to vitamin A. We found that using an alternative phytoene synthase transgene resulted in an increase of β-carotene levels by up to 23 fold. Although the nutritional benefit will depend on the uptake and bioconversion of β-carotene, the new 'Golden Rice 2' has the potential to provide a child's RDA in just over two portions of rice. The activities underway for its implementation will be discussed.

Carotenoids, most importantly β-carotene, are precursors of vitamin A in the human diet. These pro-vitamin A compounds are converted to vitamin A by the body. Carotenoids are ubiquitous in the plant world, and although rice plants produce β-carotene in green tissue there is none present in the edible part of the grain (the endosperm). In parts of Asia, where rice is the predominant part of the diet, vitamin A deficiency (VAD) is a severe problem resulting in depressed immunity and increased susceptibility to disease, permanent blindness or death. Children are particularly vulnerable, and it is estimated that up to 200 million children are affected by severe vitamin A deficiency with 1.3 – 2.5 million preschool children deaths annually. Ye et al (2000) developed Golden Rice with the intention of contributing to the alleviation of VAD by providing β-carotene in the rice endosperm. They achieved this by genetically modifying rice to produce key enzymes of the carotenoid biosynthesis pathway in the endosperm. They introduced two transgenes; phytoene synthase (*Psy*) from daffodil and a multifunctional carotene desaturase (*crtI*) from the soil bacterium *Erwinia uredovora*. Both were specifically expressed in the seed endosperm to bridge the gap in the production of β-carotene. A maximum level of 1.6 $\mu g\ g^{-1}$ total carotenoids was achieved, and although this was a major breakthrough with substantial benefit, it was recognised that higher levels of β-carotene would be more advantageous. At a higher carotenoid content, better meeting the daily requirement, the contribution of Golden Rice to combating VAD would be considerably improved.

Through this ground breaking work it was known that the absence of phytoene synthase and carotene desaturase prevented β-carotene accumulation in the rice endosperm, but it was not known what was limiting yet higher levels being accumulated in Golden Rice. It was possible that one of the two enzymes introduced was still limiting the pathway or there may have been other restrictions, for instance in the precursors supply. To study this more easily, we established a model for carotenoid biosynthesis using maize callus.

We systematically tested seven *Psy* genes from alternative sources in the maize model to determine if any resulted in an increase to the inherent carotenoid content (Paine et al, 2005). There was a dramatic effect on carotenoid levels in the maize callus. *Psy* from carrot, bell

pepper, tomato, maize, arabidopsis and rice all out-performed daffodil demonstrating that *Psy* was a major control in the pathway. We tested the most effective phytoene synthases (bell pepper, tomato, rice and maize) in rice plants, again using daffodil *Psy* as a reference. As found in the maize model, the choice of phytoene synthase transgene greatly affects carotenoid accumulation in transgenic rice grains; some grains were deeply coloured with an orange hue. Up to 16μg g^{-1} of coloured carotenoids were achieved in the rice endosperm using a phytoene synthase from maize with the *Erwinia uredovora* carotene desaturase gene. Beneficially, using the more active phytoene synthases resulted in the preferential accumulation of β-carotene (80-90%) compared to that found when using daffodil phytoene synthase (60-70%).

Using the knowledge gained from the experimental rice plants, a larger transformation experiment was undertaken to generate a second generation Golden Rice using the maize *Psy* with *crtI*. 'Golden Rice 2' plants with levels of coloured carotenoids up to 37μg g^{-1} were obtained. By utilising the alternative *Psy* it proved possible to increase the carotenoid content of the Golden Rice endosperm by 23-fold.

GOLDEN RICE HUMANITARIAN PROJECT

A few years ago Ingo Potrykus and Peter Beyer, the inventors of the original Golden Rice, established a humanitarian project with the vision of making Golden Rice freely available to resource poor farmers and their communities in developing countries where vitamin A deficiency is a significant problem (www.goldenrice.org). Through a licensee network of public sector research institutes in Asia, Golden Rice is to be traditionally bred into local varieties more suited to existing conditions. Syngenta has been a close supporter of this work from the outset through a public/private partnership, and has donated technologies, product development and other expertise to help achieve this. In 2004 field trials were conducted with 'Golden Rice 1' plants generated and donated by Syngenta. It has also donated the 'Golden Rice 2' plants to the project so that under the governance of the GoldenRice Humanitarian Board a rice variety that has the potential to provide a child's RDA of pro-vitamin A in just over two portions can be made available to those that need it. Of course several years work lies ahead to determine the performance of the technology in different varieties and growing conditions, to undertake bioavailability studies, taste trials, and meet the hurdles of regulatory and consumer acceptance. But whatever the challenges ahead, introgression of 'Golden Rice 2' from laboratory to plate is a magnificent opportunity for a sustainable contribution to alleviation of vitamin A deficiency.

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