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Identification and quantification of glucosinolate and flavonol compounds in rocket salad (*Eruca sativa*, *Eruca vesicaria* and *Diplotaxis tenuifolia*) by LC–MS: Highlighting the potential for improving nutritional value of rocket crops

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ABSTRACT

Liquid chromatography mass spectrometry (LC–MS) was used to obtain glucosinolate and flavonol content for 35 rocket accessions and commercial varieties. 13 glucosinolates and 11 flavonol compounds were identified. Semi-quantitative methods were used to estimate concentrations of both groups of compounds. Minor glucosinolate composition was found to be different between accessions; concentrations varied significantly. Flavonols showed differentiation between genera, with *Diplotaxis* accumulating quercetin glucosides and *Eruca* accumulating kaempferol glucosides. Several compounds were detected in each genus that have only previously been reported in the other. We highlight how knowledge of phytochemical content and concentration can be used to breed new, nutritionally superior varieties. We also demonstrate the effects of controlled environment conditions on the accumulations of glucosinolates and flavonols and explore the reasons for differences with previous studies. We stress the importance of consistent experimental design between research groups to effectively compare and contrast results.
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1. Introduction

The groups of crops collectively known as rocket (or arugula, rucola, roquette) are all members of the *Brassicaceae* family, and are native to the areas surrounding the Mediterranean Sea (Martinez-Sanchez et al., 2006). Rocket crops belong to two genera, *Eruca* and *Diplotaxis*, and are increasingly important in the salad vegetable market (Pasini, Verardo, Cerretani, Caboni, & D'Antuono, 2011). The species are now grown commercially all over the world in countries as diverse as the USA, UK, Italy, Spain, Morocco, Israel, India and Australia (Bozokalfa, Esiyok, & Yagmur, 2011).

Previous studies have highlighted rocket as a rich source of glucosinolate (GSL) compounds (Kim, Jin, & Ishii, 2004). Virtually all other members of the *Brassicaceae* contain GSLs as secondary metabolites that act as part of plant defence mechanisms (Schranz, Manzaneda, Windsor, Clauss, & Mitchell-Olds, 2009). GSLs and their hydrolysis products have also been implicated in giving rocket its characteristic pungent aromas and flavours (Bennett et al., 2002) and volatiles (such as isothiocyanates (ITCs) and indoles) have been consistently linked with anticarcinogenic

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activity in mammalian tissues (Lynn, Collins, Fuller, Hillman, & Ratcliffe, 2006).

Both *Eruca* and *Diplotaxis* species contain similar profiles of GSLs within the leaf tissue, the most prominent of which are glucosativin (4-mercaptobutyl-GSL), glucoerucin (4-(methylthio)butyl-GSL) and glucoraphanin (4-(methylsulfinyl)butyl-GSL). Glucosativin and glucoerucin breakdown products are thought to contribute most to pungency and flavour in rocket (Pasini, Verardo, Caboni, & D'Antuono, 2012). Numerous other GSLs have also been identified within rocket tissue, for example diglucothiobeinin (4-(β-D-glucopyranosyldisulfanyl)butyl-GSL) (Kim et al., 2007), 4-hydroxyglucobrassicin (4-hydroxy-3-indolymethyl-GSL) (Cataldi, Rubino, Lelario, & Bufo, 2007) and 4-methoxyglucobrassicin (4-methoxy-3-indolymethyl-GSL) (Kim & Ishii, 2006).

Rocket species also contain large concentrations of polyglycosylated flavonol compounds, which are known to infer numerous beneficial health effects in humans and other animals. Particularly of note are their effects on the gastrointestinal tract and in cardiovascular health (Bjorkman et al., 2011; Traka & Mithen, 2011). Several studies in rocket have identified and quantified polyglycosylated flavonols, which belong to three core aglycones: isorhamnetin, kaempferol and quercetin (Bennett, Rosa, Mellon, & Kroon, 2006).

Prolonged intake of *Brassicaceae* vegetables and leaves has a demonstrably beneficial impact on human health (D'Antuono et al., 2009); however much of the world's population do not consume enough of them to have these benefits, as is highlighted in several studies (Casagrande, Wang, Anderson, & Gary, 2007). Therefore, instead of only promoting increased consumption of leafy vegetables such as rocket, we propose increasing the nutritional quality and phytochemical density of varieties by using advanced screening and plant breeding methods, whilst still maintaining the sensory and visual acceptance of the consumer. This has already been achieved in broccoli with the production of varieties such as Beneforte which accumulates high concentrations of glucoraphanin (Traka et al., 2013).

In this study we draw a comparison between commercial rocket varieties available for public consumption and underutilised genetic resources. Nineteen gene bank accessions of *Eruca sativa* and sixteen commercial varieties (comprising *E. sativa*, *Eruca vesicaria* and *Diplotaxis tenuifolia*) were evaluated for GSL and polyglycosylated flavonol composition under controlled environment conditions. We hypothesise that through selective breeding for morphological traits in rocket, many important health promoting phytochemical traits may have been lost in commercial varieties, and that by breeding from underutilised accessions, nutritionally superior varieties can be produced. We also hypothesise that controlled environment growing conditions minimizes the effects of environmental stress on rocket plants, and provides a platform for comparable results between research groups and repeat experiments. We also call on other groups to consider plant maturity and time of harvest as an important factor in determining the usefulness of data to breeders and growers.

2. Materials and methods

2.1. Plant material

Rocket accessions were selected from three European gene banks based upon information provided by Elsoms Seeds Ltd. (Spalding, Lincolnshire, UK). In total 19 were sourced; 2 from the Centre for Genetic Resources in the Netherlands (CGN, Wageningen, The Netherlands), 12 from the Leibniz-Institut für Pflanzengenetik und Kulturpflanzenforschung (IPK, Gatersleben, Germany), and 5 from the University of Warwick Crop Centre Genetic Resources Unit (Wellesbourne, UK; formerly Warwick HRI). A further 16 commercial varieties were collected: 13 were independently sourced from retailers, 1 provided by Elsoms Seeds Ltd., and 2 from Bakkavor Group Ltd. (Bourne, Lincolnshire, UK).

Three biological replicates of each accession/variety were germinated under controlled environmental conditions (in Saxcil growth cabinets) after being sown in a random sequence. Long-day lighting was used (16 h light, 8 h dark) at an intensity of 200 μmol m^{-2} s^{-1} (equivalent to 10,800 Lux of sunlight). Daytime temperatures were set at 20 °C and nighttime temperatures at 14 °C. Seedlings were grown for ten days in seedling trays and then transplanted to larger trays; four plants of each replicate were grown on. Plants were grown for another twenty days and then leaves from the four plants were harvested together. Sampling for each plant took approximately one minute from the cutting of the leaves at the petiole to being placed in zip-loc freezer bags on dry ice inside a polystyrene container (with lid). For health and safety reasons it was decided that liquid nitrogen would not be used in this process.

Thirty days was chosen as the optimum point of harvest as it reflects the typical number of days commercial growers grow their crop after sowing. Bags were placed in a −80 °C freezer immediately after harvest and transport was completed (<30 min). Samples were freeze-dried in batches for three days (in a Vertis Bench-top Series). Leaves from each rep were ground into a fine powder using a combination of pestle and mortar and miniature coffee grinder (De'Longhi KG49, Treviso, Italy).

2.2. Reagents and chemicals

All solvents and chemicals used were of LC–MS grade and obtained from Sigma–Aldrich (Poole, UK) unless otherwise stated.

2.3. Glucosinolate/flavonol extraction

The following method was adapted from Pasini, Verardo, Caboni, and D'Antuono (2012), Jin et al. (2009). Three experimental replicates of each biological rep were prepared as follows: 40 mg of ground rocket powder was heated in a dry-block at 75 °C for 2 min, as suggested by Pasini, Verardo, Caboni, and D'Antuono (2012), as a precautionary measure to inactivate as much myrosinase enzyme as possible before liquid extraction. 1 ml of preheated 70% (v/v) methanol (70 °C) was then added to each sample and placed in a water bath for 20 min at 70 °C. Samples were then centrifuged for 5 min (6000 rpm, 18 °C) to collect loose material into a pellet. The supernatant was then taken and put into fresh Eppendorf tubes. The volume was adjusted to 1 ml with 70% (v/v) methanol and frozen at −80 °C until analysis by LC–MS.

2.4. LC–MS^2 analysis

Immediately before LC–MS analysis each sample was filtered using 0.25 μm filter discs with a low protein binding Durapore polyvinylidene fluoride (PVDF) membrane (Millex; EMD Millipore, Billerica, MA, USA) and diluted with 9 ml of HPLC-grade water. Samples were run in a random order with QC samples (Dunn, Wilson, Nicholls, & Broadhurst, 2012). An external reference standard of sinigrin hydrate was also prepared for quantification of GSL compounds, and isorhamnetin for flavonol compounds. Preparation was as follows: A 12 mM solution was prepared in 70% methanol. A dilution series of concentrations was prepared as an external calibration curve with HPLC-grade water (200, 150, 100, 56, 42, 28, 14 and 5.6 ng μl; sinigrin correlation coefficient: $y = 12.496x - 15.012$; $r^2 = 0.993$, isorhamnetin correlation coefficient: $y = 0.3205x - 5.3833$, $r^2 = 0.921$). Standard response factors were used in the calculation of GSL concentration where

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