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Analysis of avenanthramides in oat products and estimation of avenanthramide intake in humans

Angela A Pridal, Wiebke Böttger, Alastair B Ross*

Division of Food and Nutrition Science, Department of Biology and Biological Engineering, Chalmers University of Technology, Gothenburg, Sweden

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ABSTRACT

Avenanthramides are phenolic compounds found only in oats, and are of interest due to suggested bioactivities, including anti-inflammatory effects and induction of apoptosis. The objective of this work was to optimise a high performance liquid chromatography (HPLC) method for analysis of avenanthramides in food, and analyse the avenanthramide content in 45 oat fractions and products. The optimised HPLC method was based on triplicate extraction of 100 mg sample with 1 ml 80% ethanol in phosphate buffer (pH 2.8) and used gallacetophenone as an internal standard. Avenalumic acid-derived avenanthramide homologues $2f_d$ and $2p_d$ were also present, making up to 20% of the total avenanthramides detected in oats. The amounts of avenanthramides detected in oat products was 2–82 μg/g. It was estimated that mean avenanthramide intake among oat consumers ranges from 0.3 to 2.1 mg/day, considerably lower than the amount used in studies that have investigated biological effects of avenanthramides in humans.

1. Introduction

Of the wholegrain cereals, oats are the most strongly associated with reduced markers of cardiovascular disease (Hollaender, Ross, & Kristensen, 2015), an effect mainly associated with their β-glucan content and its effect on reduction of LDL-cholesterol. Oats also contain avenanthramides, phenolic compounds which are unique to oats, and possess several proposed bioactivities, including a strong anti-inflammatory activity, proposed to be related to their association with reduced risk of disease (Meydani, 2009). There are three main avenanthramides described in oats, which are based on 5-hydroxyanthranilic acid conjugated with p-coumaric acid, caffeic acid, or ferulic acid (Fig. 1). Two main naming systems have been proposed based on either letters or a combination of a number denoting the type of anthranilic acid and a letter denoting the type of cinnamic acid (Bratt et al., 2003; Collins & Mullin, 1988). The number '2' is used to denote an avenanthramide with a 5-hydroxyanthranilic acid moiety while the letters p, c and f are used to denote p-coumaric acid, caffeic acid and ferulic acid, respectively. In this paper the number-letter system is used, and the equivalent letter noted in the tables. Besides these three main avenanthramides (2c, 2f and 2p). Many other avenanthramides have been described (Bratt et al., 2003; Collins, 1989; Emmons, Peterson, & Paul, 1999), though these are not included in quantitative methods for avenanthramides. Among these 'minor' avenanthramides are those with an avenalumic acid substitution instead of a cinnamic acid (Collins, McLaughlin, & Blackwell, 1991), which have been described from avenanthramide-enriched oat extracts. This modification makes these avenanthramides slightly more hydrophobic than the main avenanthramides 2c, 2f and 2p, which may have important effects on their bioavailability. Avenalumic acid substituted avenanthramides are denoted by a subscript 'd' after the 'number-letter' name.

Avenanthramides are bioavailable in humans (Chen, Milbury, Collins, & Blumberg, 2007) based on detection in plasma and recently their metabolites have been determined in mice (Wang et al., 2015), and human urine (Sang, Wang, & Yerke, 2017) and plasma (Walsh et al., 2017). In rats they can be stored in skeletal muscle, hepatic and cardiac tissue, even if rapidly eliminated from circulation (Koenig, Dickman, Wise, & Ji, 2011).

Most methods for avenanthramide analysis are based on extraction in organic solvent (usually methanol or 80% ethanol) followed by liquid chromatography with either ultra-violet (UV) (Bryngelsson, Mannerstedt-Fogelfors, Kamal-Eldin, Andersson, & Dimberg, 2002; Collins & Mullin, 1988; Mattila, Pihlava, & Hellstrom, 2005) or mass spectrometry (MS) detection (Jastrebova, Skoglund, Nilsson, & Dimberg, 2006; Xie et al., 2017). Most methods have used large amounts of sample (> 1 g) and solvent (> 10 ml total extraction solvent). With the exception of one recent method using an internal standard (Xie et al., 2017), all have used external standard calibration for quantification, which relies on the assumption that the extraction procedure is close to 100% efficient.

* Corresponding author.
E-mail addresses: pridala@student.ethz.ch (A.A. Pridal), wiebke.boettger@online.de (W. Böttger), Alastair.Ross@chalmers.se (A.B. Ross).

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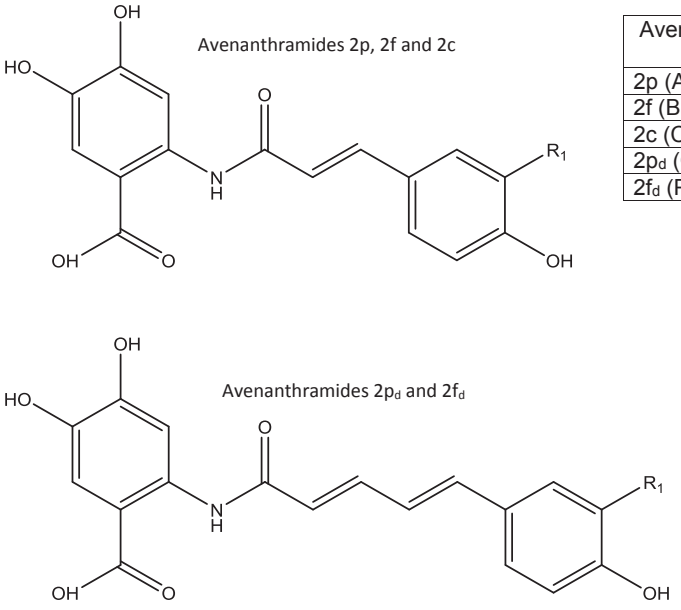

Avenanthramide name	R_1
2p (A)	H
2f (B)	OCH_3
2c (C)	OH
$2p_d$ (O)	H
$2f_d$ (P)	OCH_3

Fig. 1. Structure of avenanthramides detected in this study.

While many papers have measured the avenanthramide content of native oats, and the effect of food processing on oats in model systems (Bryngelsson, Dimberg, & Kamal-Eldin, 2002; Bryngelsson, Ishihara, & Dimberg, 2003; Redaelli et al., 2016), few studies report the amount in commercial food products (Xie et al., 2017), and none have reported their content in a wide range of oat-containing products. Measuring the amount of avenanthramides in commercial food products is important for understanding whether the postulated bioactivity of avenanthramides could be relevant at normal intakes of oat products.

In order to determine the avenanthramide content of a wide range of oat-containing products, we have optimised both the extraction procedure and an HPLC method for their analysis, and applied this to the measurement of 45 different oat products and fractions available in Sweden and the United States.

2. Materials and methods

2.1. Chemicals and samples

Oat products were purchased from local supermarkets in the Jönköping region of Sweden and Boston, Massachusetts in the United States. Additional oat flour and bran samples were provided by Swedish Oat Fiber AB (Bua, Sweden). Methanol and ethanol were obtained from VWR Chemicals (Spånga, Sweden) and Kemetyl AB (Haninge, Sweden), respectively, and were of analytical grade. HPLC grade acetonitrile was purchased from Poch S. A. (Gliwice, Poland). For the preparation of mobile phase mixtures, formic acid from Fisher Scientific (Hampton, USA) was used. The reference standards avenanthramide A, B and C (> 98%) were obtained from ReseaChem (Switzerland). Tested internal standards gallacetophenone, verapamil hydrochloride and 2-phenyl-2,4,6-tri-hydroxyacetophenone were from Sigma Aldrich (Switzerland). For the preparation of all aqueous solutions Milli-Q® purified water using a Merck Millipore (Billerica, MA, USA) purification system was used.

2.2. Sample extraction optimisation

Based on different methods used in the literature, extraction solvent (100% methanol (Bryngelsson et al., 2002) or 80% ethanol in 10 mmol/l phosphate buffer, pH 2.8 (Wise, 2011)), sample amount (100 mg or 1 g of sample) and extraction solvent volume (sample:solvent ratio 1:5 or 1:10), use of sonication, number of extract replicates (3, 4 or 5) and type of evaporation (rotary evaporation or vacuum centrifuge evaporation) were optimised. All extractions were carried out at room temperature. During optimisation, each extraction permutation was repeated five times, and the conditions that gave the highest peak area and lowest relative standard deviation were selected. The optimised method was as follows: 100 mg of sample milled to pass through a 0.25 mm screen was placed in a 1.5 ml microcentrifuge tube and 1 ml of 80% ethanol in 10 mmol/l phosphate buffer, pH 2.8, was added, along with 100 µl of internal standard mix in methanol (see HPLC method development). Samples were then vortexed in a vortex mixer to ensure that the sample and solvent were well mixed, and then sonicated for 10 min. Samples were then extracted in an end-over-end mixer for 20 min. The extracted samples were centrifuged at 1400 × *g* for 5 min in a microcentrifuge (Thermo Electron, USA) and the supernatant transferred to a second microcentrifuge tube for evaporation. The extraction procedure (solvent addition, vortexing, end-over -end mixing and centrifugation) was repeated a total of three times. The three extracts were pooled and evaporated in a vacuum centrifuge evaporator (MiVac Duo, Genevac, United Kingdom) at 40 °C. Dried extracts were resuspended in 100 µl methanol and transferred to chromatography vials with glass inserts.

2.3. High-performance liquid chromatography method development

An optimised HPLC method was developed to be run on a HPLC system with a maximum backpressure limit of 400 bar instead of an UHPLC system, and to use internal standard quantification instead of external standard calibration. Previously external standard calibration has been the main method for quantification of avenanthramides by HPLC. C_8 and C_{18} columns from different manufacturers were tested, with an Intertsil ODS-3 3 µm 150 × 3 mm column (GLSciences, Japan) providing the best peak shape and resolution between avenanthramides 2p and 2f, which were not fully resolved on most columns. The use of methanol or acetonitrile, both with 0.1% formic acid, as the strong solvent (i.e. solvent B) was tested, and acetonitrile was found to provide better peak resolution. Three different potential internal standards, gallacetophenone, verapamil and 2-phenyl-2,4,6-tri-hydroxyacetophenone were separated chromatographically from the avenanthramides. The three internal standards were tested throughout the method validation to determine which performed best overall in terms of reproducibility and recovery. The HPLC system used for this method was a Dionex - P580A LPG HPLC Pump System, a Dionex STH-585 column oven, a Dionex