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Isolation of α-linolenic acid biohydrogenation products by combined silver ion solid phase extraction and semi-preparative high performance liquid chromatography

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ABSTRACT

Polyunsaturated fatty acids typically found in cattle feed include linoleic (LA) and α-linolenic acid (ALA). In the rumen, microbes metabolize these resulting in the formation of biohydrogenation products (BHP), which can be incorporated into meat and milk. Bioactivities of LA-BHP, including conjugated linoleic acid (*cis* (*c*) 9,*trans* (*t*) 11-18:2 and *t*10,*c*12-18:2) and *trans* fatty acid isomers (*t*9-, *t*10- and *t*11-18:1) have been investigated, but effects of several BHP unique to ALA have not been extensively studied, and most ALA-BHP are not commercially available. The objective of the present research was to develop methods to purify and collect ALA-BHP using silver ion (Ag^+) chromatography in sufficient quantities to allow for convenient bioactivity testing in cell culture. Fatty acid methyl esters (FAME) were prepared from perirenal adipose tissue from a cow enriched with ALA-BHP by feeding flaxseed. These were applied to Ag^+-solid phase extraction, and eluted with hexane with increasing quantities of acetone (1, 2, 10, 20%) or acetonitrile (2%) to pre-fractionate FAME based on degree of unsaturation and double bond configuration. Fractions were collected, concentrated and applied to semi-preparative Ag^+-high performance liquid chromatography (HPLC) for the isolation and collection of purified isomers, which was accomplished using isocratic elutions with hexane containing differing amounts of acetonitrile (from 0.015 to 0.075%). Purified *trans*-18:1 isomers collected ranged in purity from 88 to 99%. Purity of the ALA-BHP dienes collected, including *c*9,*t*13-18:2, *t*11,*c*15-18:2 and *t*10,*c*15-18:2, exceeded 90%, while purification of other dienes may require the use of other complementary procedures (e.g. reverse phase HPLC).

1. Introduction

In ruminants (e.g. cattle, sheep, goat), dietary polyunsaturated fatty acids (PUFA) undergo extensive biohydrogenation by rumen microbes resulting in the production of numerous biohydrogenation products (BHP) which can subsequently accumulate in meat and milk [1–3]. Dietary PUFA typically include linoleic (LA, 18:2n-6) and α-linolenic (ALA, 18:3n-3) acids, with the types and amounts of BHP produced being affected by the diet, its influences on rumen conditions, and their influences on the microbial population [4–6]. The two prominent LA-BHP include *cis* (*c*) 9, *trans* (*t*) 11-18:2 (rumenic acid, RA) and *t*11-18:1 (vaccenic acid, VA), and these have been found to be protective against inflammation, several types of cancer, and cardiovascular disease [7–10]. Biological effects of BHP unique to ALA (e.g. BHP with double bonds past carbon 12 from the carboxyl end) have not been extensively characterized [11–15], and may represent an untapped nutraceutical resource.

Characterizing the bioactivity of naturally occurring BHP has been minimal as most are not commercially available. Comprehensive analysis of BHP can also be complex, requiring multiple gas chromatographic (GC) analyses combined with silver ion high performance liquid chromatography (Ag^+-HPLC) [16–18]. In addition, when analyzing ruminant products with complex fatty acid (FA) profiles, Ag^+-thin layer chromatography (Ag^+-TLC) or Ag^+-solid phase extraction (Ag^+-SPE) is sometimes employed for pre-fractionation 1–2 mg of fatty acid methyl esters (FAME) prior to GC analyses [16,17]. Silver ion chromatography has been used to fractionate lipids for more than 50 years. *Pi* electrons of FA double

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bonds react reversibly with silver ions to form polar complexes, and retention during Ag^+-chromatography increases with the number of double bonds, and with *cis* over *trans* double bonds [19]. Semi-preparative Ag^+-HPLC [20], and reverse-phase (RP-HPLC) [21] have been used to isolate increased quantities of *t*- and *c*-monoenes, but methods to isolate many BHP specific to ALA are still required. The objective of the present investigation was to develop semi-preparative Ag^+-chromatography methods for the isolation of BHP specific to ALA to allow for their convenient bioactivity testing in cell culture.

2. Materials and methods

2.1. Chemicals

Hexane, acetone, methanol and acetonitrile were HPLC grade, toluene and diethyl ether were ACS grade (EMD, VWR, Mississauga, ON, Canada). Glacial acetic acid was purchased from Sigma Aldrich (Oakville, ON, Canada). Sodium methoxide (0.5 M) in methanol was sourced from Supelco (Bellefonte, PA, USA). Anhydrous sodium sulfate was ACS grade (Fisher Scientific, Ottawa, ON, Canada), and 2′,7′-dichlorofluorescein (90%) was sourced from Sigma–Aldrich (Oakville, Ontario).

2.2. Preparation of fatty acid methyl esters

Perirenal adipose tissue enriched with ALA-BHP was selected from an experiment where cows were fed a diet containing 15% ground flaxseed, a rich source of ALA [22]. Adipose tissue was ground twice through a 5 mm dye (Butcher Boy meat grinder Model TCA22, Lasar MFG Co, Los Angeles, CA, USA) and then rendered in a 60 °C water bath. Fat was freeze dried for 12 h, dissolved in toluene and methylated using 0.5 M sodium methoxide [23]. Water and hexane were then added, followed by shaking and recovery of FAME in the upper hexane layer, which was then dried under $N_{2(g)}$. The extent of methylation was checked by TLC using 90:10 (v/v) hexane:diethyl ether containing 2% acetic acid as the developing solvent (Silica gel G plate, Analtech Inc., Newark, DE, USA), and visualizing under UV light after spraying the plate with 2′,7′-dichlorofluorescein in methanol.

2.3. Fractionation of FAME by Ag^+-SPE

Related groups/families of FAME were fractionated using a modification of the method described by Kramer et al. [17]. Discovery® Ag^+-SPE columns (750 mg/6 ml, Supelco, Bellefonte, PA, USA) were activated with 4 ml actetone, followed by 4 ml hexane. Twenty mg of FAME, as opposed to the 1 mg of FAME applied by Kramer et al. [17], were added to columns in hexane and eluted with combinations of hexane:acetone (H:AO) or hexane:acetonitrile (H:AN) applied at 1.0 p.s.i. using a Cerex System 48 Positive Pressure II Manifold (Canadian Life Science, Peterborough, ON, Canada). Saturated fatty acids (SFA) were eluted with 6 ml of 99:1 H:AO (v/v). From this point forward fractions collected were numbered consecutively. The *t*-monounsaturated fatty acid (MUFA) and *t*,*t*-conjugated linoleic acid (CLA) isomers were collected with 5 × 2 ml elutions (fractions 1–5) of 98:2 H:AO (v/v). The *c*-MUFA and several *c*,*t*-CLA isomers were collected with 6 ml (fraction 6) of 90:10 H:AO (v/v), and the majority of non-conjugated 18:2 BHP were recovered with 5 × 2 ml (fractions 7–11) of 80:20 H:AO (v/v). Residual 18:2 and 18:3 BHP were collected in 6 × 2 ml (fractions 12–17) of 98:2 H:AN (v/v). After the final 98:2 H:AN elution, 20 p.s.i. was applied to remove residual solvent, and columns were reconditioned by adding 4 ml acetone, drying for 15 min using $N_{2(g)}$ at 1.0 p.s.i., then reactivated as initially described. Columns were reused until noticeable reduction in solvent flow or visible changes to packing. Collected FAME were dried under $N_{2(g)}$, suspended in hexane and stored at −20 °C until further analyses/fractionation.

2.4. GC analysis of collected fractions

Fatty acid methyl esters were analysed by GC as described by Kramer et al. [17]. The FAME (1 μl) were injected at 0.25 mg/ml using a 20:1 split onto a CP-Sil88 column (100 m, 25 μm ID, 0.2 μm film thickness) in a CP-3800 gas chromatograph equipped with an 8600-series auto-sampler (Varian Inc., Walnut Creek, CA, USA). Hydrogen was used as the carrier gas under constant pressure (25 p.s.i., initial flow rate of 1.9 ml/min). Injector and flame ionization detector temperatures were held at 250 °C. Analysis of FAME was completed using one temperature program to analyze most FAME, follow by a second analysis using a different temperature program to separate peaks co-eluting during the first analysis. For the first temperature program, the initial column temperature was 45 °C, held for 4 min, increased to 175 °C at 13 °C/min, held for 27 min, increased to 215 °C at 4 °C/min and held for 35 min. For the second temperature program, the initial temperature 45 °C, held for 4 min, increased to 150 °C at 13 °C/min, held for 47 min, increased to 215 °C at 4 °C/min and held for 35 min. Peaks were identified based on retention times of reference standards (GLC-465 &-603, Nu-Chek Prep. Inc., Elysian, MN, USA) and previously published retention times and elution orders [13,17,24,25].

2.5. Individual BHP separation and collection by Ag^+-HPLC

Fractions collected during SPE fractions were applied to a semi-preparative 250 mm × 10 mm Chrompack ChromSpher 5 Lipids Ag^+-ion column attached to a Prostar 230 HPLC, equipped with a Prostar 410 autosampler, a Prostar 335 Photo-Diode-Array detector and a Prostar 701 series fraction collector (Varian Inc., Walnut Creek, CA, US). Aliquotes (5 μl) were injected at a concentration of 60 mg/ml, with the detector set at 205 nm for monoenes and polyenes with methylene interrupted double bonds, and 233 nm for FA with conjugated double bonds. Peak collection was triggered by slope sensitivity. The concentration of acetonitrile in the mobile phase was adjusted for each fraction to optimise resolution. Fractions 2 and 3 from Ag^+-SPE which contained *trans* monoenes were eluted using 0.04% acetonitrile in hexane at a flow rate of 6 ml/min. Fraction 4 was eluted using 0.03% acetonitrile in hexane at a flow rate of 6 ml/min, which improved the resolution of *t*12-18:1 from later eluting peaks (i.e. *t*10-, *t*11-18:1). Due to co-elution of *t*15/*t*14-18:1 when eluting with 0.04 and 0.03% acetonitrile in hexane, these isomers were collected, dried, resuspended in hexane, and re-eluted using 0.015% acetonitrile in hexane at 8 ml/min. Collection of non-conjugated dienes in fractions 8 and 9 as well as partially-conjugated trienes in fractions 15 and 16 were eluted with 0.075% acetonitrile in hexane at a flow rate of 8 ml/min. No solvent gradients were used for Ag^+-HPLC, which enabled direct recycling of solvents and eliminated the need for re-equilibrating of the system between injections.

3. Results and discussion

3.1. Ag^+-SPE fractionation

3.1.1. Collection of t-18:1 fractions

Kramer et al. [17] reported a method for the pre-analytical fractionation of FAME groups/families using 1 mg FAME applied to Ag^+-SPE (750 mg resin), and this was successfully adapted to accommodate 20 mg of FAME (Table 1). The majority of SFA were first eluted with 6 ml of 99:1 H:AO, with a trace eluting in fraction 1 (i.e. the first 2 ml elution with 98:2 H:AO). This was not problematic because SFA are well separated from *t*-18:1 during subsequent