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Effect-directed analysis of cold-pressed hemp, flax and canola seed oils by planar chromatography linked with (bio)assays and mass spectrometry

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ABSTRACT

Cold-pressed hemp, flax and canola seed oils are healthy oils for human consumption as these are rich in polyunsaturated fatty acids and bioactive phytochemicals. However, bioactive information on the food intake side is mainly focused on target analysis. For more comprehensive information with regard to effects, single bioactive compounds present in the seed oil extracts were detected by effect-directed assays, like bioassays or an enzymatic assay, directly linked with chromatography and further characterized by mass spectrometry. This effect-directed analysis is a streamlined method for the analysis of bioactive compounds in the seed oil extracts. All effective compounds with regard to the five assays or bioassays applied were detected in the samples, meaning also bioactive breakdown products caused during oil processing, residues or contaminants, aside the naturally present bioactive phytochemicals. The investigated cold-pressed oils contained compounds that exert antioxidative, antimicrobial, acetylcholinesterase inhibitory and estrogenic activities. This effect-directed analysis can be recommended for bioactivity profiling of food to obtain profound effect-directed information on the food intake side.

1. Introduction

Cold-pressed oils are oils that are extracted from oilseeds, *e.g.*, with a screw press or hydraulic press. Cold-pressing is used to extract oils from plant seed instead of conventional solvent extraction, as organic solvent or external heat is not required in cold-pressing. Hence, the bioactive compounds such as essential fatty acids, phenolics, flavonoids and tocopherols in the cold-pressed oils are not significantly affected by the processing condition. This ensures the high quality of cold-pressed oils, although the extracted oil quantity is lower than the quantity obtained by conventional solvent extraction.

Hemp (*Cannabis sativa*), flax (*Linum usitatissimum*) and canola (*Brassica napus*) seed oils are examples of oils that are extracted by cold-pressing in New Zealand. The cold-pressed seed oils are usually sold in health food stores with premium price as supplements for human health. Cold-pressed oils are important to human nutrition due to their favorable polyunsaturated fatty acid content, notably α-linolenic acid and linoleic acid (Teh & Birch, 2013). The cold-pressed seed oils are not further refined prior to bottling in dark bottles with nitrogen blanketing. Hemp, flax and canola seeds contain approximately 30–40% oils. However, yields are strongly dependent on the processing (*e.g.*, preconditioning, temperature during pressing and pressure involved).

Studies on physicochemical and quality characteristics of cold-pressed seed oils such as hemp, flax, and canola seed oils comprised the fatty acid, tocopherol, and tocotrienol compositions, color, chlorophyll pigments, total phenolic acids, total flavonoids, moisture and volatile matter content, free fatty acids, acid value, unsaponifiable matter, peroxide value, conjugated dienoic acids, *p*-anisidine value, specific extinction, melting and crystallization characteristics (Choo, Birch, & Dufour, 2007; Teh & Birch, 2013). Canola, hemp, and flax seed oils contained as dominant fatty acids oleic, linoleic and linolenic acids, respectively, which led to the different melting and crystallization profiles (Teh & Birch, 2013). Hemp seed oil contained the highest tocopherol, chlorophyll, flavonoid and phenolic acid contents (Teh & Birch, 2013).

Up to now, a detailed research study on the bioactivity of single compounds in the extracts of these cold-pressed seed oils was not available, except for target analysis with regard to tocopherols and

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tocotrienols (Choo et al., 2007; Teh & Birch, 2013). Although there were studies on the effectiveness of flax seed oils in improving human health using animal models, the type of studies was expensive and time-consuming (Williams et al., 2007). Phytochemicals are the bioactive, non-nutrient, and naturally occurring plant compounds that are present in all sorts of plants, which can be categorized into various groups (Lampe & Chang, 2007). The phytochemicals in cold-pressed seed oils that would exert bioactive properties could be beneficial for human health. However, residues or contaminants left in the food chain can be bioactive as well. Thus, further research is of interest using non-target analysis methods.

The most advanced subgroup of planar chromatography is high-performance thin-layer chromatography (HPTLC). HPTLC-bioautography-mass spectrometry was recently demonstrated as a powerful technique for the discovery of estrogen-effective compounds in food (Klingelhöfer & Morlock, 2014; Morlock & Klingelhöfer, 2014) or bioactive compounds in extracts from grape pomace, wine, bilberries and blueberries powders (Cretu & Morlock, 2014; Krüger, Urmann, & Morlock, 2013). The advantages of HPTLC include the separation of crude extracts and short analysis time for simultaneous analysis of several complex samples. There are many bioactive compounds existent in plant oils. In contrast to sum parameter assays, the hyphenation HPTLC-UV/Vis/FLD-effect-directed analysis (EDA)-ESI-MS is able to discover and characterize single compounds that exert particular bioactivities in a short time. Analysis of bioactive phytochemicals from the extracts of hemp, flax and canola seed oils by HPTLC has not attained much attention yet. As it is worth to explore the compounds that exert bioactive properties in these seed oils, HPTLC linked with effect-directed detections was chosen to assign individual effective compounds and to further characterize these by mass spectrometry.

2. Materials and methods

2.1. Reagents and chemicals

Methanol, ethanol, acetonitrile, toluene, ethyl acetate, chloroform, formic acid, *n*-hexane, glacial acetic acid, glycine, sodium hydroxide, 4-methylumbelliferyl-β-D-galactopyranoside (MUG, all gradient grade), natural product reagent (99.9 %) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, ⩾98%) were purchased from Roth (Karlsruhe, Germany). Bidistilled water was produced by a Heraeus Destamat Bi-18E (Thermo Fisher Scientific, Schwerte, Germany). Potassium hydroxide pellets, polyethylenglycol (PEG) 400, 2,2-diphenyl-1-picrylhydrazyl (DPPH·), acetylcholinesterase (AChE) lyophylisate, bovine serum albumin (BSA), TRIS buffer, hydrochloric acid (HCl) 32%, α-naphthyl acetate, Fast Blue Salt B (all of analytical grade), copper sulfate and ammonium formate were manufactured by Fluka Sigma Aldrich (Steinheim, Germany). HPTLC plates silica gel 60 (also with F_{254}), HPTLC plates reversed phase (RP)-18 water-wettable (W) F_{254s} and HPTLC plates silica gel 60 F_{254} MS-grade plates were purchased from Merck (Darmstadt, Germany). *Saccharomyces cerevisiae* BJ3505 (protease-deficient, MAT, PEP4: HIS3, Prb11.6R, HIS3200, lys2-208, trp1101, ura3-52) was received from W. Schwack, University of Hohenheim, Stuttgart, Germany, who obtained it from S. Buchinger, German Federal Institute of Hydrology, Koblenz, Germany. The particular yeast strain was originally generated by McDonnell et al. (1991). Cold-pressed hemp and flax seed oils were obtained by Oil Seed Extractions Limited, Ashburton, New Zealand while cold-pressed canola seed oil was received from New Zealand Vegetable Oil Limited, Canterbury, New Zealand.

2.2. Sample preparation

Sample preparation was performed at the Department of Food Science, University of Otago, Dunedin, New Zealand. The extraction of polyphenols from cold-pressed seed oils was carried out according to Salta, Mylona, Chiou, Boskou, and Andrikopoulo (2007). Briefly, oil samples (1 g each) were extracted three times with methanol (2 mL each). The combined extracts were evaporated to dryness using a rotary evaporator and dissolved in 2 mL acetonitrile prior to washing two times with *n*-hexane (3 mL each) to remove lipid residues. Acetonitrile was evaporated under vacuum (for oversea transport) and the dry residue was dissolved in 1 mL methanol (1 g/mL).

2.3. Sample application

The methanolic extracts (5–30 μL) were sprayed as 8 mm bands with the 25-μL syringe onto the HPTLC plate (dosage speed 150 nL/s) using the Automatic TLC Sampler 4 (ATS 4, CAMAG, Muttenz, Switzerland). The first sample was applied at a distance of 15 mm from the left side and 8 mm from the bottom side, with a track distance of 20 mm. The plate size was cut to smaller pieces using the TLC Plate Cutter (CAMAG).

2.4. Chromatography, derivatization and documentation

The mobile phase development for each oil extract was performed on HPTLC plates silica gel 60 in the Twin Trough Chamber (TTC, 10 × 10 cm, CAMAG) with various mobile phases as discussed. The migration distance was 65 mm, which took mostly about 30 min. The relative air humidity and the room temperature were 40 ± 5% and 23 ± 3 °C, respectively. For some assays, the plates were prewashed by chromatography with methanol, followed by drying (100 °C, 20 min). For the planar yeast estrogen screen (pYES) assay, a mixed solvent of *n*-hexane, toluene and ethyl acetate (8:3:2, V/V/V) was used as mobile phase on the HPTLC plate RP-18 W F_{254s}. The chromatograms were dried by a cold air stream using a hair dryer. Optionally for derivatization, the chromatogram was immersed into a methanolic natural product reagent solution (5 mg/mL), followed by drying and dipping into a 10% methanolic PEG 400 solution using the TLC Immersion Device III (CAMAG, vertical speed 3.5 cm/s, immersion time 0 s). Documentation of the dried chromatograms was performed at UV 254 and 366 nm by TLC Visualizer or DigiStore 2 Documentation System, both equipped with a high-resolution 12 bit CCD camera (CAMAG). Data was processed with winCATS software, version 1.4.7.2018 (CAMAG). Before application of (bio)assays, due to residual acid traces of the mobile phase, the dried chromatogram was neutralized in the TTC with the potassium hydroxide pellets (placed in the opposite trough) for 1 h.

2.5. DPPH· scavenging activity of bioactive compounds in the extracts

The dried chromatogram was dipped into a methanolic DPPH· solution (0.2 mg/mL) using the TLC Immersion Device III (CAMAG, vertical speed 2 cm/s, immersion time 5 s). After immersion, the plate was dried for 90 s in the dark at ambient temperature and heated at 60 °C for 30 s on the TLC Plate Heater (CAMAG) prior to documentation under white light illumination (reflection and transmission mode).

2.6. Antimicrobial Aliivibrio fischeri activity of bioactive compounds in the extracts

The dried and neutralized chromatogram was immersed at an immersion speed of 3.5 cm/s for 2 s into the *A. fischeri* bacteria