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Liquid chromatography–mass spectrometry coupled with multivariate analysis for the characterization and discrimination of extractable and nonextractable polyphenols and glucosinolates from red cabbage and Brussels sprout waste streams

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ABSTRACT

Nonextractable polyphenol (NEP) fractions are usually ignored because conventional extraction methods do not release them from the plant matrix. In this study, we optimized the conditions for sonicated alkaline hydrolysis to the residues left after conventional polyphenol extraction of Brussels sprouts top (80 °C, 4 M NaOH, 30 min) and stalks (60 °C, 4 M NaOH, 30 min), and red cabbage waste streams (80 °C, 4 M NaOH, 45 min) to release and characterize the NEP fraction. The NEP fractions of Brussels sprouts top (4.8 ± 1.2 mg gallic acid equivalents [GAE]/g dry waste) and stalks (3.3 ± 0.2 mg GAE/g dry waste), and red cabbage (11.5 mg GAE/g dry waste) waste have significantly higher total polyphenol contents compared to their respective extractable polyphenol (EP) fractions (1.5 ± 0.0, 2.0 ± 0.0 and 3.7 ± 0.0 mg GAE/g dry waste, respectively). An LC-MS method combined with principal components analysis (PCA) and orthogonal partial least squares–discriminant analysis (OPLS–DA) was used to tentatively identify and discriminate the polyphenol and glucosinolate composition of the EP and NEP fractions. Results revealed that phenolic profiles of the EP and NEP fractions are different and some compounds are only found in either fraction in all of the plant matrices. This suggests the need to account both fractions when analyzing the polyphenol and glucosinolate profiles of plant matrices to attain a global view of their composition. This is the first report on the discrimination of the phenolic and glucosinolate profiles of the EP and NEP fractions using metabolomics techniques.

1. Introduction

Phenolic compounds comprise a diverse group of bioactive compounds found in nature and are widely distributed as secondary metabolites in plants and hence part of the human diet. Currently, the phenolic structure of about 10,000 compounds has been described in literature [1]. Renewed interest in the study of phenolic compounds arose when they were discovered to be powerful antioxidants, followed by numerous studies focusing on their biological and bioactive characteristics [2,3]. So far, most of the studies only refer to extractable phenolics (EP) and do not consider the nonextractable phenolic fraction (NEP) and thus are often overlooked by current literature [4,5]. The NEP fraction comprises of phenolic compounds that are bound or trapped in the plant matrix and consequently remain in the residue after extraction with aqueous–organic solvents.

Increasing food waste has been a growing concern in modern society. Efforts to reduce food waste have been the subject of many academic and non-academic fora. Valorization of agricultural wastes is thus a major step in alleviating this problem. We have previously shown that agricultural wastes also possess high amounts of polyphenols, which could be harnessed for use in food applications, such as functional ingredients, antioxidants, etc. [6,7]. If these bioactive components are recovered from the waste stream, they could be used as additives to food and/or cosmetics to create high-value products. It has earlier been reported that higher amounts of phenolics are found in the NEP fraction of agricultural waste streams compared to their EP fractions [8–10].

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Exploiting this fraction therefore results to better valorization of the waste streams. However, the differences in the phenolic profiles of the extractable and nonextractable fractions have not been deeply studied. In this paper, we investigate the EP and NEP fractions of two different waste streams belonging to the *Brassica* family, Brussels sprouts and red cabbage. Initially, the EP fraction was obtained by conventional solvent extraction and the phenolic composition was characterized. Thereafter, the residue left after solvent extraction was collected and the parameters for NEP extraction were optimized for each waste stream. This is the first report about the EP and NEP characterization of Brussels sprouts and red cabbage waste streams. Also in this study, we show the use ultrahigh-pressure liquid chromatography–mass spectrometry combined with metabolomics-based analysis tools, principal components analysis (PCA) and orthogonal partial least squares–discriminant analysis (OPLS–DA), to discriminate the phenolic profile of the EP from NEP fractions and to determine which compounds cause their distinction. This provides a rapid and convenient analytical method for screening and characterizing EP and NEP without the need for quantification of the individual components or manual integration of each chromatographic peak.

2. Materials and methods

2.1. Reagents and plant material

U(H)PLC–MS grade methanol and formic acid were acquired from Biosolve (Valkenswaard, the Netherlands) whereas analytical grade methanol, HCl and NaOH were purchased from VWR International (Leuven, Belgium).

Red cabbage (*Brassica oleracea* var. *capital* f. *rubra*) and Brussels sprouts (*Brassica oleracea* var. *gemmifera*) waste were harvested in November 2013. For Brussels sprouts, the top leafy part was separated from the stalks and analyzed separately due to their big structural difference, while the sample material of red cabbage consisted only of the external leaves. The plant materials were cut, blended into smaller pieces and immediately stored in a freezer at −20 °C. Approximately 250 g of each plant material were freeze-dried and ground into a fine powder with an IKA-M20 Werke Grinder and stored at −20 °C until further analysis.

2.2. Solvent extraction of EP and collection of residues containing NEP

The solvent extraction protocol was based on the method by Olsen et al. [11]. Initially, 2 g of the freeze-dried plant powder was weighed and placed in 50 mL centrifuge tubes with 15 mL of 100% MeOH and homogenized using an IKA T25 digital Ultraturrax at 10,000 rpm for 45 s. The tubes were then placed on ice for 15 min and centrifuged at 13,000 × *g* for 10 min at 4 °C. The supernatant (1) was collected in a 50 mL volumetric flask while the residue left in the tube was re-extracted with 10 mL 80% MeOH and re-homogenized at 10,000 rpm for 45 s, placed on ice for 15 min and then centrifuged at 13,000 × *g* for 10 min at 4 °C. The supernatant was added to supernatant (1) and the volume was adjusted to 50 mL using 100% MeOH. Subsequently, the residue left in the centrifuge tubes after extraction was dried under reduced pressure and was used to extract NEP.

2.3. Measurement of total phenolic content (TPC)

The total phenolic content (TPC) of the extracts was determined with the colorimetric Folin–Ciocalteau assay previously optimized [7]. Briefly, 1.5 mL cuvettes were filled with 1200 μL distilled water, 50 μL of the plant extract and 100 μL Folin–Ciocalteau phenol reagent (diluted 10 times in distilled water). For making the calibration curves, 50 μL gallic acid was placed instead of the sample with concentrations of 0–250 μg gallic acid mL^{-1}. Thereafter, the cuvettes were incubated in the dark for 5 min and the solution was then mixed with 150 μL of sodium carbonate (Na_2CO_3). Finally, the cuvettes were incubated for 2 h in the dark at room temperature, immediately followed by measuring the resulting chromophores with a Varian Cary 50 Series spectrophotometer at a wavelength of 760 nm. Total phenolic content was then expressed as mg gallic acid equivalents (GAE) per gram dried plant material.

Table 1
Parameters for the optimization of NEP extraction.

NaOH concentration (M)	Temperature (°C)	Sonication time (min)
1	40	15
2	60	30
4	80	45

2.4. Sonicated alkaline hydrolysis of the residue left after solvent extraction

The optimization of the parameters for the sonicated alkaline hydrolysis is summarized in Table 1, comprising of 27 combinations, which were analyzed in triplicates.

Briefly, 0.1 g of the residue was placed in a tube and mixed with 2 mL of NaOH (1, 2 or 4 M). The tubes were flushed with nitrogen for 30 s and sealed to prevent the oxidation of the phenolic compounds. Furthermore, the samples were incubated in a temperature-controlled ultrasonic water bath in an Elmasonic S60H unit with a frequency of 37 kHz and a nominal power of 180 W. The temperatures (40, 60 and 80 °C) and incubation times (15, 30 and 45 min) were varied depending on the set-up as earlier described in Table 1. Due to heating during sonication, the temperature in the water bath was checked every 5 min and adjusted if necessary.

After hydrolysis, the samples were neutralized by adding HCl. The liberated NEP was then extracted using 4 mL of MeOH (0.1% formic acid) followed by vortexing for 2 min. The tubes were centrifuged at 10,000 × *g* for 10 min at 4 °C. Extraction was performed twice and the final volume was adjusted to 20 mL using 100% methanol.

2.5. Solid phase extraction (SPE)

Aliquots (1 mL) of the polyphenol fractions were diluted in 20 mL of 0.1% (vol) formic acid (in ultrapure water) and loaded into a preconditioned C18 solid phase extraction (SPE) cartridge (500 mg per 4 mL) (Davison Discover Science, Deerfield, IL, USA). Columns were preconditioned by loading 2 × 3 mL methanol and 2 × 3 mL water, wherein each solvent was allowed to stand for 2 min prior to use. After loading the sample, the columns were washed with 5 mL of MilliQ water (0.1% (vol) formic acid). The polyphenols were recovered using 3 mL MeOH (0.1% (vol) formic acid). The samples were then dried using light stream of nitrogen and re-dissolved in 1 mL of 10% dimethyl sulfoxide (DMSO) in acidified water prior to LC-MS analysis.

2.6. Identification of compounds using U(H)PLC-ESI-MS

LC-MS analysis was performed with a Waters Acquity UPLC system (Waters Corp., Milford, MA, USA) connected to a Synapt HDMS-TOF-MS (Waters Corp., Milford, MA, USA). Plant extract components were separated using a Waters Acquity BEH C18 column (2.1 mm × 150 mm, 1.7 μm particle size) attached to a Waters VanGuard Pre-column (2.1 mm × 5 mm) during gradient