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Separation of alk(en)ylresorcinols from rye bran with saturated, monoenoic, dienoic, trienoic and hydroxylated monoenoic side chains using an octyl phase in ultra-high performance liquid chromatography and their differentiation by tandem mass spectrometrie

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ABSTRACT

Alkylresorcinols (ARs) occur in bran of cereals and in fruits from the Anacardiaceae family. Their separation by liquid chromatography is challenging, especially in rye (*Secale cereale* L.) that has a complex AR composition. An octyl phase (C8) with 1.8 μm particles was used for the analysis of an acetone extract of rye bran. The ARs were detected by UV at 205 and 275 nm and by MS applying selected ion monitoring (SIM) of known and hypothetical *m/z* values in positive and negative mode. The compounds found were subjected to product ion scans in a triple quadrupole mass spectrometer. The C8 UHPLC column has a suitable selectivity for the analysis of ARs from rye. In combination with the sub–2 μm particles, baseline separation of most ARs was achieved. The MS^2 spectra in positive mode show diagnostic fragments that allow identifying the ARs subclasses (saturated, monoenoic, dienoic, trienoic and hydroxylated monoenoic) unambiguously. Several minor ARs were detected for the first time: $C_{23:3}$, $C_{27:1}OH$, $C_{20:0}$, $C_{22:0}$, $C_{24:0}$ and some minor alkenylresorcinol isomers. The chromatographic resolution on the C8 column is unprecedented in the field of rye ARs. Thus, isolation and quantification using non-mass-selective detectors is now possible for each AR. Since rye bran has the most complex AR composition, this method is expected to facilitate the analysis of ARs also in other samples.

1. Introduction

Alkylresorcinols (ARs) occur in bran of cereals like wheat, rye, barley, and spelt but also in foods like mango, cashew and quinoa and some other organisms such as algae, mosses and fungi [1–3]. The most complex variety of ARs has been found in rye (*Secale cereale* L.) containing saturated ($C_{n:0}$), monoenoic ($C_{n:1}$), dienoic ($C_{n:2}$), trienoic ($C_{n:3}$) and hydroxylated monoenoic ($C_{n:1}OH$) ARs of various chain lengths. In reversed-phase liquid chromatography, quadruples of $C_{n:0}$, $C_{n+2:1}$, $C_{n+4:1}OH$, and $C_{n+4:2}$ ARs with $n = 15–27$ elute closely or co-elute. Unsaturated and hydroxylated ARs have not been observed for every *n* in that range. Typically but not consistently, *n* is an odd number. With increasing *n*, the compounds within the quadruples could not be separated so far [4,5].

Up to now, C18 phases have been used for the analysis of ARs with poor resolution, even when sub–2 μm and solid-core phases were used. For example, Knödler et al. achieved a baseline separation of the quadruple $C_{15:0}$, $C_{17:1}$, $C_{19:1}OH$, and $C:_{19:2}$ on a C18 Aqua column (150 mm × 3.0 mm, 3 μm) from Phenomenex. In the next quadruple, the peaks of $C_{17:0}$ and two isomers of $C_{19:1}$ overlapped; these signals were separated from the overlapping pair $C_{21:1}OH$ and $C_{23:2}$. The situation got worse in all following quadruples [6]. A similar resolution was obtained by Geerkens et al. on a Synergi Hydro-RP C18 column (150 mm × 3.0 mm, 4 μm) also from Phenomenex [5]. A promising method was published by Ziegler et al. using a Phenomenex Kinetex C18 (250 mm × 4.6 mm, 5 μm) column with solid-core particles. Thus, $C_{17:0}$, $C_{19:1}$, $C_{21:1}OH$ and $C_{21:2}$ as well as $C_{19:0}$, $C_{21:1}$, $C_{23:1}OH$, and $C_{23:2}$ from einkorn (*Triticum monococcum*) were baseline separated. Among the higher homologues, only $C_{23:0}$, $C_{25:1}$, $C_{25:0}$, and $C_{27:1}$ were detected in that cereal. These two pairs were fairly separated [7].

Two articles show chromatograms of AR from rye bran run on sub–2 μm columns: In a previous study, we used a Nucleodur

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C18 Pyramid (150 mm × 2.0 mm, 1.8 μm) from Macherey + Nagel [8]. Resolution was not increased compared to the aforementioned methods, but total time for chromatography was only 15 min. Ross tested various columns and selected a Phenomenex Kinetex (150 mm × 2.0 mm, 1.7 μm) with solid-core particles as best for purpose. The peak separation was enhanced compared to the chromatograms of the methods cited above, but starting from C17:0, the saturated and monounsaturated ARs were not baseline separated. The hydroxylated alkenylresorcinols, dienes with $n > 21$ and trienes were not detected [4].

Interestingly, Ross & Kochnar tested an octyl phase (Zorbax C8, 150 mm × 4.6 mm, 3.5 μm; by Agilent) and stated that this column "provided adequate resolution, although with some peak tailing" [9] without giving details. Further analyses in this article were run on a Zorbax Extend C18 (150 mm × 3.0 mm, 3.5 μm) from Agilent with poor resolution [9].

UV and fluorescence detection are most common for the quantitation of ARs, but also coulometric electrode array detection (CEAD) has been applied successfully with low detection limits [4]. Mass spectrometry after chromatography was first used after GC as recapped in two articles by Ross et al. [10,11]. In the last decade, HPLC has become the method of choice for AR analysis. All LC–MS publications in the field of ARs originate from Carle's group [2,5–7], who used an ion-trap MS for structure elucidation. The common main fragments with m/z 111 and 123 of saturated and unsaturated ARs, respectively, were described. A fragmentation pathway of polyunsaturated ARs was proposed.

ARs have been reported to show several bioactivities in vitro (i.e., inhibition of enzymes, antioxidative capacity, inhibition of human colon cancer cell growth), but there is missing evidence for the bioavailability of AR homologues in humans or animals [12]. The activity of these phenolic lipids is correlated to their amphiphilic character and their ability to interact with biological membranes [13]. It is expected that the bioactivity of unsaturated AR homologues might have a wider array than the saturated AR molecules [6].

In this article, a method is presented allowing near baseline separation even of the heaviest homologues of rye ARs on a C8 column with 1.8 μm particles. Such a resolution has not been reached so far. Furthermore, characteristic mass spectra of each homologue series obtained by a triple quadrupole mass spectrometer (TQ-MS) are shown. This allows an identification of the different ARs by MS^2. Several minor ARs were detected in rye for the first time.

2. Material and methods

2.1. Chemicals

MS grade acetonitrile and MS grade water were obtained from Chemsolute (Renningen, Germany). MS grade formic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA). The $C_{15:0}$ alkylresorcinol (1,3-dihydroxy-5-pentadecylbenzene) was provided by ReseaChem (Burgdorf, Switzerland) at a purity of >98%. Oleic and linoneic acid were supplied by Riedel-de Haën/Sigma Aldrich (Seelze, Germany) and Fluka/Sigma-Aldrich (Steinheim, Germany), respectively, at 60% purity.

2.2. Sample extraction

The extraction is described in detail in a previous work [8]. Briefly, in a first extraction step, *n*-hexane was used for defatting of rye bran. Then, the dried bran was extracted twice with acetone in an Ultrasonic Bath (VWR, Darmstadt, Germany). This acetone extract was used for this investigation.

2.3. UHPLC/MS

Instrumentation was the same as described elsewhere [14], i.e., an Acquity UPLC system with a UV detector (PDA) and a triple quadrupole mass spectrometer (TQD) from Waters (Milford, MA, USA) with MassLynx 4.1 software (Waters). A Nucleodur C8 Gravity column (150 mm × 2.1 mm, 1.8 μm) from Macherey + Nagel (Düren, Germany) was used. The mobile phases consisted of water (eluent A) and acetonitrile (eluent B) with and without the addition of formic acid (99.9 + 0.1, v + v) using the following gradient elution: 0 min, 55% B; 15 min, 100% B; 17 min, 100% B; 17.8 min, 55% B; 19.5 min, 55% B. The flow rate was 0.4 mL/min, the column temperature was 40 °C, and the injection volume was 1–5 μL.

The mass spectrometer was tuned using a solution of 1,3-dihydroxy-5-pentadecylbenzene, and the following parameters were applied for positive and negative ionization: capillary voltage 3.0 kV/−2.8 kV, cone voltage −30 V/55 V, extractor voltage −1.0 V/1.0 V, RF voltage −0.1 V/0.1 V, source temperature 150 °C, desolvation temperature 450 °C, cone gas (nitrogen) flow 50 L/h, and desolvation gas (nitrogen) flow 800 L/h. The collision gas (argon) flow used in MS/MS experiments was 0.22 mL/min, and collision energies of 18–35 V was applied. The ARs were detected by selected ion monitoring (SIM) of known and hypothetical m/z values. The compounds found were subjected to product ion scans. The selected reaction monitoring (SRM) methods used the following target fragments: 85 ($C_{n:0}$), 123 ($C_{n:1}$, $C_{n:2}$, $C_{n:1}OH$), 95 ($C_{n:3}$) in positive mode and 81 ($C_{n:0}$, $C_{n:1}$, $C_{n:3}$) and 122 ($C_{n:2}$), 123 ($C_{n:1}OH$) in negative mode.

3. Results and discussion

3.1. Separation

Chromatograms of the rye bran extract are shown in Fig. 1 (chromatographic conditions see subsection 2.3). Baseline separation of the ARs was achieved up to the quadruple $C_{21:0}$, $C_{23:1}$, $C_{25:1}OH$, $C_{25:2}$. The following and last quadruple is still satisfactorily separated as well as the following $C_{25:0}$ and $C_{27:1}$. The latter two compounds might be completed to a quadruple by $C_{29:1}OH$ and $C_{29:2}$, but these two molecules could not be detected. A partial separation of isomeric alkenylresorcinols as shown by Knödler et al. could not be realized on the C8 column [6]. Finally, steryl ferulates were eluted, which can be seen at 325 nm. Their identity was confirmed by the m/z of their $[M-H]^-$ ions in comparison with published data [7,15]. These peaks interfere with the $C_{27:0}$ AR. Some of the minor ARs coelute with major ARs or with each other. Further, $C_{17:1}OH$, and two minor $C_{17:1}$ ARs could not be separated from the fatty acids linoleic, linolenic and oleic acid, respectively. This is of concern when a low detection wavelength, e.g., 205 nm, is chosen; at 275 nm the fatty acids do not absorb. The UV spectra of linoleic acid, oleic acid and $C_{15:0}$ are shown in Fig. S1 (supplement). The identities of the fatty acids were confirmed by comparison of their retention times and mass spectra to those of standard compounds. Free fatty acids are known to occur in rye bran [16].

Obviously, the high peak capacity of sub–2 μm and solid-core columns cannot compensate for the lack of selectivity of C18 for the various ARs from rye. The C8 column used in this study combines selectivity and the separation power of 1.8-μm particles, resulting in unprecedented resolution of these compounds except for some minor alkenylresorcinol isomers.

3.2. UV detection

ARs have UV absorption maxima at 205–210 and 275 nm. Formic acid in the eluents impairs detection especially at lower wave-