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# Comparative plant sphingolipidomic reveals specific lipids in seeds and oil

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ABSTRACT

Plant sphingolipids are a highly diverse family of structural and signal lipids. Owing to their chemical diversity and complexity, a powerful analytical method was required to identify and quantify a large number of individual molecules with a high degree of structural accuracy. By using ultra-performance liquid chromatography with a single elution system coupled to electrospray ionization tandem mass spectrometry (UPLC–ESI-MS/MS) in the positive multiple reaction monitoring (MRM) mode, detailed sphingolipid composition was analyzed in various tissues of two Brassicaceae species *Arabidopsis thaliana* and *Camelina sativa*. A total of 300 molecular species were identified defining nine classes of sphingolipids, including Cers, hCers, Glcs and GIPCs. High-resolution mass spectrometry identified sphingolipids including amino- and N-acylated-GIPCs. The comparative analysis of seedling, seed and oil sphingolipids showed tissue specific distribution suggesting metabolic channeling and compartmentalization



## Introduction

Plant sphingolipids is a complex family of lipids with a large structural diversity (Lynch and Dunn, 2004; Pata et al., 2010; Sperling and Heinz, 2003). The basic building block of sphingolipids is an amino alcohol long-chain base (LCB), which is composed predominantly of 18 carbon atoms. The LCBs are characterized by the presence of an amino group on the second carbon (C2) and at least, two hydroxyl groups on the first (C1) and third carbons (C3) to form sphinganine (d18:0). The LCB moiety is also often hydroxylated at position C4 leading to 4-hydroxysphinganine (t18:0). Finally, LCBs can also be desaturated (Δ4 and/or Δ8) to form for instance, 8-sphingenine (d18:1, Δ8) or 4-hydroxy-8-sphingenine (t18:1, Δ8) (Markham et al., 2006). The LCBs are N-acylated with fatty acids (FAs) to form ceramides (Cers). The fatty acid moieties are diverse in structure with chain lengths ranging from C16 to C26, α-hydroxylation (hCers) and/or desaturation (Markham and Jaworski, 2007). The attachment of a polar head group to the primary hydroxyl group of the LCB moiety of Cers and hCers produced complex sphingolipids such as glucosylceramides (Glcs) and glycosylinositolphosphoceramides (GIPCs) (Blaas and Humpf, 2013; Buré et al., 2011). The various FAs, LCBs, and head groups generate an important structural diversity and complexity of sphingolipids, with at least 168 individual molecular species of sphingolipids reported in Arabidopsis (Markham and Jaworski, 2007). Recently, additional identifications (Blaas and Humpf, 2013; Buré et al., 2011) increased this number in plants to about a thousand molecular species which makes sphingolipids to the most structurally diverse group of lipids.

Plant sphingolipids are bioactive compounds that not only serve as structural components of cellular membranes but are also involved in different physiological functions, such as cell polarity, programmed cell death, guard cell closure and different stress responses (Berkey et al., 2012; Lynch and Dunn, 2004; Markham et al., 2013; Pata et al., 2010; Sperling and Heinz, 2003). In plants, sphingolipids are important for plant development, adaptation to abiotic stress and the response to pathogens (Chao et al., 2011; Chen et al., 2008, 2012; Markham et al., 2011; Saucedo-Garcia et al., 2011; Wang et al., 2008). However, the molecular determinants of sphingolipid functions are still poorly understood. Because of their structural complexity, powerful analytical tools are required to identify a large number of individual sphingolipid molecules with a high degree of structural accuracy. One has to precisely determine the structure of the LCB, the FA as well as the polar head. It has been showed for instance that the FA length or the LCB C4-hydroxylation were essential for plant sphingolipid functions (Chen et al., 2008; Markham et al., 2011). The method of choice for sphingolipid analysis is liquid chromatography coupled to electrospray ionization, tandem mass spectrometry (LC–ESI-MS/MS) that provides the necessary sensitivity, structural specificity and relative high-throughput. It could also be carried

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out for small sample quantities and in complex matrices. This method was previously applied with success to animal (Bielawski et al., 2006; Bui et al., 2012; Masood et al., 2012; Merrill et al., 2005; Shaner et al., 2009) and plant (Bartke et al., 2006; Islam et al., 2012; Markham and Jaworski, 2007) sphingolipids. In plants, the GIPCs class was also specifically analyzed by matrix-assisted laser desorption/ionization (MALDI) MS/MS (Buré et al., 2011) and by LC–Fourier Transform MS (Blaas and Humpf, 2013).

The objective of this work was to develop a simplified ultraperformance liquid chromatography (UPLC) separation coupled to ESI-MS/MS method to quickly obtain a complete profiling of different classes of sphingolipids including Cers, hCers, Glcs and GIPCs in structurally and functionally very different plant tissues. This method was applied to the comparative sphingolipidomic of the three different plant tissues: the actively growing seedlings, the quiescent seeds and its storage fraction, the pressed oil. The presence of sphingolipids in plant oil was never precisely documented before and was a matter of debate. The oil fraction was also of interest to validate our analytical method in presence of a high matrix effect in the ESI-MS/MS mode.

## Results and discussion

### *Multiple reaction monitoring (MRM) methods for sphingolipids*

High-throughput sphingolipidomic approaches require simplified and optimized analytical methods. Plant samples were extracted according to the method described by Markham and Jaworski (2007). Lipid samples were then analyzed by high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry without prior purification except methylamine treatment to improve ionization. The method was simplified by using a mobile phase made of THF/$CH_3CN$/ammonium formate with a single elution gradient for UPLC separation of all the different classes of sphingolipids. The different classes of lipids were separated with a single gradient thanks to the high efficiency of the UPLC separation. UPLC has the advantage of a better chromatographic resolution and brings higher sensitivity in comparison to HPLC.

The different sphingolipid species were identified and quantified by UPLC–ESI-MS/MS using multiple reaction monitoring (MRM) mode, a method in which the eluate was continuously scanned for selected precursor-product ion pairs to enhance the sensitivity and specificity of the analysis. The method has the advantage to be suitable to relatively small samples (about several mg of plant material). The MRM analysis mode was defined by selecting both precursor and characteristic product ions. For the analyses of Cers, hCers and Glcs, the product ions corresponded to the long-chain base moiety as previously reported (Bartke et al., 2006; Merrill et al., 2005). Therefore, the product ions used for the LCBs, t18:1, t18:0, d18:1 and d18:0 were, respectively *m*/*z* 262, 264, 264 and 266. For GIPCs, the product ions corresponded to the ceramide moiety (Markham and Jaworski, 2007). The different classes of sphingolipids (Cers, hCers, Glcs and GIPCs) were first determined on the basis of results previously reported by Markham and Jaworski (2007). The analysis was subsequently carried out over a wide range of theoretical modifications including the fatty acyl-chain length (from 16 to 30 carbons both odd and even numbers), hydroxylation and insaturation. Only compounds present in significant amounts were analyzed thereafter. The results of these MRM screenings are given in Supplementary data (Tables S1–S4).

The major drawback of MRM mode was the presence of isotopic and isobaric sphingolipid species detected for a same MRM transition. Notably, when these molecules are present in high amounts in the sample, their $^{13}C$ isotopic component may overlap with sphingolipids with higher masses (+1 or +2, corresponding to simple or double $^{13}C$ isotope). This problem was observed in particular, for Glcs and GIPCs with t18:0, since the M+2 $^{13}C$ isotope of sphingolipids with t18:1 could also be detected. The presence of isobaric compounds was observed mainly for GIPCs because the product ion used was not very selective since it corresponded to the ceramide moiety and not to the LCB. For instance, a GIPC with three hydroxyl groups would have two groups on the LCB chain and the third group could be either on the same chain or the FA chain and hence would be detected as single species in MRM mode. However, the two molecules could be easily discriminated by their elution time because the trihydroxylated LCB would be more polar than the hydroxylated FA.

### *High-resolution mass determination for sphingolipids*

In some cases, the UPLC–ESI-MS/MS set-up could not discriminate between two sphingolipid species that were associated in the

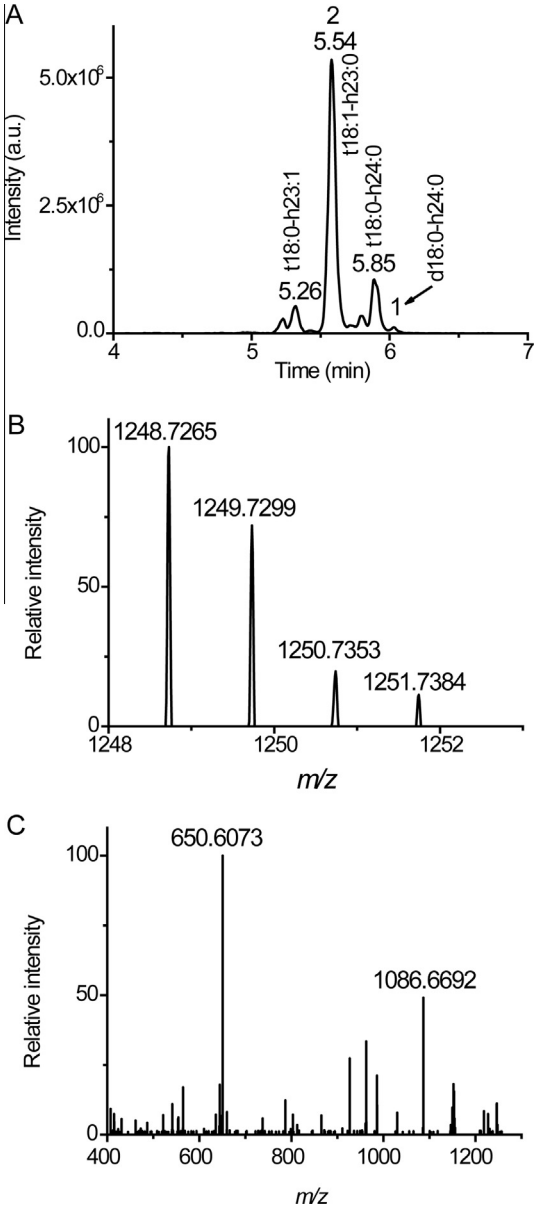


**Fig. 1.** Identification of GIPCs with d18:0 LCB. (A) Chromatogram of MRM transition 1248.7 > 650.6, seedling extract. (B) HRMS spectrum of product **2**, resolution fixed at 60,000. (C) $MS^2$ spectrum of compound **2**.

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