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Analysis of phospholipids in bio-oils and fats by hydrophilic interaction liquid chromatography–tandem mass spectrometry

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ABSTRACT

A new, sensitive and selective liquid chromatography–electrospray ionization–tandem mass spectrometric (LC–ESI-MS/MS) method was developed for the analysis of Phospholipids (PLs) in bio-oils and fats. This analysis employs hydrophilic interaction liquid chromatography–scheduled multiple reaction monitoring (HILIC–sMRM) with a ZIC–cHILIC column. Eight PL class selective internal standards (homologs) were used for the semi-quantification of 14 PL classes for the first time. More than 400 scheduled MRMs were used for the measurement of PLs with a run time of 34 min. The method's performance was evaluated for vegetable oil, animal fat and algae oil. The averaged within-run precision and between-run precision were ≤10% for all of the PL classes that had a direct homologue as an internal standard. The method accuracy was generally within 80–120% for the tested PL analytes in all three sample matrices.

1. Introduction

A variety of oils and fats are typical raw materials for the production of renewable diesel fuels. The raw materials for Neste Corporation's renewable NEXBTL diesel include vegetable oils, such as rapeseed oil (RSO), soya bean oil (SBO), technical corn oil, and palm oil; wastes; residues; and side streams (second-generation biofuel raw materials), such as Animal Fats (AF), Used Cooking Oil (UCO) and Palm Fatty Acid Distillate (PFAD), as well as third-generation biofuel raw materials, such as algae oil. In the NEXBTL diesel manufacturing process, oils and fats are catalytically hydrogenated. The final product is mostly composed of alkanes that originate from fatty acyls of different glycerolipids, mainly mono-, di- and triacylglycerides and free fatty acids. In addition to these lipids, oils and fats contain varying amounts of other lipids, such as phospholipids (PL), which contain phosphorus in their phosphate group.

Catalysts are required in the hydrodeoxygenation (HDO) and isomerization steps of the NEXBTL process. Phosphorus is a catalyst poison in the NEXBTL process; therefore, PLs must be removed from the feedstock using different pretreatment processes, such as degumming and bleaching. The suitability of the feedstock for the NEXBTL process and the efficiency of the pretreatment processes can be evaluated by determining the amount of PLs in each PL class. Information about the exact PL fatty acyl composition is typically not required.

PLs in oils and fats have traditionally been measured using high-performance liquid chromatography (HPLC) with ultraviolet (UV) [1] or evaporative light scattering (ELSD) [2,3] detection employing normal phase (NP) chromatography [4]. Sample preparation is typically performed by solid phase extraction (SPE) [5–7]. These methods are excellent for high concentrations and for the specific analytical problems for which they were created [8]. However, these methods have limited selectivity and sensitivity. In addition, it is difficult to use homologues and stable-isotope-labelled compounds as internal standards in these methods because of the risk of co-elution with the analytes.

Liquid chromatography tandem mass spectrometry (LC–MS/MS) and direct-infusion tandem mass spectrometry (MS/MS), the so-called shotgun lipidomics approach, offers more general solutions for PL analytics [9–11]. Because PLs are polar and non-volatile, electrospray ionization (ESI) is normally employed in conjunction with MS. Quantitative ESI-MS has been well reviewed by Yang and Han [12]. In practice, ESI requires internal standards for quantitative analysis due to its susceptibility to matrix effects and temporal signal variation. Bio-oils can typically contain tens to hundreds of PL species, and their identity varies between sample types. It is therefore very challenging to have separate calibration curves and internal standards for each PL species. However, individual lipid species within a PL class can possess similar response factors in the low concentration range that is covered by modern ESI-MS/MS instruments for a limited (typical) range of acyl chain lengths and

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number of double bonds [9,12–15]. Therefore, signal normalization with lipid class selective internal standards (IS), lipid homologues or stable isotope labelled homologues, can lead to quantitative results if the experimental conditions are carefully selected.

The advantage of shotgun lipidomics is that all of the lipid species within a lipid class are simultaneously introduced into the ESI source and both the analytes and IS experience the same ionization conditions. On the other hand, almost the same conditions can be reached in LC–MS/MS if normal phase (NP) or hydrophilic interaction liquid chromatography (HILIC) is used. Furthermore, LC separation provides additional selectivity for the analysis that can be beneficial if the sample is complex. Due to the MS/MS overlap, certain PL species of phosphatidylcholines (PC) and sphingomyelins (SM) may not be distinguished from each other without LC [13]. Of the two HPLC modes, HILIC mobile phases tend to be more compatible with ESI than are NP mobile phases [16]. In the NP and HILIC modes, the PLs are retained on the HPLC column from their polar head groups; thus, the PLs of the same PL class, including the IS, elute out of the column at approximately the same time. Therefore, it can be expected that the analytes and the lipid class selective IS experience similar, if not identical, ionization conditions. The ionization conditions can be further improved (matrix effects reduced) by carefully purifying the sample prior to LC–MS/MS. LC–MS/MS may yield better sensitivity and detection of minor components than the shotgun approach due to the sequential ionization of the analytes and may reduce the matrix background and lead to a more accurate determination of the noise and ionic background [8,9,12].

Several groups have used HILIC–ESI-MS or HILIC–ESI-MS/MS for PL analysis. Schwalbe-Herrmann et al. used a silica-column-based HILIC method for the separation of five PL classes [17]. Desoubzdanne et al. used a silica column, quadrupole linear ion trap in Multiple Reaction Monitoring (MRM) mode and a PL homologue as an internal standard for the quantification of phosphatidylcholines from cell samples [18]. There are at least three published methods in which zwitterionic HILIC column Sequant ZIC–HILIC [19–21] is used. In this work, we used a Sequant ZIC–cHILIC column. ZIC–cHILIC (where c stands for choline) is interesting for PL separation because it contains a phosphorylcholine functional group that is attached to the phosphate group into the stationary phase. The phosphorylcholine group is the polar head group of PC and SM. In this orientation, the positive charge (choline) is more accessible to the analytes than is the negative charge (phosphate), which should be beneficial for the retention of PLs via ionic interaction because all of the PLs possess a negatively charged phosphate group. Two of the methods that apply ZIC–HILIC were for the qualitative profiling of six to eleven PL classes [19,20], and in the third method [21], the semi-quantitation of six PL classes was performed using a triple quadrupole without internal standards.

Examples of the methods in which NP chromatography were applied to PL analytics include the works of Boukhchina et al. [22] and Harrabi et al. [23], where the PL concentrations in vegetable oils and oil seeds were measured by negative mode LC–ESI-MS/MS. In these studies, the concentrations were estimated without using internal standards. Boselli et al. used a 3D ion trap and ELSD in parallel for pork meat analysis [24]. This approach allowed for the quantification of both of the PL classes and the lipid species (fatty acid composition) within a PL class. Ivanova et al. described a negative mode LC–ESI-MS linear ion trap method for the analysis of PLs in cell cultures and tissues [25]. These authors applied ion trap scans and PL class selective internal standards for the quantification of PLs.

In this work, we present for the first time an HILIC–sMRM method that applies a ZIC–cHILIC HPLC column and eight PL class selective internal standards (homologs) for the semi-quantification of 14 PL classes. The suitability of the method for the intended purpose, the analysis of PLs in bio-oils and fats, was evaluated by studying the method linearity, precision and accuracy. All of the phospholipid concentrations that are reported in this work are expressed in milligrams of phosphorus per kilogram because the method was especially developed for the speciation of phosphorus.

2. Experimental

2.1. Materials

Acetonitrile, 2-propanol, methanol (all LC–MS Chromasolv), ammonium formate and formic acid (both eluent additives for LC–MS) were purchased from Fluka (Steinheim, Germany); diethylether, the 25% ammonia water solution, and anhydrous citric acid (all 'Baker analysed') were purchased from J.T. Baker (Deventer, The Netherlands). *n*-Hexane (HiPerSolv Chromanorm) was purchased from VWR.

Chloroform with ethanol as a stabilizer was purchased from VWR chemicals (Fontenay-Sous-Bois, France). LC–MS-grade water was produced by the Millipore Integral 10 Milli-Q system equipped with Millipore LC-Pak polisher.

PL analyte standards and IS were purchased from Avanti polar lipids (Alabaster, AL). See Table 1 for the definition of PL abbreviations. Individual PL species are annotated according to their fatty acid composition [26], e.g., palmitoyl oleoyl glycerophosphocholine is annotated as PC(16:0/18:1) or PC 34:1. The latter is used when the exact composition of the fatty acyls is not or does not need to be known, which is the case when the MS/MS is operated in such a way that the exact fatty acyl composition is not revealed. The PLs that were used as analytes (one per lipid class) were PA(17:0/17:0) sodium salt, PC(18:0/18:0), PE(17:0/17:0), PG(18:0/18:0) sodium salt, PI(17:0/20:4) ammonium salt, PS(16:0/16:0) sodium salt, SM(d18:1/17:0), LPA(17:0) sodium salt, LPC(18:0), LPE(18:0), LPG(18:0) sodium salt, and LPS(18:1) sodium salt. All of the analyte materials, except for PI(17:0/20:4), were purchased as solid powders, while the PI analyte and internal standards were obtained as methanol solutions in 1-mL ampoules. The internal standards that were included in the partial method validation of PC, PE, PG and PI had fatty acyl compositions of 25:0 (12:0/13:0) and 43:6 (21:0/22:6). Composition 25:0 (12:0/13:0) was used for PS, 31:1 (17:0/14:1) for PA, 13:0 and 17:1 for LPA and 13:0 for LPC.

2.2. Solution preparation

The individual calibration stock solutions and spiking stock solutions containing 0.15–0.30 mg P/mL of the PL analyte standards, except for PI, were prepared in chloroform–methanol–water (30:19:3, v/v/v) by weighing approx. 15 mg of the solid compound into a 4-mL deactivated glass vial with PTFE/Silicone septum (Waters 186000838DV), adding 4 mL of the solution (weight recorded and considered in the calculations) and dissolving by vortexing.

The analyte standard working solution was prepared from individual calibration stock solutions at the 0.3–4.5 mg P/L level by pipetting 40–500 μL of individual stock solutions (depending on the measurement sensitivity) to a 20-mL volumetric flask. The flask was filled with chloroform–methanol (85:15, v/v). This solution did not contain PI because of the low concentration of the standard solution in the ampoule (Section 2.1), but it was added directly to the highest-calibration level. The intermediate dilution of the spiking solution (accuracy study) was prepared the same way as the analyte standard working solution, but the concentrations of the analytes in the solution were at the 1–18 mg P/L level (by pipetting 80–1000 μL of individual stock solutions), and the solution was poured into a 10-mL volumetric flask. The spiking working solution was prepared from intermediate dilution by pipetting 800 μL of