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Analysis of Phospho- and Sphingolipids in Dairy Products by a New HPLC Method

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ABSTRACT

Dairy phospho- and sphingolipids are gaining interest due to their nutritional and technological properties. A new HPLC method, using an evaporative laser light-scattering detector, was developed, which enabled excellent separation of glucosylceramide, lactosylceramide, phosphatidic acid, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, phosphatidylcholine, sphingomyelin, and lysophosphatidylcholine in less than 21 min, including the regeneration of the column. No loss of column performance was observed after 1500 runs because an acid buffer was used. The output signal of the evaporative laser light scattering detector was highly dependent of the flow of the carrier gas and the temperature of the nebulizer, and was maximized by means of a response surface experimental design. Finally, raw milk, cream, butter, buttermilk, Cheddar whey, quarg, and Cheddar cheese were analyzed for their polar lipid content. The absolute values varied substantially (0.018 to 0.181 g/100 g of product). Significant differences were found in the relative content of each polar lipid class among the analyzed products.
(**Key words:** evaporative laser light scattering detection, high-performance liquid chromatography, phospholipid, sphingolipid)

Abbreviation key: ELLSD = evaporative laser light-scattering detector, **GLUCER** = glucosylceramide, **LACCER** = lactosylceramide, **LPC** = lysophosphatidylcholine, **PA** = phosphatidic acid, **PC** = phosphatidylcholine, **PE** = phosphatidylethanolamine, **PI** = phosphatidylinositol, **PL** = polar lipids, **PS** = phosphatidylserine, **SM** = sphingomyelin.

INTRODUCTION

Phospho- and sphingolipids are amphiphilic molecules with lipophilic acyl chains and a hydrophilic head. The phospholipids contain a phosphate residue onto which different organic groups may be linked. Sphingolipids can contain a similar organophosphate group or a mono- or disaccharide (glycosphingolipids). In dairy products, important phospholipids are phosphatidylethanolamine (**PE**), phosphatidylinositol (**PI**), phosphatidylserine (**PS**), and phosphatidylcholine (**PC**). Important sphingolipids in dairy products are sphingomyelin (**SM**), glucosylceramide (**GLUCER**), and lactosylceramide (**LACCER**). Lysophosphatidylcholine (**LPC**) and phosphatidic acid (**PA**) are rarely reported in dairy products; when they do appear, it is likely due to careless sample preparation or to phospholipase activity (Christie et al., 1987). In recent years, phospho- and sphingolipids have received renewed interest because of the positive biological activities they exert in the human body. In particular, their ability to reduce blood cholesterol levels (Eckhardt et al., 2002) and to enhance brain functioning (Pepeu et al., 1996), their antioxidative properties (Saito and Ishihara, 1997), their bacteriostatic properties (Sprong et al., 2002), and the inhibitory effect of sphingolipids on colon cancer have been studied intensively (Vesper et al., 1999). Moreover, polar lipids (**PL**) are extensively used for their functionality and emulsifying qualities in several food systems (Gunstone, 2001; Schneider, 2001).

Dairy products are a good source of these PL (Vesper et al., 1999). The biological membrane of native milk fat globules consists of about one-third phospho- and sphingolipids, stabilizing the milk fat globules in the serum phase of the milk. Analysis of these lipids can be accomplished by means of ^{31}P-nuclear magnetic resonance, HPLC, TLC, Fourier transform infrared, and by measuring total phosphorous content (Vanhoutte et al., 2004).

Over the course of the past few decades, HPLC has become the preferred method for the determination of PL, as quantitative and qualitative analysis can readily be obtained at a relatively low cost compared with ^{31}P-nuclear magnetic resonance.

Critical points in the analysis of PL in food products are the method of extraction, separation, and detection. Often, little attention is given to the first of these. The majority of PL in food products are present in membra-

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nous structures, interacting with compounds of a complex food matrix, making them difficult to extract. Standard methods of extraction like Röse–Gottlieb and Werner–Schmidt are used extensively for dairy products. Because these methods use a base or acid in combination with heat, they can lead to oxidation and hydrolysis of PL. Therefore cold-extraction procedures like those of Folch et al. (1957) and Bligh and Dyer (1959) using chloroform-methanol or like Hara and Radin (1978), using hexane-isopropanol should be used. After extraction, often a tedious purification or fractionation step using solid phase extraction cartridges or column chromatography is applied. However, the combination of an inadequate extraction procedure and purification step can result in low and variable recoveries and thus in unreliable and inaccurate results.

Several HPLC methods are described for the separation of lecithin and derivatives (Mounts et al., 1992; Abidi et al., 1996; Carelli et al., 1997; Bonekamp and Fiebig, 1999). However, these methods are less applicable to dairy products. Phosphatidylserine, which is only present in trace amounts in lecithin fractions, is often poorly separated from other PL. Moreover, these methods do not consider the presence of SM and cerebrosides.

Most of the recent chromatographic methods used for the separation of PL in dairy products are based on the method of Becart (1990), using a gradient mixture of chloroform, methanol, and a buffer at high pH (>7) with an alkali modifier like triethylamine or ammonium hydroxide on a plain silica column (Becart et al., 1990; Vaghela and Kilara, 1995; Caboni et al., 1996). The modifier is used to enhance peak shape and resolution. Although enabling a fair separation of most of the PL, the high pH quickly dissolves the silica packing, thereby seriously reducing column life.

For the chromatographic analysis of fats and oils, the use of evaporative light scattering detection is generally preferred. In this type of detector, the elution solvent from the column is nebulized by the aid of a pressurized gas (compressed air, helium, or nitrogen) in a heating tube. The analyte is not evaporated and passes as an aerosol through a beam of conventional or laser light, which is reflected and refracted. The scattered light is detected by a photomultiplier or a photodiode, which is placed at a fixed angle and is directly related with the quantity of the analyte and the droplet size. The evaporative light-scattering detector is a universal detector that responds to any analyte that is less volatile than the mobile phase. It has a low background signal, is compatible with a broad range of solvents, it allows gradient elution (unlike the refractive index detector), and the signal is independent of the degree of saturation and chain length of an acyl chain (unlike the UV detector). However, the droplet size (and thus the response) is highly dependent on the flow of the nebulizing gas, the temperature of the evaporating tube and the flow rate, and on the composition and physical characteristics of the mobile phase. Therefore, the working conditions should be optimized to ensure the highest possible detector sensitivity and should be reproduced rigorously each time. Otherwise, a recalibration is indispensable when working quantitatively. The mobile phase should be of the highest quality, as nonvolatile impurities would result in an increased background signal, and could alter analyte droplet formation and consequently detector sensitivity. In the last decade, detectors equipped with a laser as a light source came commercially available. These evaporative laser light scattering detectors (**ELLSD**) outperform other evaporative light-scattering detector models in sensitivity, stability, and reproducibility over longer periods of analysis. After all, compared with conventional light sources, a laser light source is characterized by higher intensity, increased life span, no decline in light intensity over longer periods, minimal equilibration time, and minimal variation between source to source (Onken and Berger, 1998; Koropchak et al., 2000).

This study was performed to develop an HPLC-ELLSD method, enabling a quick separation of the most abundant PL classes present in dairy products, by means of an acid modifier, without the need of a purification/fractionation step.

MATERIALS AND METHODS

Materials

Acid whey and Cheddar whey were obtained from a local dairy plant (Büllinger Butterei, Büllingen, Belgium and Belgomilk, Langemark, Belgium, respectively). Raw milk was obtained from a local farmer. Skimmed milk, buttermilk, Gouda cheese, quarg, butter, and cream samples were obtained from the local supermarket. All samples were stored at <4°C prior to analysis.

Chloroform, hexane, isopropanol and methanol used for extraction were of 99+ grade and obtained from Chem-Lab NV (Zedelgem, Belgium).

Chloroform, methanol, and formic acid used as mobile phase were of HPLC grade and obtained from Acros Organics (Geel, Belgium). Triethylamine (HPLC grade) and PL standards of GLUCER, LACCER, PA, PE, PI, PS, PC, SM, and LPC were obtained from Sigma-Aldrich NV (Bornem, Belgium).

Chromatographic Analysis

Polar lipid separations were performed on a Thermo Finnigan Surveyor HPLC system with 4 solvent lines,