Journal of Chromatography A, xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Characterization of E 471 food emulsifiers by high-performance thin-layer chromatography–fluorescence detection

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ARTICLE INFO

Article history:
Received 2 April 2018
Received in revised form 3 May 2018
Accepted 6 May 2018
Available online xxx

Keywords:
Monoacylglycerols
Diacylglycerols
Triacylglycerols
Free fatty acids
Food emulsifiers
High-performance thin-layer chromatography–fluorescence detection (HPTLC–FLD)

ABSTRACT

Mono- and diacylglycerol (MAG and DAG) emulsifiers, also known as food additive E 471, are widely used to adjust techno-functional properties in various foods. Besides MAGs and DAGs, E 471 emulsifiers additionally comprise different amounts of triacylglycerols (TAGs) and free fatty acids (FFAs). MAGs, DAGs, TAGs and FFAs are generally determined by high-performance liquid chromatography (HPLC) or gas chromatography (GC) coupled to mass selective detection, analyzing the individual representatives of the lipid classes. In this work we present a rapid and sensitive method for the determination of MAGs, DAGs, TAGs and FFAs in E 471 emulsifiers by high-performance thin-layer chromatography with fluorescence detection (HPTLC–FLD), including a response factor system for quantitation. Samples were simply dissolved and diluted with *t*-butyl methyl ether before a two-fold development was performed on primuline pre-impregnated LiChrospher silica gel plates with diethyl ether and *n*-pentane/*n*-hexane/diethyl ether (52:20:28, *v*/*v*/*v*) as the mobile phases to 18 and 75 mm, respectively. For quantitation, the plate was scanned in the fluorescence mode at UV 366/>400 nm, when the cumulative signal for each lipid class was used. Calibration was done with 1,2-distearin and amounts of lipid classes were calculated with response factors and expressed as monostearin, distearin, tristearin and stearic acid. Limits of detection and quantitation were 1 and 4 ng/zone, respectively, for 1,2-distearin. Thus, the HPTLC–FLD approach represents a simple, rapid and convenient screening alternative to HPLC and GC analysis of the individual compounds. Visual detection additionally enables an easy characterization and the direct comparison of emulsifiers through the lipid class pattern, when utilized as a fingerprint.

1. Introduction

Mono- and diacylglycerols (MAGs and DAGs) are known for their surface-active properties and thus frequently used as food emulsifiers to adjust techno-functional properties such as viscosity, creaming and foaming stability, mainly during the production of bread, pastry, margarines, ice cream and other milk products [1]. According to Regulation (EC) No 1333/2008 [2], emulsifiers of the type E 471 (MAG and DAG emulsifiers) as well as derivatives of MAGs and DAGs (E 472a–E 472f) with acetic acid (Acetem), lactic acid (Lactem), citric acid (Citrem) and tartaric acid (Datem) are approved as additives for food stuffs.

MAG and DAG emulsifiers are generally produced by transesterification of triacylglycerols (TAGs), exemplarily obtained from hydrogenated palm oil, with glycerol or by direct esterification of glycerol with fatty acids [1], when the latter technique is used if a specific fatty acid composition is aimed. By-products of the transesterification are unprocessed glycerol, TAGs and free fatty acids (FFAs). The relative composition of the products in the product-mixture is hardly controllable *via* the raw material and stoichiometric ratio of the reactants [1], resulting in varying contents of MAGs between 10 and 60%. MAGs can be enriched (>95%) by a molecular distillation under vacuum at temperatures of 140–170 °C. Nevertheless, emulsifiers of the type E 471 are generally not defined single substances but mixtures of MAGs, DAGs, TAGs and FFAs with diverse and strongly varying composition, additionally containing by-products such as glycerol and inorganic components used for neutralization. Commission Regulation (EU) No 231/2012 [3] defines E 471 emulsifiers as mixtures of mono-, di- and triesters of fatty acids of edible oils with glycerol including low amounts of FFAs, when the amount of mono- and diesters has to be at least 70%.

The composition of the emulsifier directly defines the techno-functional properties of the product, when deviations in the relative composition and the dosage of the emulsifier distinctly influence the product structure, especially the viscosity properties. There-

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https://doi.org/10.1016/j.chroma.2018.05.010
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Please cite this article in press as: C. Oellig, et al., Characterization of E 471 food emulsifiers by high-performance thin-layer chromatography–fluorescence detection, J. Chromatogr. A (2018), https://doi.org/10.1016/j.chroma.2018.05.010

fore, a constant composition of the applied emulsifier is essential to guarantee the long life stability and high product quality. Thus, reliable and simple methods are required to control the composition of technical emulsifiers.

Several methods are available for the determination of MAGs and DAGs, when the main focus is on the analysis of natural lipids ("lipidomics"), such as animal and vegetable fats, blood and membrane lipids [4–6], or biodiesel [7,8]. Most commonly, gas chromatography (GC) or high-performance liquid chromatography (HPLC) are used to separate the compounds, and detection is mainly performed by mass spectrometry to gain high selectivity and sensitivity. In addition, one- or two-dimensional thin-layer chromatography (TLC) is frequently mentioned to separate and quantitate natural lipid classes like phospholipids, cholesterol(esters) and glycerides [9–14], or to analyze MAGs and DAGs in biodiesel [7,15]. TLC methods for the analysis of food emulsifiers, however, are not available. For quantitation, all published methods suggest to separate the individual representatives of the lipid classes and quantitate them individually, which includes time-consuming and costly calibration with numerous standards.

Thus, the aim of the present study was to develop a sensitive, selective and automated planar chromatographic method for the analysis of MAGs, DAGs, TAGs and FFAs in E 471 emulsifiers with a straightforward determination strategy. With the numerous separation possibilities in high-performance thin-layer chromatography (HPTLC), the efficient and group-selective separation of the lipid classes should be possible. Derivatization with a fluorescence marker will offer sensitive analyte detection. For quantitation, an easy strategy based on only a single calibration standard should be established to detect and cumulatively quantitate the individual lipid classes. With the possibility of visual detection, HPTLC will offer an immediate overview of the emulsifier composition and the fast and smart characterization of emulsifiers and their direct comparison in one run on a single plate.

2. Material and methods

2.1. Chemicals and materials

1-Lauryl-*rac*-glycerol (>99%), 1-myristyl-*rac*-glycerol (>99%), 1-palmitoyl-*rac*-glycerol (>99%), 1-stearoyl-*rac*-glycerol (>99%), 1-oleoyl-*rac*-glycerol (>99%), 1,2-dipalmitoyl-*rac*-glycerol (>99%), 1,2-distearoyl-*rac*-glycerol (>99%), 1,3-distearoylglycerol (>99%), lauric acid, myristic acid, palmitic acid, stearic acid, oleic acid (all analytical standard grade, >99.5%), glyceryl trilaurate (>99%), glyceryl trimyristate (>99%), glyceryl tripalmitate (>99%), glyceryl tristearate (>99%) and glyceryl trioleate (>99%) were obtained from Sigma-Aldrich (Steinheim, Germany). 1,2-Dilauroyl-*rac*-glycerol (99%), 1,3-dilauroylglycerol (99%), 1,2-dimyristoyl-*rac*-glycerol (99%), 1,3-dimyristoylglycerol (99%), 1,3-dipalmitoylglycerol (99%), 1,2-dioleoyl-*rac*-glycerol (98%), and 1,3-dioleoylglycerol (>99%) were purchased from Larodan (Malmö, Sweden). Methanol (LC–MS, Chromasolv), diethyl ether (≥99.5%, GC, puriss.), *n*-pentane (≥99% for residue analysis, Chromasolv) and *t*-butyl methyl ether (TBME) (≥99.8%, HPLC, Chromasolv) were obtained from Sigma-Aldrich. *n*-Hexane (95%, for pesticide residue analysis, Chemsolute) was purchased from Th. Geyer (Renningen, Germany). Formic acid (>98%, analytical reagent grade) was obtained from Fisher Scientific (Schwerte, Germany). Primuline (dye content 50%) and coralyne chloride hydrate were from Sigma-Aldrich. Berberine chloride (~95%) was purchased from Fluka-Sigma-Aldrich (Steinheim, Germany). Ultrapure water (>18 MΩ cm) was supplied by a Synergy System (Millipore, Schwalbach, Germany). HPTLC silica gel LiChrospher F_{254}s plates from Merck (Darmstadt, Germany) were used without pre-washing. Emulsifiers of the type E 471 were provided from different producers.

2.2. Standard solutions

For the preparation of standard-mix stock solutions, 2.5 mg of mono-, di-, triacylglycerols and free fatty acids (MAGs, DAGs, TAGs and FFAs) with equal carbon chain lengths were dissolved in 10 mL of TBME (250 mg/L). The stock solutions were stored at 4 °C. For HPTLC–FLD method development, the determination of response factors and visual comparison of emulsifiers, the standard-mix stock solutions were diluted 1:10 with TBME, resulting in a concentration of 25 ng/μL for MAGs, DAGs, TAGs and FFAs in the standard-mix working solutions. For the determination of limit of detection and quantitation (LOD/LOQ) and the analysis of emulsifiers of the type E 471, a calibration standard stock solution of 1,2-distearin (250 mg/L) was prepared. As working standard for LOD/LOQ studies, this standard stock was diluted 1:166 with TBME, resulting in a concentration of 1.5 ng/μL. For the analysis of emulsifiers, the calibration standard solution (25 ng/μL) was achieved by dilution of the standard stock 1:10 with TBME.

2.3. Sample preparation

Different emulsifiers of the type E 471 (distilled MAG emulsifiers and mixtures of MAGs and DAGs (MAG/DAG emulsifiers)) were used as samples. Emulsifiers (12.5 mg) were dissolved in 10 mL of TBME in an ultrasonic bath for 2 min (1.25 mg/mL). For HPTLC–FLD method development and the visual comparison of emulsifiers, the emulsifier stock was diluted to 40 and 100 ng/μL with TBME for the distilled MAG and the MAG/DAG emulsifiers, respectively. For quantitation of the emulsifiers, the emulsifier stocks were diluted 1:40 with TBME for the distilled MAG emulsifiers (31.25 ng/μL), and 1:5 and 1:25 for the MAG/DAG emulsifiers (250 and 50 ng/μL).

2.4. High-performance thin-layer chromatography–fluorescence detection (HPTLC–FLD)

Before application, HPTLC plates were dipped into a solution of primuline (250 mg/L in methanol including 0.1% formic acid) with the TLC Chromatogram Immersion Device III (CAMAG, Muttenz, Switzerland), immersion speed 1, immersion time 20 s, immersion depth 90 mm), dried in a fume hood for 5 min, followed at 50 °C for 20 h. Pre-impregnated plates were stored in a SICCO Star-Vitrum desiccator (Bohlender, Grünsfeld, Germany) until use to prevent contamination. An Automatic TLC Sampler 4 (ATS 4, CAMAG) was used to apply samples and standards as 6-mm bands with the following settings leading to 22 tracks on a 20 cm × 10 cm plate: 8 mm distance from the lower edge, 15 mm distance from the left edge, and 8 mm track distance. Application parameters were: 16 μL/s filling speed, 200 nL predosage volume, 200 nL retraction volume, 250 nL/s dosage speed, 7 s rinsing vacuum time and 1 s filling vacuum time. TBME was used as the rinsing solvent with 2 rinsing cycles and 1 filling cycle. The application volume for LOD/LOQ determinations was 1–15 μL of the individual working standard solution, resulting in 1.5–22.5 ng/zone for 1,2-distearin. Sample application volumes for the determination of response factors were 5 and 20 μL of the standard-mix working solutions. After the application, a drying step for 10 min in a fume hood followed. HPTLC plates were first developed in a 20 cm × 10 cm twin-trough chamber (CAMAG) with diethyl ether to a migration distance of 18 mm. After drying the plate in a chamber over phosphorous pentoxide for 20 min, a second development was performed in the Automatic Developing Chamber (ADC2, CAMAG) equipped with a 20 cm × 10 cm twin-trough chamber (CAMAG). Before development, the plate activity was controlled to 33% relative humidity by satu-

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