Identification and Quantification of Aminophospholipid-Linked Maillard Compounds in Model Systems and Egg Yolk Products

Carmen M. Utzmann and Markus O. Lederer*

Institut für Lebensmittelchemie (170), Universität Hohenheim, Garbenstr. 28, D-70593 Stuttgart, Germany

While the Maillard reaction of free amino acids and proteins is a well-established process, no defined structures from the nonenzymatic browning of aminophospholipids in foodstuffs have been described so far. Phosphatidylethanolamine (PE)-linked glucosylamines (Schiff-PE), Amadori products (Amadori-PE), 5-hydroxymethylpyrrole-2-carbaldehydes (Pyrrole-PE), and carboxymethyl (CM-PE) as well as carboxyethyl (CE-PE) derivatives were detected and quantified by liquid chromatography−electrospray mass spectrometry (LC−(ESI)MS). Model incubations of soy-PE and D-glucose were employed to firmly establish the LC−(ESI)MS procedure. Analyses of spray-dried egg yolk powders and lecithin products derived therefrom show one-fourth of the native D-glucose content of egg yolk to be transformed to Amadori-PE, corresponding to a PE derivatization quota of 11−15.5 mol %. Schiff-PE and Pyrrole-PE were present only in low amounts, no CM-PE and CE-PE could be identified in any of the investigated samples. The high glycation rate of egg yolk PE will influence the emulsifying properties and perhaps even the oxidation resistance of the respective products.

Keywords: *Maillard reaction; phosphatidylethanolamine; lecithin; egg yolk; Amadori compound; advanced glycation end products (AGEs)*

INTRODUCTION

The Maillard reaction is initiated by the nonenzymatic condensation of glucose with amino groups under formation of glucosylamines, which as a rule rearrange to the more stable aminoketoses (Amadori products) (Ledl and Schleicher, 1990; Friedman, 1996). Aminoketoses have been identified in a number of foodstuffs (Wittmann and Eichner, 1989) as well as in the human organism (Day et al., 1979). They are further converted into deoxyosones that enter into a complex series of reactions, producing a heterogeneous group of amine-bound moieties termed "advanced glycation end products" (AGEs) (Monnier and Cerami, 1981). The numerous studies, addressing such subjects, so far have been focused on the Maillard reaction of proteins. Several recent reports indicate, however, that aminophospholipids may likewise be targets for nonenzymatic browning. These membraneous functional lipids are vital for the maintenance of cellular integrity and survival. Therefore, the in vivo significance of glycated phosphatidylethanolamine (PE) and phosphatidylserine (PS) has mainly been investigated (Bucala et al., 1993; Pamplona et al., 1995; Ravandi et al., 1995; Ravandi et al., 1996; Lertsiri et al., 1998; Requena et al., 1997; Pamplona et al., 1998). No defined structures from such reactions have been reported to date in foodstuffs, although nonenzymatic browning of aminophospholipids has early been recognized in dried egg (Lea, 1957).

From model reactions, we have for the first time definitely established the structure of a PE-linked Amadori product and pyrrolecarbaldehyde (Lederer et al., 1997; Lederer and Baumann, 2000); formation of carboxymethylated PE has been unequivocally proven by Requena et al. (1997). So far, identification and quantification of phospholipid-linked Maillard products is based on complex workup and/or derivatization procedures and allows monitoring of one single compound only. We have therefore independently synthesized the following compounds from 1,2-dipalmitoyl-3-*sn*-phosphatidylethanolamine (**1**, PE (16:0−16:0)): the Amadori product 4-hydroxy-4-oxo-1-[(palmitoyloxy)-methyl]-9-(2,3,4,5-tetrahydroxytetrahydro-2*H*-pyran-2-yl)-3,5-dioxa-8-aza-4λ^5-phosphanon-1-yl palmitate (**2**), the pyrrolecarbaldehyde 2-{[{2-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]ethoxy}(hydroxy)phosphoryl]-oxy}-1-[(palmitoyloxy)methyl]ethyl palmitate (**3**), the carboxymethyl (CM) derivative 7-hydroxy-7,13-dioxo-10-(palmitoyloxy)-6,8,12-trioxa-3-aza-7λ^5-phosphaoctacosan-1-oic acid (**4**), and the carboxyethyl (CE) derivative 7-hydroxy-2-methyl-7,13-dioxo-10-(palmitoyloxy)-6,8,12-trioxa-3-aza-7λ^5-phosphaoctacosan-1-oic acid (**5**) (see Figure 1). With these reference materials, a method based on coupled liquid chromatography−electrospray ionization mass spectrometry (LC−(ESI)MS) has been developed for the simultaneous determination of such Maillard compounds in complex biological matrixes (Utzmann and Lederer, 2000).

Formation of compound **3** can be rationalized by reaction of **1** with 3-deoxyglucosone, which represents a key intermediate during the degradation of hexoses in the course of Maillard processes (Ledl and Schleicher, 1990; Jurch and Tatum, 1970; Lederer and Baumann, 2000). CM-derivatives may be derived from oxidative degradation of Amadori products (Glomb and Monnier, 1995), termed as glycoxidation, or reactions of amines with glyoxal that can stem from D-glucose autoxidation (Wells-Knecht et al., 1995) as well as lipid peroxidation (Loidl-Stahlhofen and Spiteller, 1994). CE-derivatives are follow-up products of the C-3 unit methylglyoxal formed, for example, by reverse aldol reaction of 3-deoxy-

* To whom correspondence should be addressed. Fax: +49 (0)711 459 4096. E-mail: ledererm@uni-hohenheim.de.

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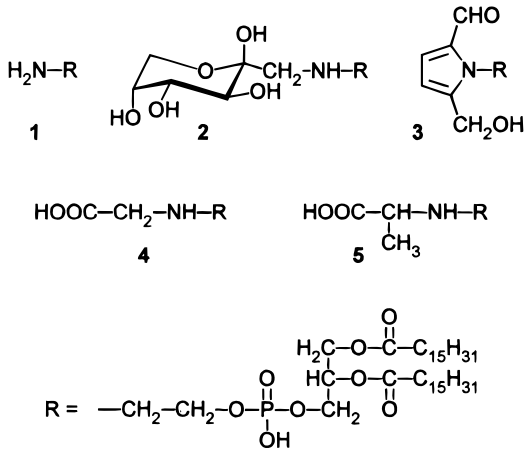

Figure 1. Structural formulas of compounds **1**−**5**.

glucosone. The lysine analogue of **5** was first described by Buettner et al. (1996).

We now report on the identification and quantification of PE-linked Maillard products, structurally analogous to **2**−**5**, in model systems as well as in spray-dried egg yolk and lecithin products derived therefrom. It will be shown that egg yolk PE derivatives undergo extensive glycation reactions during industrial processing.

MATERIALS AND METHODS

Materials. Ultrapure water from a Milli-Q 185 plus apparatus (Millipore, Eschborn, Germany), HPLC grade methanol and tetrahydrofuran (Fluka, Neu-Ulm, Germany), and aqueous ammonia (NH_3, 25%, Merck) were used for all LC separations. Phosphatidylethanolamine from soy bean (≥95%, soy-PE) was purchased from Larodan (Malmö, Sweden); sodium cyanoborohydride, trifluoroacetic acid, trifluoromethanesulfonic anhydride, and 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) from Fluka; Chromabond solid-phase extraction cartridges (SPE, C18, 1000 mg, 6 mL) from Macherey-Nagel (Düren, Germany). The test combination for the enzymatic D-glucose/D-fructose quantification was obtained from Boehringer (Mannheim, Germany). D-Glucose and D-fructose were generously supplied by Südzucker AG (Mannheim, Germany). Spray-dried egg yolk was provided by Lucas Meyer GmbH & Co. (Hamburg, Germany) and Wolff & Olsen GmbH & Co. (Hamburg, Germany), the lecithin samples by Lucas Meyer GmbH & Co.

Preparation of Amadori Compounds from Soy-PE, According to the Method of Utzmann and Lederer (2000). Di-*O*-isopropylidene-2,3:4,5-β-D-fructopyranose was prepared, following a procedure given by Brady (1970), and transformed into its triflate derivative as described by Xenakis et al. (1983). The triflate derivative (125 mg, 0.32 mmol) was dissolved in DMF (2 mL, dried over 4 Å molecular sieve), and soy-PE (200 mg, ∼0.29 mmol) and 1,8-diazabicyclo[5.4.0]undec-7-ene (85 μL, 0.57 mmol) were added. The solution was refluxed for 30 min, the solvent removed in high vacuum (5×10^{-2} mbar), and the residue taken up in a $CHCl_3$/MeOH mixture (3 mL, 2:1, v/v) and purified by preparative thin-layer chromatography (PTLC) [mobile phase, $CHCl_3$/MeOH/NH_3 (25%) 60:20:0.8 v/v/v]. The plates were covered except for 2 cm margins and sprayed with 2,7-dichlorofluorescein solution (0.1% (w/v) in MeOH). The band at $R_f = 0.6$ showing fluorescence on the margins was scraped out and the silica gel extracted with MeOH (1 × 75 mL, 2 × 50 mL). The solvent was stripped off, the residue dissolved in MeOH (10 mL) and passed through a membrane filter (0.45 μm), and the filtrate evaporated to dryness, yielding the isopropylidene-protected Amadori products of the various PE-species (76 mg, ∼0.08 mmol, ∼28%). The product was dissolved in trifluoroacetic acid/water (2 mL, 9:1, v/v) and the solution kept at room temperature for 4 h. The solvent was removed in high vacuum, and the residue was taken up in $CHCl_3$ (2 mL) and purified by column chromatography [2 (i.d., ∅) × 8 (height) cm, $CHCl_3$/MeOH/H_2O 95:35:2 (v/v/v), 400 mL]. Fractions (7 mL each) were tested for the Amadori products by TLC [mobile phase, $CHCl_3$/MeOH/H_2O 95:35:2 (v/v/v); $R_f = 0.34$; detection, spray reagent 0.1% (w/v) 2,7-dichlorofluorescein in MeOH]. From the respective fractions, the solvent was evaporated, the residue dissolved in MeOH (10 mL) and passed through a membrane filter (0.45 μm). The MeOH was stripped off, and the product dried in high vacuum, yielding phospholipid-linked Amadori products with different combinations of fatty acid substituents (15 mg, ∼0.017 mmol, ∼21%). LC−(ESI)MS (Amadori products of the major soy-PE derivatives): Amadori-PE (18:2−18:2), t_R = 18.4 min, *m*/*z* (relative intensity) 900.7 (100, $[M - H]^-$), 832.7 (13); Amadori-PE (16:0−18:2), **12**, t_R = 19.3 min, *m*/*z* 876.7 (100, $[M - H]^-$), 808.6 (13); Amadori-PE (18:1−18:2), t_R = 20.2 min, *m*/*z* 902.7 (100, $[M - H]^-$), 834.6 (14); Amadori-PE (16:0−18:1), **13**, t_R = 21.5 min, *m*/*z* 878.7 (100, $[M - H]^-$), 810.7 (18); Amadori-PE (18:0−18:1), t_R = 24.1 min, *m*/*z* 906.6 (100, $[M - H]^-$), 838.5 (14).

Incubation of Soy-PE with D-Glucose at 65 °C for the Identification of Schiff-PE 6 and Amadori-PE 12. Soy-PE (36 mg, ∼0.05 mmol) and D-glucose (367 mg, 2.04 mmol) were suspended in EtOH/phosphate buffer 0.1 M, pH 7.4 (4 mL, 3:2, v/v), sonicated, and kept at 65 °C for 2 h. The incubation mixture was purified by SPE: the C18 SPE cartridge was attached to a vacuum manifold, fitted with an adapter and a 20 mL reservoir, and conditioned with MeOH/H_2O (5 mL, 1:1, v/v). The incubation mixture was transferred to the SPE column, vacuum applied, and the eluent discarded. The cartridge was rinsed with MeOH/H_2O (2 × 2 mL, 1:1, v/v) and MeOH (1 mL) and the eluent again discarded. The phospholipid fraction was eluted with $CHCl_3$/MeOH (4 × 2 mL, 2:1, v/v), the eluate divided into two portions, and the solvent removed in vacuo. One aliquot (21 mg) was dissolved in MeOH (10 mL), passed through a membrane filter (0.45 μm), and injected into the LC−MS system. To the other aliquot was added $NaBH_3CN$ (21 mg, 0.33 mmol) and the mixture suspended in EtOH/H_2O (3 mL, 3:1, v/v), sonicated, and kept at 65 °C for 19 h. The solvent was stripped off, the residue taken up in eluent (1 mL), and purified by column chromatography [0.8 ∅ × 10 cm, $CHCl_3$/MeOH/H_2O 65:25:4 (v/v/v), 50 mL]. Fractions (2 mL each) were tested for phospholipids by TLC [mobile phase, $CHCl_3$/MeOH/H_2O, 65:25:4 (v/v/v); $R_f = 0.47-0.19$; detection, spray reagent 5% (w/v) phosphomolybdic acid in EtOH/5 min at 60 °C]. From the fractions containing phospholipids, the solvent was stripped off and the residue taken up in MeOH (10 mL), passed through a membrane filter (0.45 μm), and evaporated to dryness. The phospholipid residue (21 mg) was dissolved in MeOH (10 mL) and the solution injected into the LC−MS system. LC−(ESI)MS: **6**, t_R = 18.8 min, *m*/*z* 876.6 (100, $[M - H]^-$), 756.5 (4); **12**, t_R = 19.3 min, *m*/*z* 876.7 (100, $[M - H]^-$), 808.6 (8); 4,10,11,12,13,14-hexahydroxy-4-oxo-1-[(palmitoyloxy)methyl]-3,5-dioxa-8-aza-4λ^5-phosphatetradec-1-yl (9*Z*,12*Z*)-9,12-octadecadienoate, t_R = 20.0 min, *m*/*z* 878.6 (100, $[M - H]^-$).

Quantification of Schiff-PE 6 and Amadori-PE 12 in Soy-PE/D-Glucose Incubations at 65 °C. Soy-PE (20 mg, ∼0.03 mmol) and D-glucose (185 mg, 1.03 mmol) were suspended in EtOH/phosphate buffer 0.1 M, pH 7.4 (2 mL, 3:2, v/v), sonicated, and kept at 65 °C for 2 h. The incubation mixture was purified by SPE as described above. The phospholipid residue was taken up in MeOH (10 mL), passed through a membrane filter (0.45 μm), diluted with MeOH (1:200), and injected into the LC−MS system.

Quantification of Schiff-PE 6, Amadori-PE 12, Pyrrole-PE 14, CM-PE 16 and CE-PE 18 in Soy-PE/D-Glucose Incubations at 100 °C. Soy-PE (20 mg, ∼0.03 mmol) and D-glucose (185 mg, 1.03 mmol) were suspended in EtOH/phosphate buffer 0.1 M, pH 7.4 (2 mL, 3:2, v/v), sonicated, and kept at 100 °C for 3 h. The incubation mixture was purified by SPE as described above. The phospholipid residue was taken up in MeOH (10 mL), passed through a membrane filter (0.45 μm), and diluted for LC−MS analysis (1:5 for full scan mode, 1:50 for quantification in the SIM mode). LC−(ESI)-

MS: **14**, t_R = 20.4 min, *m/z* 822.5 (100, $[M - H]^-$), UV: λ_{max} = 296 nm; **16**, t_R = 11.6 min, *m/z* 794.5 (20), 772.5 (100, $[M - H]^-$); **18**, t_R = 12.2 min, *m/z* 808.5 (23), 786.5 (100, $[M - H]^-$).

Preparation of Lyophilized Egg Yolk. The egg yolk of two fresh white chicken eggs (market grade A) was carefully separated from the albumen, diluted with water (100 mL), and lyophilized, yielding 9.2 g (EY1) and 9.8 g (EY2) of dried egg yolk, respectively.

Isolation of the PE and Glycated PE (PE + *g* PE) Fraction from Lyophilized and Spray-Dried Egg Yolk. Egg yolk powder (2 g) was suspended in a $CHCl_3$/MeOH mixture (10 mL, 2:1, v/v) and stirred for 15 min. After filtration, the solution was purified by column chromatography [2 ∅ × 10 cm, $CHCl_3$/MeOH/H_2O 290:100:5 (v/v/v), 350 mL]. Fractions (7 mL each) were tested for PE and *g* PE by TLC [mobile phase, $CHCl_3$/MeOH/H_2O 65:25:4 (v/v/v); R_f = 0.44 (PE) and 0.35 (*g* PE); detection, spray reagent 5% (w/v) phosphomolybdic acid in EtOH/5 min at 60 °C]. From the fractions containing PE and *g* PE, the solvent was stripped off in vacuo and the residue dissolved in MeOH (10 mL), passed through a membrane filter (0.45 μm), and evaporated to dryness. For LC−MS analysis, the isolated PE + *g* PE fraction (60 mg) was dissolved in MeOH (5 mL), passed through a membrane filter (0.45 μm), and either injected directly into the LC−MS system (full scan mode) or diluted with MeOH; 1:100 (v/v) for quantification of Pyrrole-PE **15**; 1:1000 (v/v) for quantification of Schiff-PE **11** and Amadori-PE **13**. LC−(ESI)MS: **11**, t_R = 21.1 min, *m/z* 878.5 (100, $[M - H]^-$), 758.6 (5); **13**, t_R = 21.5 min, *m/z* 878.6 (100, $[M - H]^-$), 810.5 (11); **15**, t_R = 22.5 min, *m/z* 824.5 (100, $[M - H]^-$), UV: λ_{max} = 296 nm.

Quantification of D-Glucose in Dried Egg Yolk. Dried egg yolk (7 g) was suspended in water (~80 mL) and the mixture sonicated for 15 min. Solutions of Carrez I [4 mL, $K_4[Fe(CN)_6] \times 3\ H_2O$ (150 g/L)] and Carrez II [4 mL, $Zn(CH_3COO)_2 \times 2\ H_2O$ (230 g/L)] were added, and the mixture was shaken vigorously and filled with water to a final volume of 100 mL. After filtration, the solution was used for enzymatic quantification of D-glucose following directly the standard procedure included in the Boehringer test combination.

Quantification of Schiff-PE 11, Amadori-PE 13, and Pyrrole-PE 15 in Lecithin Samples. Egg yolk derived lecithin (150 mg), dissolved in MeOH (10 mL), was either used directly for LC−MS analysis (detection of Pyrrole-PE **15**), or diluted with MeOH (1:100) for the quantification of Schiff-PE **11** and Amadori-PE **13**.

Isolation of *g* PE from Egg Yolk Lecithin. Egg yolk lecithin (EYL1, 300 mg) was dissolved in eluent (3 mL) and separated by column chromatography [2 ∅ × 8 cm, $CHCl_3$/MeOH/H_2O 65:25:4 (v/v/v), 100 mL]. Fractions (5 mL each) were tested for *g* PE by TLC [mobile phase, $CHCl_3$/MeOH/H_2O 65:25:4 (v/v/v); R_f = 0.30; detection, spray reagent 5% (w/v) phosphomolybdic acid in EtOH/5 min at 60 °C]. From fractions containing *g* PE, the solvent was stripped off, the residue dissolved in MeOH (2 mL), and the solution passed through a membrane filter (0.45 μm) and subjected to LC−MS analysis.

High-Performance Liquid Chromatography−Electrospray Mass Spectrometry (LC−(ESI)MS). LC−(ESI)MS was run on an HP1100 HPLC system (Hewlett-Packard; Waldbronn, Germany) that comprised an HP1100 autosampler, HP1100 gradient pump, HP1100 column thermoregulator, and HP1100 diode array detector (DAD) module, coupled to a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer equipped with an ESI interface. Chromatographic conditions were as follows: column, YMC-Pack Pro C18, 120 Å, 5 μm (YMC Europe, Schermbeck, Germany; guard column 10 × 4.6 mm, column 150 × 4.6 mm); column temperature = 25 °C; flow rate = 1.0 mL/min; injection volume = 10 μL; eluent mixtures: (A) MeOH/H_2O/NH_3 (25%) 80:20:0.5 (v/v/v), (B) MeOH/THF/H_2O/NH_3 (25%), 65:25:10:0.5 (v/v/v/v); gradient: % B (*t* (min)) 0(0), 100(30−40), 0(43−50); postcolumn splitting ratio 1:20. MS parameters were as follows: ESI^-; source temperature 120 °C; capillary 3.0 kV; HV lens 0.5 kV; cone voltage 60 V. The MS system was operated either in full scan (*m/z* 200−1000) or single ion mode (SIM; span 0.5 Da, dwell time 0.5 s). For data acquisition and processing, the MassLynx 3.2 software was used.

Liquid Chromatography. Silica gel 60 F_{254}, 0.2 mm (Merck, Darmstadt, Germany) was used for TLC, silica gel 60 F_{254}, 2 mm for PTLC, and silica gel, 63−200 μm (Baker, Gross-Gerau, Germany) for column chromatography.

Lyophilization. A Leybold-Heraeus (Köln, Germany) Lyovac GT 2 was applied.

RESULTS

Our previously developed LC−(ESI)MS method (Utzmann and Lederer, 2000) was first employed to determine aminophospholipid glycation in model systems using a PE-isolate from soy bean (soy-PE), which contains derivatives with various combinations of fatty acid substituents. A highly endcapped C18 material that is stable toward alkaline eluents, up to a pH of 9.0, and a water/ammonium hydroxide/methanol/tetrahydrofuran gradient were used for LC analysis; the MS was operated in the negative electrospray ionization (ESI^-) mode using either full-scan or single-ion monitoring (SIM).

Calibration. For quantification, the LC−MS system was calibrated in the ESI^-−SIM mode with the reference compounds **1**−**5**. The respective quasimolecular ($[M - H]^-$) ion traces at *m/z* 690.6 (**1**), 852.6 (**2**), 798.6 (**3**), 748.6 (**4**), and 762.6 (**5**) were used for monitoring; calibration graphs, which are best described by second-order polynomials, and corresponding equations are given in Figure 2. Limits of detection (LOD) and limits of quantitation (LOQ) were calculated according to the recommendations of the Deutsche Forschungsgemeinschaft (DFG) (Walter et al., 1991) using the quasilinear region of the calibration graphs, which comprises the five lowest concentration levels for each analyte. The following LOD/LOQ values have been determined: 0.19/0.28 μmol/L (**1**), 0.097/0.14 μmol/L (**2**), 0.028/0.041 μmol/L (**3**), and 0.057/0.085 μmol/L (**4**, **5**). Since ion generation in ESI primarily depends on the polar functions of the molecule, structures analogous to **1**−**5** differing only in the fatty acid composition may be presumed to give a molar response virtually identical to that of the respective reference compound. All quantifications described below rest upon this assumption, which is validated by the findings of Kim et al. (1994). These authors have shown ESI sensitivity to be almost exclusively affected by the nature of the phospho headgroup, with the fatty acid substituents exhibiting only negligible effects. Consequently, the calibration functions for compounds **1**−**5** can be employed straightforwardly for quantification of other PE-derivatives and their respective Maillard products. The effect of the polar headgroup on ESI sensitivity is directly reflected by the different slopes of the calibration graphs for **1**−**5** in Figure 2. Since the PE-nitrogen in **3** is incorporated in a pyrrole ring and has lost its basic character, formation of negatively charged ions from **3** is a priori favored. The carboxyl groups in **4** and **5** also enhance $[M - H]^-$ generation; both compounds show almost identical responses. Thus, as expected, a higher sensitivity is obtained for compounds **3**−**5** relative to **1** and **2** in the ESI^- mode.

Model Studies. Soy-PE (~15 mM) and D-glucose (500 mM) were reacted in 3:2 ethanol-phosphate buffer (pH 7.4) mixtures for either 2 h at 65 °C or 3 h at 100 °C; the ethanol is required for better solubilization of the phospholipids. 2-Linoleoyl-1-palmitoyl-3-*sn*-phosphatidylethanolamine (**9**, PE (16:0−18:2), see Figure 7), one

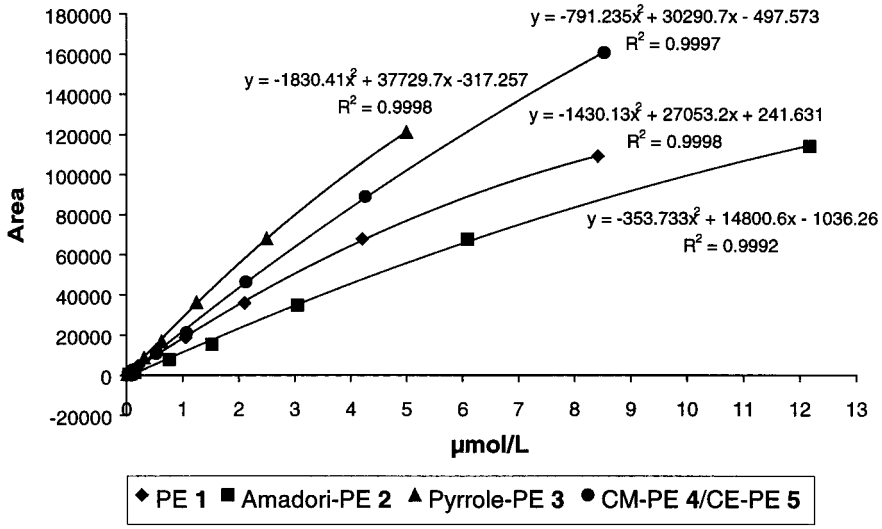

Figure 2. LC−(ESI)MS calibration graphs and corresponding equations for compounds **1**−**5**.

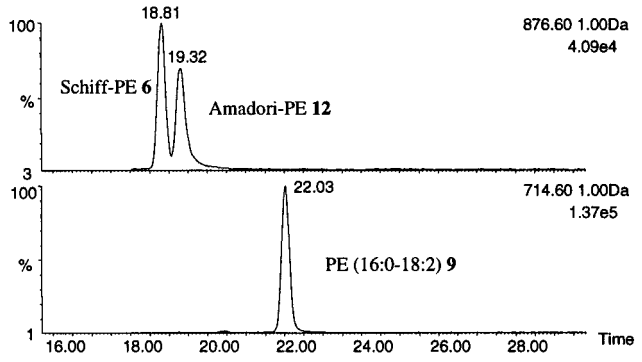

Figure 3. SIM traces for the PE derivative **9** (*m*/*z* 714.6) and the corresponding Maillard products **6** and **12** (*m*/*z* 876.6).

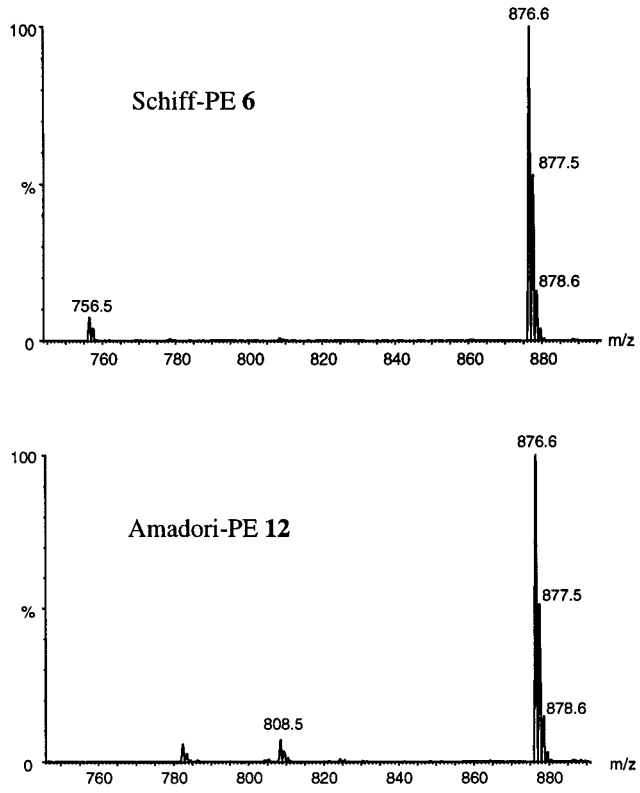

Figure 4. ESI mass spectra showing characteristic fragment and/or adduct ions for Schiff-PE **6** and Amadori-PE **12**.

of the major soy-PE derivatives, was used as a probe to evaluate glycation in these mixtures. This procedure is based on the fact that all PE species are glycated to more or less the same extent. In Figure 3 typical SIM chromatograms are given for the 65 °C incubation, with ion traces for **9** (*m*/*z* 714.6) and the corresponding Amadori compound 4-hydroxy-4-oxo-1-[(palmitoyloxy)-methyl]-9-(2,3,4,5-tetrahydroxytetrahydro-2*H*-pyran-2-yl)-3,5-dioxa-8-aza-4λ^5-phosphanon-1-yl (9*Z*,12*Z*)-9,12-octadecadienoate (**12**, Amadori-PE, *m*/*z* 876.6). Two peaks were observed in the *m*/*z* 876.6 ion trace; the respective mass spectra, obtained from a full scan run, are outlined in Figure 4. The signal at 19.3 min displays a fragmentation pattern almost identical to that of reference compound **2** and therefore is assigned to the Amadori product **12**. This assignment is confirmed unequivocally by co-injection of PE-linked Amadori products, independently synthesized from soy-PE according to Utzmann and Lederer (2000). From the fragmentation of the [M − H]$^-$ parent ion for the peak at 18.8 min, the glucosylamine structure 2-{[hydroxy-(2-{[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl]amino}ethoxy)phosphoryl]oxy}-1-[(palmitoyloxy)methyl]ethyl (9*Z*,12*Z*)-9,12-octadecadienoate (**6**, Schiff-PE) was derived for this compound. It represents a precursor of **12** in the course of Maillard processes. The characteristic loss of 120 Da from [M − H]$^-$ is rationalized by a reverse aldol reaction yielding deprotonated 4-hydroxy-4,10-dioxo-1-[(palmitoyloxy)-methyl]-3,5-dioxa-8-aza-4λ^5-phosphadec-1-yl (9*Z*,12*Z*)-9,12-octadecadienoate (**8**, *m*/*z* 756.5, see Figure 5) under elimination of erythrose (**7**). For the reaction mixture, reduced with sodium cyanoborohydride ($NaBH_3CN$), LC−(ESI)MS analysis gives a single peak with *m*/*z* 878.6 for [M − H]$^-$ and the signals for glucosylamine **6** and Amadori compound **12** are no longer observed. Hence, both products have been transformed to 4,10,-11,12,13,14-hexahydroxy-4-oxo-1-[(palmitoyloxy)methyl]-3,5-dioxa-8-aza-4λ^5-phosphatetradec-1-yl (9*Z*,12*Z*)-9,12-octadecadienoate; the diastereoisomers obtained from **12** by this procedure are not separated chromatographically.

From the 65 °C incubation, only compounds **6** and **12** were identified. The quantitative results of both the 65 °C and the 100 °C reaction are shown in Figure 6. The molar percentage given for **6** is tentative since the

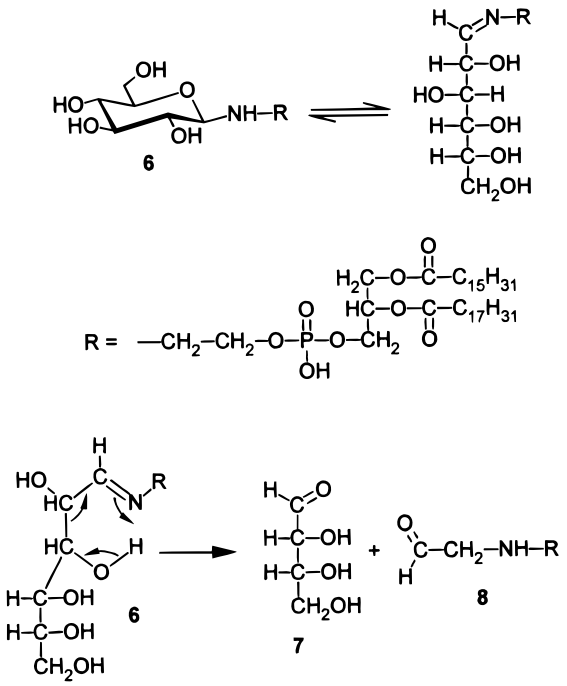

Figure 5. Fragmentation pathway for the formation of deprotonated **8** (*m*/*z* 756.5) from the quasimolecular ion ([M − H]$^-$, *m*/*z* 876.6) of Schiff-PE **6** by elimination of erythrose (**7**).

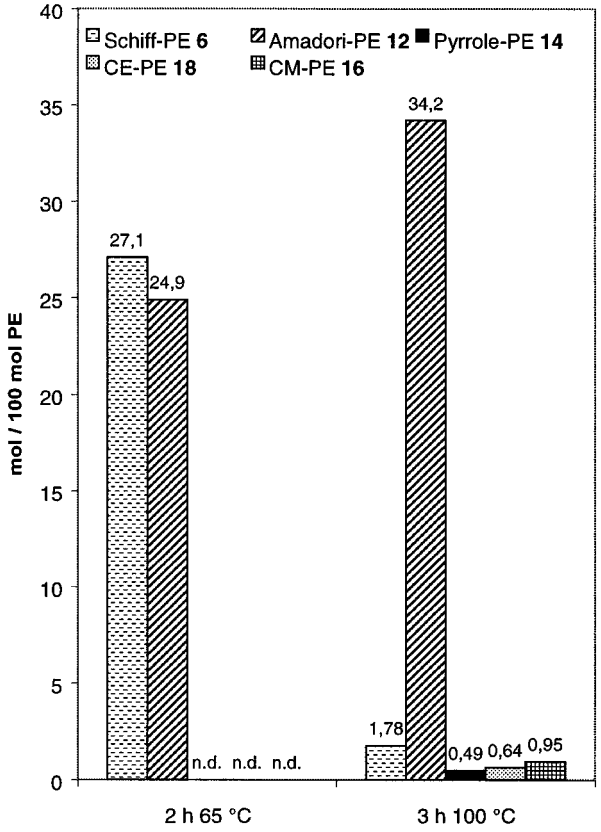

Figure 6. Quantitative results for the soy-PE/D-glucose model incubations, with overall formation of Schiff-PE, Amadori-PE, Pyrrole-PE, CE-PE, and CM-PE assumed to be equivalent to that of the Maillard products **6**, **12**, **14**, **16**, and **18**, which were used as PE-glycation indicators. The given values represent means of two replicates for each sample.

calibration graph for the Amadori product **2** was employed for quantification. This reservation holds for all glucosylamine data described below. Due to the reasons stated above, an identical molar response cannot be expected for compounds **2** and **6**, despite the closely related structures. In the 100 °C incubation, all monitored Maillard products were in fact detected: Schiff-PE **6**, Amadori-PE **12**, 2-{[{2-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]ethoxy}(hydroxy)phosphoryl]oxy}-1-[(palmitoyloxy)methyl]ethyl (9*Z*,12*Z*)-9,12-octadecadienoate (**14**, Pyrrole-PE), (20*Z*,23*Z*)-7-hydroxy-7,12-dioxo-10-[(palmitoyloxy)methyl]-6,8,11-trioxa-3-aza-7λ^5-phospha-20,23-nonacosadien-1-oic acid (**16**, CM-PE), and (20*Z*,23*Z*)-7-hydroxy-2-methyl-7,12-dioxo-10-[(palmitoyloxy)methyl]-6,8,11-trioxa-3-aza-7λ^5-phospha-20,23-nonacosa-dien-1-oic acid (**18**, CE-PE). As expected, the higher temperature and extended incubation time favor the formation of the AGEs **14**, **16**, and **18**, whereas the amount of glucosylamine **6** is significantly reduced compared to the 65 °C reaction.

Egg Yolk and Lecithin Studies. With the LC−MS procedure thus firmly established for model systems, we looked for Maillard products in lyophilized and spray-dried egg yolk in the next step. Egg yolk contains about 44% (w/w) triglycerides, 14% (w/w) phosphatidylcholine (PC), and 2.8% (w/w) PE in dry weight (dry wt) (Privett et al., 1962). From all samples, the phospholipid fraction was separated from neutral lipids by column chromatography on silica gel and analyzed by LC−(ESI)MS. Glycation was determined by monitoring the [M − H]$^-$ ion traces of 2-oleoyl-1-palmitoyl-3-*sn*-phosphatidylethanolamine (**10**, PE (16:0−18:1), see Figure 7) and of the corresponding Maillard compounds **11**, **13**, **15**, **17**, and **19**. In the lyophilized samples (EY1 and EY2), which we have prepared ourselves from fresh chicken eggs, only 0.5−1 mol % of PE was identified in form of the Schiff base as shown in Figure 8. This low glycation level corresponds to the enzymatically determined value of about 4 g/kg for free D-glucose in dry weight, agreeing well with literature data for the native D-glucose content in egg yolk (Tumann and Silberzahn, 1961). In contrast, both commercial egg yolk powders (EY3 and EY4), obtained from pasteurized egg yolk and spray-dried at 160 °C, contain only 0.3 and 0.6 g/kg D-glucose but considerable amounts (12.7 and 13.5 mol %) of PE-linked Amadori products. About one-fourth of the D-glucose starting material has thus been transformed into aminoketoses such as 4-hydroxy-4-oxo-1-[(palmitoyloxy)methyl]-9-(2,3,4,5-tetrahydroxytetrahydro-2*H*-pyran-2-yl)-3,5-dioxa-8-aza-4λ^5-phosphanon-1-yl (*Z*)-9-octadecenoate (**13**). Contents of ∼0.6 and ∼0.04 mol %, respectively, were determined for the glucosylamine 2-{[hydroxy(2-{[3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2*H*-pyran-2-yl]amino}ethoxy)phosphoryl]oxy}-1-[(palmitoyloxy) methyl]ethyl (*Z*)-9-octadecenoate (**11**) and the pyrrolecarbaldehyde 2-{[{2-[2-formyl-5-(hydroxymethyl)-1*H*-pyrrol-1-yl]ethoxy}(hydroxy)phosphoryl]-oxy}-1-[(palmitoyloxy)methyl] ethyl (*Z*)-9-octadecenoate (**15**). Hence, we have for the first time definitively established the formation of aminophospholipid-linked Maillard products in foodstuffs. Although the AGEs **17** and **19** could not be detected, the presence of Pyrrole-PE **15** indicates that egg yolk PE-derivatives likewise are prone to advanced glycation reactions in the course of industrial processing.

Since aminophospholipids are highly glycated in spray-dried egg yolk, lecithin products derived therefrom are also expected to contain considerable amounts of Amadori compounds, such as **13**. We have therefore

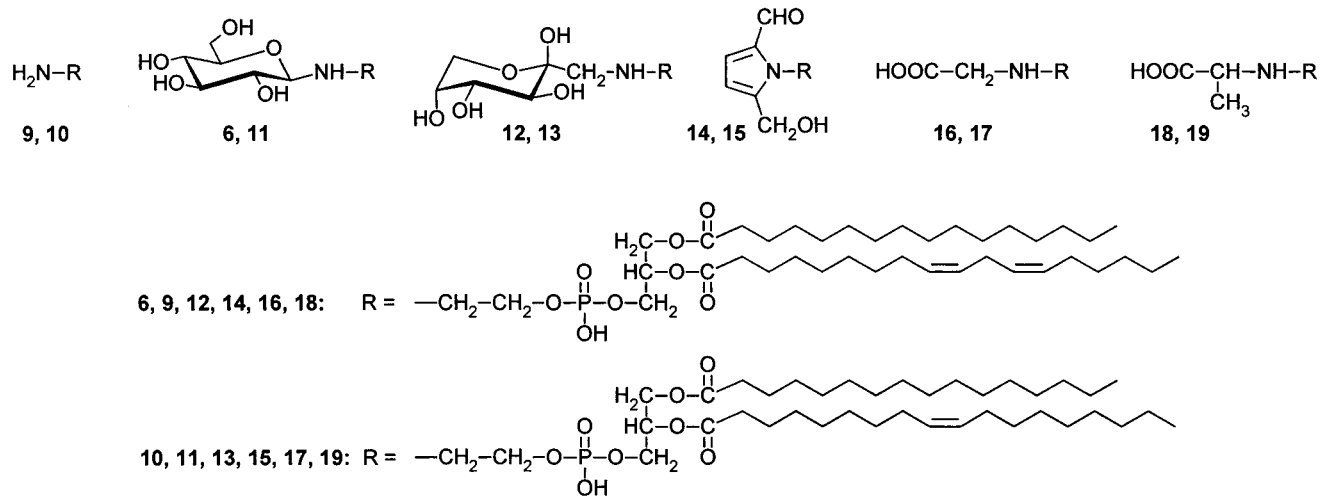

Figure 7. Structural formulas of PE (16:0−18:2) **9**, PE (16:0−18:1) **10**, and the corresponding Maillard compounds **6** and **11**−**19**.

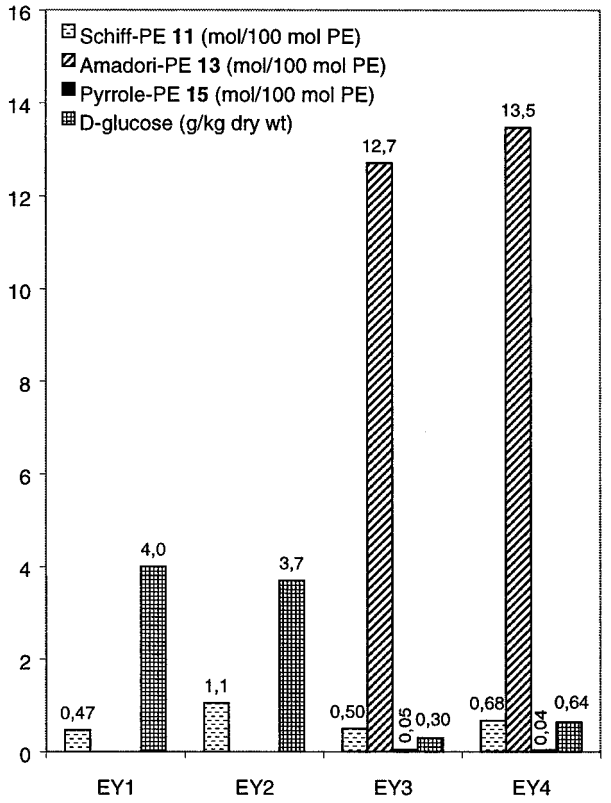

Figure 8. Glycation quota and D-glucose content of lyophilized (EY1, EY2) and spray-dried (EY3, EY4) egg yolk samples, with overall formation of Schiff-PE, Amadori-PE, and Pyrrole-PE assumed to be equivalent to that for the Maillard products **11**, **13**, and **15**, which were used as PE-glycation indicators. The given values represent means of two replicates for each sample.

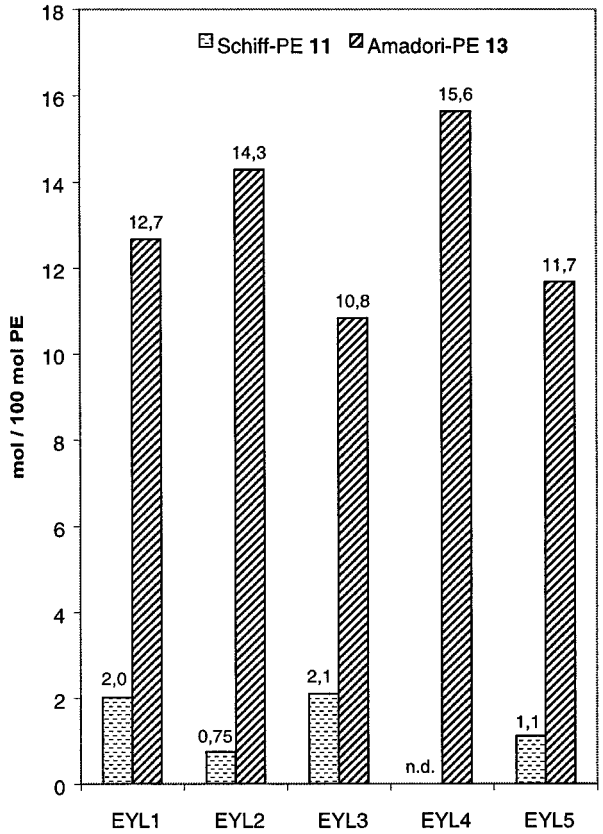

Figure 9. Glycation quota of the egg yolk lecithin samples EYL1-EYL5, with overall formation of Schiff-PE and Amadori-PE assumed to be equivalent to that for the Maillard products **11** and **13** which were used as PE-glycation indicators. The given values represent means of two replicates for each sample.

analyzed five samples (EYL1-EYL5) that have been prepared from egg yolk, spray-dried under identical conditions as described for EY3 and EY4. Products EYL1-EYL4 were obtained from egg yolk by extraction with ethanol; from the residue of the ethanol extract the neutral lipids were removed with acetone. In the case of EYL5, in contrast, the egg yolk was extracted with supercritical carbon dioxide, and the residue of this process being virtually free from neutral lipids, extracted with ethanol. All samples have been analyzed directly by LC−MS, without any cleanup step. The bars in Figure 9 show that the molar percentage determined for **11** and **13** is in the same range as that of the egg yolk starting material (see Figure 8). The different extraction procedures apparently have no or only negligible effects on the Maillard product content. Trace amounts of pyrrolecarbaldehyde **15** were also identified in EYL1-EYL5. Figure 10 demonstrates that other PE derivatives, such as PE (18:0−18:1) and PE (18:0−20:4), are glycated to more or less the same extent as PE (16:0−18:1) **10**, which we have used as a probe for the quantification of the overall PE derivatization by Maillard compounds in egg yolk products. Analogous results were obtained for all other investigations described

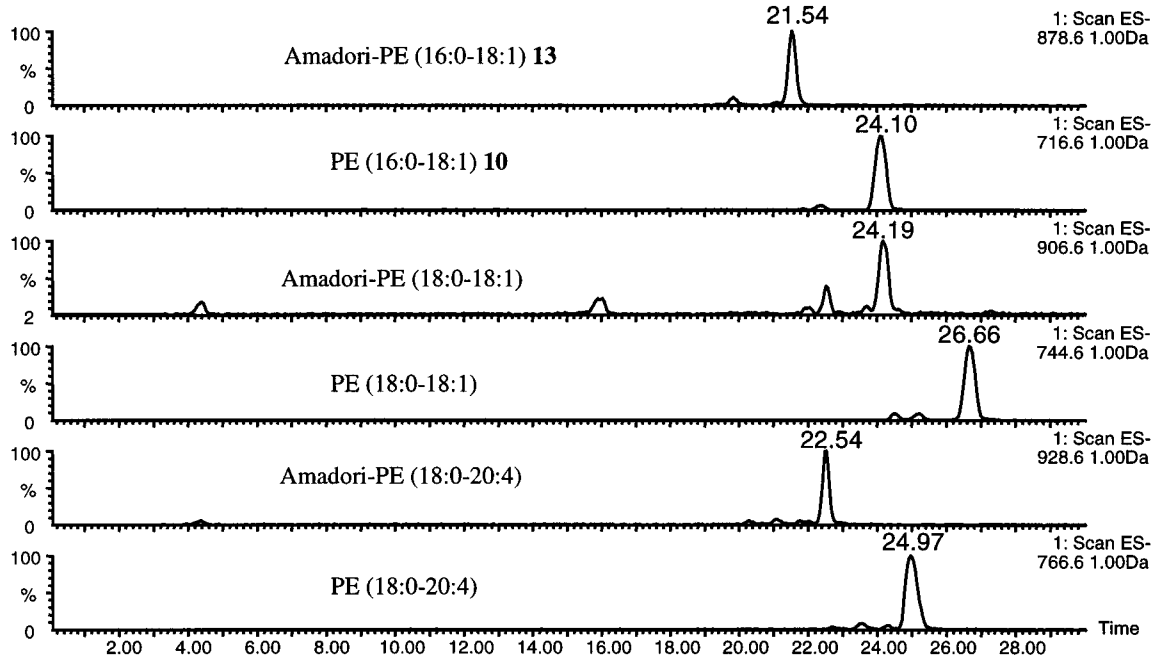

Figure 10. $[M - H]^-$ Ion traces for the major egg yolk PE derivatives PE (16:0−18:1) **10**, 2-oleoyl-1-stearoyl-3-*sn*-phosphatidylethanolamine (PE (18:0−18:1)), and 2-arachidoyl-1-stearoyl-3-*sn*-phosphatidylethanolamine (PE (18:0−20:4)) as well as for the corresponding Amadori products.

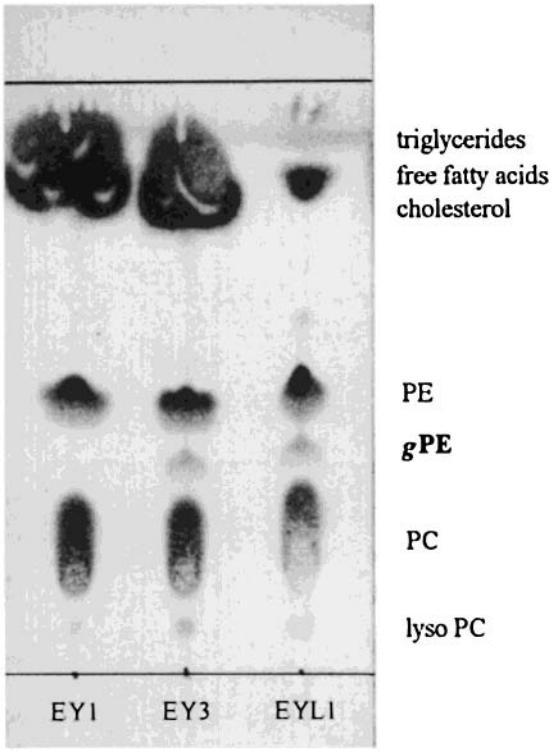

Figure 11. TLC separation for EY1, EY3, and EYL1 showing the glycated PE (*g* PE) spot between the PE and PC fraction.

above. The ion traces show a characteristic difference in retention time of about 2.5 min between the respective PE derivative and the corresponding Amadori product.

Figure 11 outlines a TLC separation for EY1, EY3, and EYL1. The traces for EY3 and EYL1 show an additional spot with an R_f value between PE and PC. We have isolated this fraction by column chromatography on silica gel, and analyzed the products by LC−(ESI)MS. The mass spectra clearly prove that the TLC fraction (*g* PE) contains both the Schiff base and the Amadori product, which is glycated PE. Hence, the existence of *g* PE in egg yolk products can also be established by a simple TLC analysis.

DISCUSSION

The worldwide lecithin market is dominated by soy lecithin (SL; 200 000 tons/year). Egg yolk derived lecithin (EYL; 400 tons/year) is applied specifically in pharmaceuticals, infant nutrition products, and cosmetics. The preferential use of EYL instead of injectable grade SL in fat infusions (for example, Lipofundin and Lipovenös) containing 6−12 g/L EYL, 50−200 g/L soy oil, and optionally 50−100 g/L middle-chain triglycerides (MCT) is based on at least two arguments: On one hand, EYL is in some cases tolerated better by patients than SL. On the other hand, EYL contains considerable amounts of highly unsaturated long-chain fatty acids such as arachidonic acid (AA, C20:4n − 6) and docosahexaenoic acid (DHA, C22:6n − 3), whereas SL bears only fatty acids up to C18. Recent clinical and epidemiological studies have indicated that long-chain n − 3 polyunsaturated fatty acids such as DHA are important in the treatment of atherosclerosis, cancer, rheumatoid arthritis, psoriasis, and diseases of old age like Alzheimer's disease and age-related macular degeneration (Drevon et al., 1993).

Both AA and DHA are also present in human milk, and are important for infant development (Bondia-Martinez et al., 1998; Simopoulos and Salem, Jr., 1992; Connor et al., 1992); it is thus reasonable to utilize EYL for infant nutrition products.

In most cases, lecithin is employed because of its emulsifying properties. Keenan and Berridge (1973) reported that gangliosides, representing glycosphingolipids, may also contribute to the emulsion-forming characteristics of egg yolk, although they are present only in very low concentration. The EYL products EYL1-EYL5 investigated in this study contain about 70% (w/

w) PC and 15% (w/w) PE, 11−15.5 mol % of which have been transformed to Amadori products. Since the polarity of the phospho headgroup is effectively changed by the glycation process, it seems fully justified to expect that such PE derivatives, just like gangliosides, are potent surfactants and influence the emulsifying properties of EYL. We are currently investigating this aspect by comparing products EYL1-EYL5 with an EYL derived from desugarized egg yolk, which definitely contains no Maillard products, as preliminary experiments have shown.

Several recent reports indicate that amino acid derived Amadori compounds are able to generate reactive oxygen species, such as the superoxide radical anion ($O_2^{\bullet-}$), from triplet oxygen ($^3O_2$). Triplet oxygen itself is only a weak oxidant since reactions with molecules having singlet multiplicity like fatty acids are spin-forbidden. Metal ion catalysis is regarded as essential for oxidations mediated by aminoketoses (Sakurai and Tsuchiya, 1988; Kawakishi et al., 1990). However, Mossine et al. (1999) recently described an alternative "metal-free" $O_2^{\bullet-}$ formation. PE-linked Amadori products such as **12** and **13** are likely to have a similar potential. Bucala et al. (1993; 1994) have shown that the *g* PE content in low-density lipoprotein (LDL) correlates with its oxidation level. From these results it may be expected that the high glycation quota (11−15.5 mol % Amadori-PE) which we have found in spray-dried egg yolk (EY3 and EY4, see Figure 8) and EYL (EYL1-EYL5, see Figure 9) enhances oxidation reactions in these products. Polyunsaturated fatty acids such as AA and DHA will be especially affected by such processes. This may lead to degradation of these fatty acids as well as to oral or intravenous intake of oxidized lipids which has been shown to stimulate the formation of reactive oxygen species by human blood monocytes (Gorog, 1991). At present, we are attempting to prove the prooxidative effect of Amadori-PE with the help of our authentic reference compound **2** and by comparing the oxidation resistance of glycated EYL with that of EYL derived from desugarized egg yolk.

Since formation of *g* PE most likely depends on processing (for example pasteurization, spray drying) and storage conditions of egg yolk products, the content of Amadori compounds such as **13** may be used as a quality criterion. Furosine and pyridosine, formed during acid protein hydrolysis from aminoketoses, are established indicators for thermally stressed milk and milk powders (Henle et al., 1991; Vanrenterghem and Deblock, 1996). We hope that Amadori-PE turns out to be an equivalent marker in the case of egg yolk products.

CONCLUSION

Formation of aminophospholipid-linked Maillard products has been definitely established in foodstuffs. Spray-dried egg yolk and lecithin products derived therefrom contain considerable amounts of PE-linked Amadori compounds (11−15.5 mol %). Future investigations will have to prove whether this high glycation rate in fact influences the emulsifying properties and lipid peroxidation processes of the respective products. If a prooxidative effect can be established for Amadori-PE, it should be considered to employ desugarized egg yolk especially for the preparation of EYL for fat infusions.

ABBREVIATIONS USED

AA, arachidonic acid; AGEs, advanced glycation end products; CE, carboxyethyl; CM, carboxymethyl; DHA, docosahexaenoic acid; dry wt, dry weight; EY, egg yolk; EYL, egg yolk lecithin; *g* PE, glycated phosphatidylethanolamine; LOD, limit of detection; LOQ, limit of quantitation; PC, phosphatidylcholine; PE, phoshatidylethanolamine; PS, phosphatidylserine; SIM, single ion monitoring; SL, soy lecithin; SPE, solid-phase extraction.

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