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Rapid determination of cholesterol oxidation products in milk powder based products by reversed phase SPE and HPLC-APCI-MS/MS

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ABSTRACT

A rapid and sensitive HPLC-APCI-MS/MS method for the determination of cholesterol oxidation products (COPs) in milk powder based foods is reported. The method consists in the direct saponification of the sample and purification of oxysterols by reversed phase C18-SPE followed by HPLC-MS/MS analysis. By this procedure, the extraction and enrichment of oxysterols are combined in a unique step, reducing sample manipulation and the possible formation of artifacts. LOD and LOQ were in the concentration ranges of 2–8 ng g^{-1} and 8–30 ng g^{-1}, respectively. The precision (CV%) was in the range 10–36% in fresh samples with a total COPs amount from 212 to 645 ng g^{-1} and 6–14% for an oxidized sample with a higher amount (3651 ng g^{-1}). The recovery ranged from 74 ± 8% for 7-ketocholesterol to 101 ± 12% for 7α-hydroxycholesterol at 200 ng g^{-1} and from 82 ± 2% for 7-ketocholesterol to 117 ± 10% for 5α,6α-epoxy cholesterol at 500 ng g^{-1} spiked levels, respectively.

1. Introduction

Cholesterol is the most important animal sterol and is present in the lipid fraction of all the foods of animal origin such as milk, eggs, meat, fish and all their derivatives. Cholesterol is a monounsaturated molecule and, due to the presence of a double bond in position 5,6 of the B ring, it can be oxidized following similar oxidative pathways as monounsaturated fatty acids (Yin, Xu, & Porter, 2011). Cholesterol oxidation products (COPs) can be formed by autoxidation, photosensitized and enzymatic oxidation (Smith, 1996) and the factors that can mostly determine the extension of the oxidation are temperature, oxygen, light, trace elements, water activity. The main detected COPs in food are 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, 5α,6α-epoxycholesterol, 5β,6β-epoxycholesterol, cholestan-3β,5α,6β-triol, and 25-hydroxycholesterol (Guardiola, Bou, Boatella, & Codony, 2004a; Otaegui-Arrazola, Menéndez-Carreño, Ansorena, & Astiasarán, 2010; Savage, Dutta, & Rodriguez-Estrada, 2002).

The presence of COPs in fresh food is generally negligible but technological treatments involving relatively high temperature, like spray-drying and frying, and inappropriate storage conditions of the product increase significantly their amount (Derewiaka & Molińska, 2015; Savage et al., 2002; Sieber, 2005). Both cholesterol and COPs in the human organism can be of endogenous or exogenous origin. In the latter case, they come from the diet and in particular from the consumption of cholesterol-rich foods, such as meat (Boselli, Cardenia, & Rodriguez-Estrada, 2012), fish (Saldanha, Sawaya, Eberlin, & Bragagnolo, 2006), eggs (Boselli, Bonoli, Caboni, & Lercker, 2002; Obara, Obiedziński, & Kołczak, 2006), milk and dairy products (Calderón-Santiago, Peralbo-Molina, Priego-Capote, & Luque de Castro, 2012; Sieber, 2005). The presence of COPs in food products has been studied in depth, as they have been linked to the development of several chronic and degenerative diseases (Otaegui-Arrazola et al., 2010). Many different analytical methods have been used for the determination of COPs in food. Generally, the analytical procedure involve an initial extraction of the lipids from the food matrix, followed by saponification/transesterification, extraction of the unsaponifiable, purification/enrichment of COPs by TLC, SPE, HPLC, derivatization to trimethylsilyl ethers, gas-chromatographic separation and detection by mass spectrometry (GC-MS) (Caboni et al., 2005;

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Calderón-Santiago et al., 2012; Georgiou, Constantinou, & Kapnissi-Christodoulou, 2014; Guardiola et al., 2004a; Przygonski, Jelen, & Wasowicz, 2000; Rose-Sallin, Huggett, Bosset, Tabacchi, & Fay, 1995; Schmarr, Gross, & Shibamoto, 1996; Szterk & Pakuła, 2016; Ubhayasekera, Verleyen, & Dutta, 2004). More recently, methods based on liquid-chromatographic separation coupled to tandem mass spectrometry with atmospheric pressure chemical ionization (HPLC-APCI-MS/MS) (Manini, Andreoli, Careri, Elviri, & Musci, 1998; Raith et al., 2005; Razzazi-Fazeli, Kleineisen, & Luf, 2000; Saldanha et al., 2006) or electrospray ionization (HPLC-ESI-MS/MS) (Georgiou et al., 2016) have been reported. In particular, APCI provides a higher ionization efficiency compared to ESI for the analysis of these relatively non-polar compounds without derivatization. HPLC coupled to refractive index (Saldanha et al., 2006) or UV–Vis (Lee & Myung, 2013) detector have also been used. Due to the complexity of the food matrices, the low levels of COPs and the possible formation of artifacts or degradation of COPs, the entire analytical procedure is lengthy and laborious with the saponification and purification steps being the critical points in order to obtain a sample suitable for analytical detection (Guardiola, Garcia-Cruset, Bou, & Codony, 2004b). Cold saponification for about 18 h is carried out to avoid extensive degradation of COPs and in particular of 7-ketocholesterol (Busch & King, 2010; Park, Guardiola, Park, & Addis, 1996). In the case of milk powders, Dionisi, Golay, Aeschlimann, and Fay (1998) showed that the direct saponification of the sample, without preliminary lipid extraction, was more efficient in terms of repeatability, accuracy and lower artifacts formation.

In products containing milk powder, which production requires high temperatures, the presence of oxysterols is inevitable and moreover, due to the high surface/volume ratio, they are prone to the formation of COPs during storage (Calderón-Santiago et al., 2012; Chudy, Pikul, & Rudzińska, 2015; Dionisi et al., 1998; Lee & Myung, 2013; Mc Cluskey et al., 1997; Przygonski et al., 2000; Rose-Sallin et al., 1995; Sieber, 2005).

The aim of our study was to develop a rapid and sensitive HPLC-APCI-MS/MS method to quantify oxysterols in products containing milk powder without the unsaponifiable extraction and the derivatization steps. One key point in this work is the clean-up procedure using reversed phase C18 SPE for the simultaneous extraction and enrichment of the oxysterols prior to HPLC-MS/MS analysis. This reduces the sample manipulation and the possible artifacts formation.

2. Materials and methods

2.1. Samples

The following commercial products, containing milk powder, were analyzed: a sample of skimmed milk infant formula powder 18 months old and opened (A); a sample of skimmed milk infant formula powder (B); a sample of skimmed milk powder (C); a sample of instant formula for ginseng coffee (D) and a sample of instant formula for cream coffee (E). With the exception of sample A, all the other samples were freshly opened.

2.2. Chemicals and materials

Cholesterol (Chol), 5α,6α-epoxycholesterol (α-Epoxy), 7-ketocholesterol (7-Keto), cholestan-3β,5α,6β-triol (Triol), 6-ketocholestanol (6-Keto; internal standard; I.S.), potassium hydroxide (KOH), methanol (MeOH) and acetonitrile (ACN) for HPLC-MS, and 85% phosphoric acid (H_3PO_4) were purchased from Sigma-Aldrich (Milan, Italy). 7α-Hydroxycholesterol (7α-OH), 7β-hydroxycholesterol (7β-OH) and 5β,6β-epoxycolesterol (β-Epoxy) were from Sterealoids (Newport, RI, USA). Solid phase extraction (SPE) columns ISOLUTE C18, 1 g, 6 mL were from Biotage (Milan, Italy).

2.3. Saponification

40 μL of a methanolic solution of 6-ketocholestanol (20 μg mL^{-1}) as internal standard and 5 mL of a 2 M solution of KOH in MeOH were added to 0.50 g of sample, in a 10 mL glass test tube with Teflon lined screw cap. The sample was vortexed for about 30 s and then maintained in agitation by an orbital shaker for about 16–18 h at room temperature.

2.4. SPE purification

The saponified sample was partially neutralized (pH 7–8) by the addition of 1.7 mL of H_3PO_4 diluted 1:5 with water, obtaining a final MeOH:H_2O ratio of about 75:25 (v/v) in the sample. The sample was centrifuged at 3000 rpm for 10 minutes and the supernatant loaded on a C18 SPE column previously conditioned with 5 mL of MeOH and 5 mL of a mixture of MeOH:H_2O (75:25, v/v). After loading, the column was washed with 5 mL of H_2O, 9 mL of a mixture of MeOH:H_2O (75:25, v/v) and finally with 9 mL of H_2O. The oxysterols were then eluted with 10 mL of MeOH and 1 mL of this solution was transferred to an autosampler vial for the HPLC-MS/MS analysis.

2.5. HPLC-MS/MS analysis

An HPLC Ultimate 3000 (Thermo Scientific, San Jose, CA, USA), equipped with a thermostated autosampler and a column oven, coupled to a Finnigan LXQ linear trap mass spectrometer (Thermo Scientific, San Jose, CA, USA) fitted with an atmospheric pressure chemical ionization (APCI) source operating in positive mode was used. To optimize the fragmentation conditions, a methanol solution of each oxysterol standard was infused into the ion source using a syringe pump at a flow rate of 20 μL min^{-1}. The conditions used for the APCI source were: temperature of the transfer line capillary 275 °C, source temperature 400 °C, discharge current 5 μA; the gas flow rates (N_2) for sheath, auxiliary and sweep were 20, 30 and 0 arbitrary units, respectively. The fragmentation spectra (MS^n) were obtained by collision-induced dissociation (CID) of the precursor ion with He at a pressure of 0.133 Pa. The collision energy was selected in order to maintain the signal of the precursor ion at around 5%.

The chromatographic separation was performed with a column Synergi Hydro, 4 μm, 250 × 2.0 mm (Phenomenex, Italy), thermostated at 30 °C. Elution was carried out at a flow rate of 0.1 mL min^{-1}, using as mobile phase a mixture of MeOH (solvent A) and ACN (solvent B) with the following gradient: 0–12 min 20% A, 14 min 90% A, 14–39 min 90% A, 44 min 20% A, 44–55 min 20% A. The injection volume was 10 μL. A time programmed valve was used to divert the effluent from the column to waste after 25 minutes in order to prevent excess cholesterol from contaminating the source of the mass spectrometer.

The HPLC-MS/MS analysis was performed using the optimized conditions described above. The acquisition was carried out in full scan (m/z 50–1500) and in full scan MS^2 (m/z 50–500) selecting the precursor ion at m/z 401.5 $[M+H]^+$ for 7-Keto and at m/z 385.5 for 6-Keto (I.S.), 7α-OH, 7β-OH, α-Epoxy, β-Epoxy and Triol, due to in-source loss of H_2O from $[M+H]^+$ ion (Table 1) (Manini et al., 1998; Raith et al., 2005; Saldanha et al., 2006). For the quantitative analysis, the area of the chromatographic peaks obtained from the extraction of the $[M+H-H_2O]^+$ fragment ion (MS^2) at m/z 383.5 for 7-Keto, and $[M+H-2H_2O]^+$ at m/z 367.5 for 6-Keto (I.S.), 7α-OH, 7β-OH, α-Epoxy, β-Epoxy and Triol were used.