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Effective application of freezing lipid precipitation and SCX-SPE for determination of pyrrolizidine alkaloids in high lipid foodstuffs by LC-ESI-MS/MS

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are naturally occurring plant toxins associated with serious hepatic disease in humans and animals. In this study, rapid and sensitive analytical method was developed for the determination of 9 toxic PAs in popularly high lipid foodstuffs by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). PAs in lipid foodstuff were effectively purified by freezing lipid precipitation (FLP) and strong cation exchange (SCX)-solid-phase extraction (SPE). Especially, FLP could easily remove the large amounts of triacylglycerols in the lipid sample extract and effectively combine with SPE cleanup. During the FLP procedure, over 77% of the lipids in the foodstuff extracts were rapidly eliminated without any significant loss of the PAs with over 81% recovery. The elimination efficiency of lipids by FLP was tested with LC-atmospheric chemical ionization (APCI)-MS. For further purification, SCX-SPE cartridge could successfully purify PAs from the remaining interfering substances by the variation pH with 5% NH_4OH in methanol. For precise quantification and confirmation of PAs in complicate sample matrices, appropriate transition ions in LC-MS/MS-multiple-ion reaction monitoring (MRM) mode were selected on the basis of MS/MS fragmentation pathways of PAs. The established analytical method was validated in terms of the linearity, limits of detection (LOD), and quantification (LOQ), precision, and accuracy. The method was deemed satisfactory by inter- and intra-day validation and exhibited both high accuracy and precision (relative standard deviation < 11.06%). Overall limits of detection and quantitation of PAs were approximately 0.06–0.60 ng/mL at a signal-to-noise ratio (S/N) of 3 and were about 0.20–1.99 ng/mL at a S/N of 10 for all foodstuffs. The established method was successfully applied for the monitoring of toxic PAs in several types of high lipid foodstuffs such as soybeans, seed oil, milk, and margarine.

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1. Introduction

Pyrrolizidine alkaloids (PAs) are known secondary plant metabolites that are expressed as a chemical defense mechanism in some plant families [1]. They have been the focus of causing genotoxic, carcinogenic, and hepatotoxic reactions in humans and animals [2,3]. Human exposure can be caused by the direct consumption of PA-containing plants such as *Asteraceae, Boraginaceae and Fabaceae* or indirectly by the consumption of foodstuffs processed from animal origin [4–6]. Due to the serious toxic effects from PAs even at a trace amount, an analytical method for the highly sensitive detection of PAs in high lipid foodstuffs is still developing. Especially for high lipid foodstuffs such as soybeans, seed oil, milk, and margarine which are popular foodstuffs, it is necessary to develop an analytical method for the determination of trace PAs.

Numerous analytical methods have been reported for the measurement of PAs in various foodstuffs to determine their concentrations in samples with high precision and accuracy. Liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS) have been reported

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for the detection of PAs due to the high sensitivity and selectivity of these methods [7–10]. GC-MS combined with chemical derivatization has also been used for the sensitive detection of PAs in foodstuffs [11–13]. However, some of the PAs can undergo thermal decomposition to form monoesters from diesters in the hot injection port or during the derivatization of the PAs [10]. Recently, LC-MS/MS-multiple-ion reaction monitoring (MRM) methods combined with simple sample preparation procedures such as direct dilution method, liquid–liquid partition, and/or ion exchange SPE have been popularly introduced for the sensitive and selective detection of PAs in foodstuffs [7–9]. The MRM mode can significantly improve the sensitivity and selectivity for the analysis of PAs in various foodstuffs with complex matrices. However, for high lipid food samples, a large amount of lipid components tend to contaminate parts of chromatographic and mass spectrometric systems, such as the injection port, column, and ion source, resulting in poor reproducible quantitation results in repetitive analyses. Due to these reasons, the rapid and accurate analysis of PAs in high lipid foodstuffs is still challenging task.

The analysis of PAs is relatively complicated due to high matrix effects and their low concentrations in high lipid foodstuffs. First, during the extraction of PAs from foodstuffs, large quantities of lipids may inevitably get co-extracted with the targets due to their high solubility in organic solvents. In general, the lipid contents of foodstuffs such as milk, cheese, and oil seeds are over 3%, 10%, and 20% of the net mass, respectively, and are mainly composed of triacylglycerols, phospholipids, and sterols as well as unsaturated and saturated fatty acids [14]. Among these, triacylglycerols and phospholipids cannot be easily separated from relatively mid-polar analytes in organic extracts by conventional cleanup methods [15–18] including simple liquid–liquid partitioning, gel permeation chromatography, and the SPE cartridge methods. Thus, these methods are still insufficient for the determination of PAs in high lipid foodstuffs due to the complicate matrices. Secondly, the detection of trace amounts of PAs can easily be masked by a high concentration of the sample matrix. The co-extracted components could negatively affect the ionization efficiency of PAs due to ion suppression and signal enhancement, resulting in poor sensitivity and selectivity. Thus, the analytes of interest remain undetected for insufficient cleanup sample.

To overcome these analytical problems, we have attempted to apply a freezing lipid precipitation (FLP) method and strong cation exchange (SCX)-SPE for the effective cleanup of the samples. The FLP method has been successfully applied to eliminate the large amounts of lipids extracted from biota samples with high lipid contents [19,20]. During the FLP procedure, lipids in organic extracts are precipitated frozen at −24 °C in the refrigerator, and then, the frozen lipids can be easily removed by just filtering the extracts. Moreover, the FLP method has several advantages over conventional methods with respect to effective sample cleanup and the relative ease in which it can be combined with SPE cleanup. In this study, the elimination efficiency of lipids by freezing lipid precipitation was also examined by LC-atmospheric pressure chemical ionization (APCI)-MS. For further cleanup, a SCX-SPE cartridge using a specific eluent was applied. The SCX-SPE method has been used for the separation of basic analytes from co-extracted contaminants [21,22].

For the sensitive and selective detection of the target PAs, LC-ESI-MS/MS-MRM mode was applied, and specific transition ions were selected to reduce any potential matrix effects. The selection of an appropriate transition ion in the MRM mode could provide unequivocal identification of the analytes and avoid false positives. For the selection of transition ions in the MRM mode, MS/MS fragmentations of PAs were preferentially investigated according to their structural characteristics. The monitoring of a specific precursor-product ion transition in the MRM mode could significantly enhance the analytical sensitivity and selectivity for trace amounts of PAs in complex food matrices.

The aim of this study was to develop an analytical method for the rapid quantification of PAs in high lipid foodstuffs by LC-MS/MS. This study included the following: (i) high efficiency of FLP for the rapid elimination of lipids, (ii) elution profiles of PAs on SCX-SPE cartridge with variation pH of eluent for cleanup of PAs, (iii) plausible MS/MS fragmentation pathways of PAs for the selection of appropriate transition ions in MRM mode, (iv) precise quantification and identification of PAs in complex matrices by LC-ESI-MS/MS-MRM mode, (v) method validation in terms of linearity, limits of detection and quantification, and precision and accuracy, and (vi) a demonstration of the effectiveness of the established method applied for the monitoring of PAs in high lipid foodstuffs.

2. Experiments

2.1. Chemicals

Organic solvents of HPLC grade (acetonitrile, methanol, and chloroform) were purchased from J.T. Baker (Rockford, IL, USA). Formic acid and ammonium hydroxide were obtained from Sigma Chemical (St. Louis, MO, USA).

Authentic pyrrolizidine alkaloids, crotaline, and retrorsine were provided by Sigma Chemical (St. Louis, MO, USA). Echimidine, heliotrine, intermedine, lasiocarpine, and senkirkine were purchased from PhytoLab (Vestenbergsgreuth, Germany). Senecionine, seneciphylline, and isoproturon-d_6 used as a recovery internal standard, were obtained from Fluka (Bűchs, Switzerland). The purities of these compounds were greater than 95%.

Stock solutions were prepared by dissolving 1 mg of the individual standards (crotaline, retrorsine, senecionine, seneciphylline, echimidine, heliotrine, intermedine, lasiocarpine, and senkirkine) in 1 mL of methanol and then diluting them with methanol when necessary. All of the stock solutions were stored at −20 °C until analysis. The solutions were kept in amber vials to protect the materials from photooxidation.

All of the foodstuff samples were supplied by markets near Seoul, South Korea. A total of 39 high lipid foodstuffs including 11 soybean, 10 seed oils, 10 milks, and 8 margarines were examined for the determination of PA levels. Soybean samples are *Arachis hypogaea, Cier arietinum, Canavalia gladiate*, *Phaseolus radiatus*, etc. Seed oils are *Borago officininalis* oil, safflower (*Carthamus tinctorius* L.) seed oil, soya oil, and sunflower (*Helianthus annuus*) seed oil. Goat milks, daily milks and margarines were purchased from markets.

2.2. Sample extraction and delipidation

Dried soybeans were pulverized with high sped mixer and the resulting powder was screened through 30 mesh. Ten grams each of milk and soybean powder were mixed and dissolved with 50 mL of chloroform-methanol (1:1, v/v) in a 250 mL round flask. For margarine and seed oil, 10 grams of sample were mixed with 50 mL of methanol. The extraction was carried out by sonication for 40 min in a 42 KHz ultrasonic bath (Branson 5510, Branson Ultrasonic Corp., Danbury, CT, USA) at room temperature. The extract was filtered with filter paper and then transferred into a 250 mL round flask. The extracted solvent was dried, redissolved in 50 mL of methanol for the milk and soybean extracts, and stored at −24 °C for 30 min to freeze the lipids. Most of the lipids precipitated as a lump on the surface of the glassware. The cold extract at −24 °C was immediately filtered with filter paper to remove the lipids (See Fig. S1), and precipitated lipids on the surface of the glassware were re-dissolved in 50 mL of methanol to extract the PAs again by same

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