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Applications of LC-MS in the study of the uptake, distribution, metabolism and excretion of bioactive polyphenols from dietary supplements

Stephen Barnes [a,b,c,*], Jeevan K. Prasain [a,b,c], Chao-Cheng Wang [a,b], D. Ray Moore II [c]

[a] Department of Pharmacology and Toxicology, University of Alabama at Birmingham, Birmingham, AL 35294, Unites States
[b] Purdue University-University of Alabama at Birmingham Botanicals Center for Age-related Disease, Birmingham, AL 35294, Unites States
[c] University of Alabama at Birmingham Comprehensive Cancer Center Mass Spectrometry Shared Facility, Birmingham, AL 35294, Unites States

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Abstract

Specific and quantitative analyses of the bioactive components and their metabolites in body fluids are essential to assess the interaction between groups of compounds in dietary supplements and preparations of psychoactives. Reverse-phase LC separations combined with tandem mass spectrometry provide the necessary specificity and sensitivity. In this paper, applications of these methods are described for the analysis of isoflavones, salvinorin A, synephrine isomers and their metabolites in serum, urine and aqueous humor.

Keywords: Dietary supplements; Phytochemicals; Polyphenols; Psychoactives; Tandem mass spectrometry

Introduction

The use of dietary supplements in the USA has increased enormously following the passage of the Dietary Education Health and Safety Act (DSHEA) in 1994 (http://www.fda.gov/opacom/laws/dshea.html). Many of these dietary supplements are derived from plants and contain organic xenobiotics that are subject to the same issues of absorption, distribution, metabolism and excretion that control the effectiveness of synthetic pharmaceutical agents. Importantly, the plant compounds share the same transport systems and metabolizing enzymes. Therefore, interactions between dietary supplements and other pharmaceuticals are to be expected. To explore this issue it is necessary to examine the composition of dietary supplements as well as their metabolites in physiological materials (blood, urine and tissues).

The analysis of biologically relevant compounds both in their native forms in pharmaceutical or dietary supplement matrices and in physiological fluids and tissues has been enormously improved by the introduction of electrospray ionization (ESI) (Fenn et al., 1989). This method allows the facile transfer without damage of what in some cases are unstable organic compounds from the liquid to the gas phase at atmospheric pressure and at room temperature. This seemingly unlikely method has also had enormous impact in the study of peptides and proteins and deservedly earned John Fenn the 2002 Nobel Prize in Chemistry. When used in combination with reverse-phase HPLC, it offers all investigators a very powerful method for the analysis of compounds in complex biological matrices.

Many of the bioactive compounds in dietary supplements of botanical origin are conjugated with glycoside groups (Kudou et al., 1991; Coward et al., 1993). These are difficult to turn into volatile derivatives for gas chromatography analysis. LC-ESI-mass spectrometry has greatly simplified their measurement. Once the dietary supplements are ingested these conjugates are hydrolyzed either by intestinal bacteria or by enzymes in the cells lining the small intestine (Day et al., 1998, 2000). In most cases, they undergo substantial metabolism within the intestinal wall, particularly to form glucuronides (Sfakianos et al., 1997). Again, LC-ESI-mass spectrometry is highly suited to the analysis of these metabolites (Barnes et al., 1994; Sfakianos et al., 1997).

* Corresponding author. Department of Pharmacology and Toxicology, MCLM 452, University of Alabama at Birmingham, 1918 University Boulevard, Birmingham, AL 35294, Unites States. Tel.: +1 205 934-7117; fax: +1 205 934 6944.
E-mail address: sbarnes@uab.edu (S. Barnes).

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Compounds with psychoactive properties are found both in botanicals and dietary supplements as well as in pharmaceutical and illicit drug formulations. Each of these compounds are subject to similar issues regarding intestinal uptake, phase I and phase II reactions in the gut wall, liver and other tissues to form metabolites, and biliary and/or urinary excretion. Each of the metabolic forms is readily analyzed by LC-ESI-mass spectrometry.

The goal of this article is to illustrate some of the applications of LC-ESI-MS, in particular tandem mass spectrometry, to the analysis of dietary supplements and psychoactive drugs.

Materials and methods

Materials

The isoflavones daidzein and genistein were obtained as described previously (Peterson and Barnes, 1991, 1996). Glucuronidase/sulfatase from *Helix pomatia* and *m*- and *p*-synephrine were obtained from Aldrich-Sigma Chemical Co., St. Louis, MO. Salvinorin A was purchased from *Salvia divinorum* Research and Information Center, Malibu, CA. Other chemicals were the best grades available.

Methods

Blood samples were obtained from dogs fed a diet containing soy protein in an experiment performed at Nestle, St. Louis, MO. The sera (0.5 ml) were incubated in 0.3 M ammonium acetate buffer, pH 5, containing 40 units of β-glucuronidase/sulfatase overnight at 37 °C. After acidification with glacial acetic acid, the incubates were extracted twice with two volumes of n-hexane. The released aglycones were subsequently recovered by extraction with 2 ml of diethyl ether three times. The ether extracts were combined, evaporated to dryness and reconstituted with 80% aqueous methanol prior to analysis. Aliquots of the reconstituted extracts were analyzed using a 100×2.1 mm i.d. C_8 reversed-phase column with the isocratic solvent 40% acetonitrile–10 mM ammonium acetate at a flow rate of 200 µl/min. The eluate was passed into the IonSpray interface of a Sciex API-III triple quadrupole mass spectrometer (Concord, Ontario, Canada). The interface was operated in the negative mode with a needle potential of −2700 V. The orifice potential was set at −60 V. Spectra were recorded from m/z 20–500. In the multiple reaction monitoring (MRM) mode, parent ion/daughter ion pairs were observed in order to specifically measure individual isoflavones and their metabolites (Fig. 1). The transitions for the isoflavones daidzein and genistein and their metabolites dihydrodaidzein, *O*-desmethylangolensin and equol were m/z 253/223, 269/133, 255/149, 257/108 and 241/119, respectively (Coward et al., 1996; Smith et al., 1999).

The aqueous humor samples were obtained from rats treated either with AIN-76A diet without isoflavones or with the addition of 250 mg genistein to this diet. Animals were

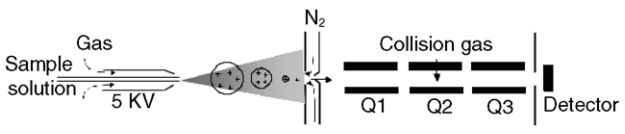

Fig. 1. Cartoon of the events in the analysis of ions in a triple quadrupole mass spectrometer. Ions formed by electrospray ionization enter the mass spectrometer through a narrow orifice. Specific molecular ions are selected by the first quadrupole filter and enter the collision cell in the second quadrupole. There they undergo fragmentation by collision with Argon. Measurement of all the resulting daughter ions in the third quadrupole produces a MS-MS spectrum. If a specific daughter ion is the only ion measured, then a quantitative MRM method can be developed.

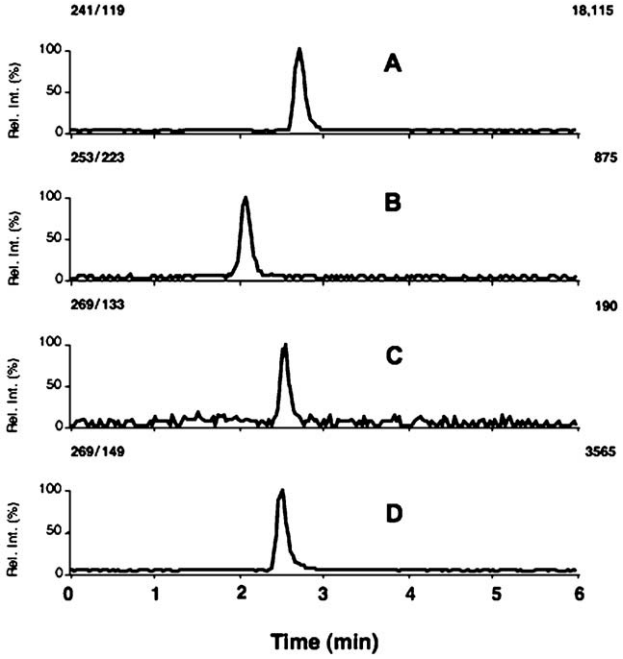

Fig. 2. MRM analysis of isoflavones in a dog serum. Following enzymatic hydrolysis and extraction, the isoflavones were subjected to isocratic LC-ESI-MRM-MS analysis. Signals from four of the channels measured are shown — the relative amounts of each compound are given by the full scale deflection value given in the top right hand corner — (A) equol, (B) daidzein, (C) genistein and (D) apigenin (internal standard).

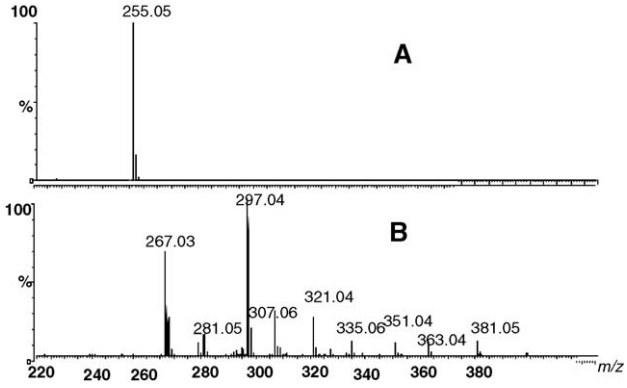

Fig. 3. The difference in fragmentation of O- and C-glycosides. Product ion spectra of protonated molecular ions of daidzin (A) and puerarin (B).

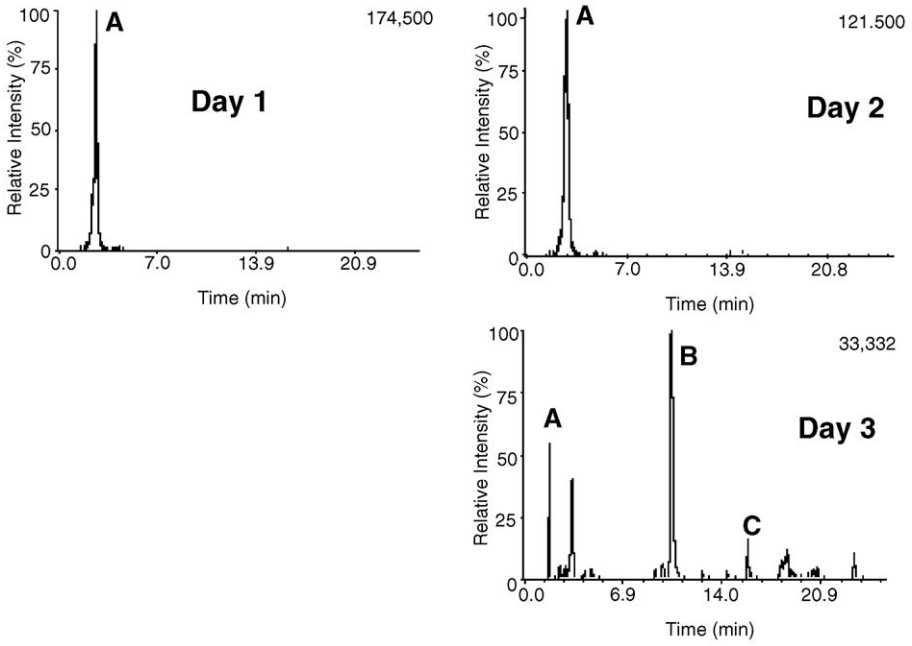

Fig. 4. LC-ESI-MRM analysis of urines obtained from rats orally administered with puerarin. Urines collected on days 1 and 2 only contain puerarin (A), whereas on day 3, daidzein (B) and its metabolite equol (C) are present in small amounts. The relative intensities are given by the figures in the top right hand corner of each total ion chromatogram.

allowed to feed and drink water ad libitum. At 75 days of age, the animals were euthanized by asphyxiation with CO_2. A flame-pulled glass capillary was inserted into the eye in front of the lens in order to collect aqueous humor. This fluid contained very low concentrations of protein, but contained physiological concentrations of NaCl. The aqueous humor (1–2 μl) was desalted using a membrane pre-concentrator placed between two pieces of 360 μm o.d./50 μm i.d. fused silica capillary and held together by a Teflon sleeve. The membrane was washed with 12 μl of 5% aqueous acetonitrile and the bound isoflavones eluted with 70% aqueous acetonitrile at 1 μl/min through a 40×0.18 mm C_{12} reverse-phase column and into the IonSpray interface of a Sciex API-III triple quadrupole mass spectrometer as described above for genistein 7-O-β-glucuronide, the transition was m/z 445/269.

A quantitative analysis procedure was established for two psychoactive compounds, *m*- and *p*-synephrine and salvinorin A. Chromatographic separation of synephrins was accomplished by gradient elution using a Develosil C_{30} column (2.0×150 mm). Solvent A and B consisted of 0.1% formic acid in water and acetonitrile, respectively. The gradient started with 0% B and was raised to 10% B over 10 min and returned back to 0% B with stop time 15 min. The ESI source was operated in the positive ion mode.

A stock solution of salvinorin A (1 mg/ml) was prepared by dissolving accurately weighed amounts in methanol. The calibration curve for serum samples was constructed by analyzing extracted control serum (extracted with diethyl ether) spiked with standard solution of salvinorin A. Calibration samples containing 20, 10, 5, 2.5, 1 and 0.1 μM were analyzed by MRM method using a Cadenza C_{18} column (4.6×150 mm)

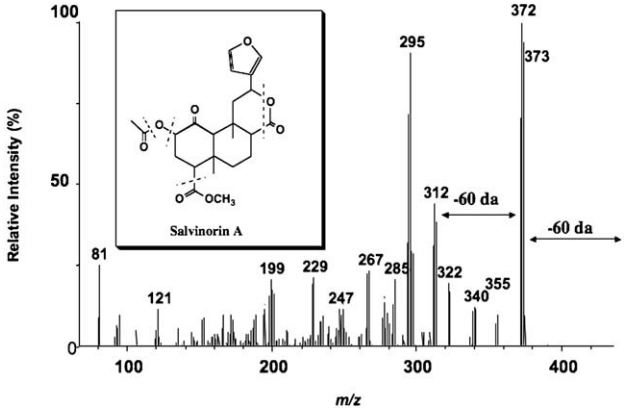

Fig. 5. Product ion spectrum of the salvinorin A protonated molecular ion at m/z 433. The sites of fragmentation are given in the figure in the inset.

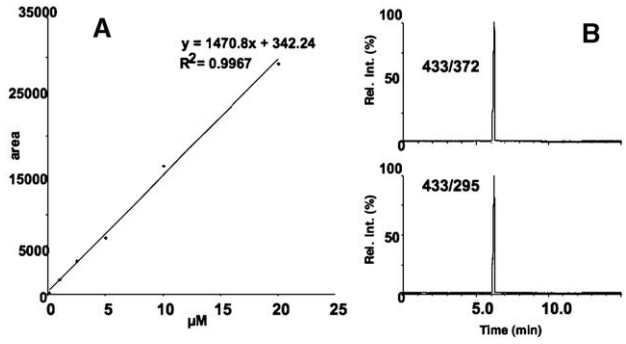

Fig. 6. Quantitative analysis of salvinorin A using LC-ESI-MRM-MS. A standard curve (A) of peak area versus concentration of salvinorin A has a correlation coefficient close to 1.00. The ion chromatograms for m/z 433/372 and 433/295 are presented in B.

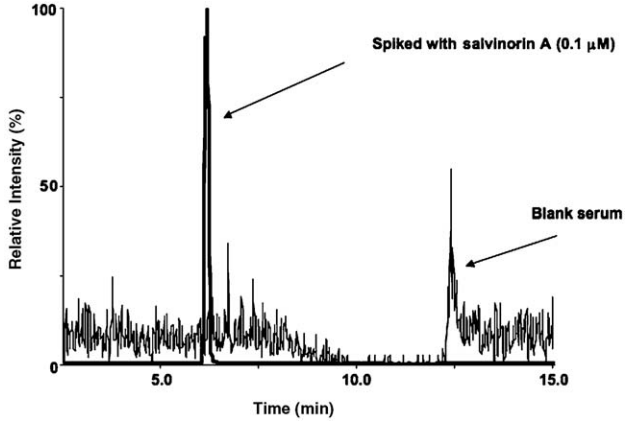

Fig. 7. A representative chromatogram of a serum sample spiked with salvinorin A showing specificity and selectivity of the method. The thicker line showing a single peak is the spiked sample containing 0.1 μM salvinorin A. The blank serum sample is presented at maximum sensitivity and contains no evidence of any signal at m/z 433/275 or m/z 433/372.

with gradient 40–100% acetonitrile containing 0.1% formic acid over 11 min.

Results

The use of the MRM approach allows for the quantitative measurement of individual isoflavones and their metabolites. The advantage of this method is that the combination of parent and daughter ions allows for specific detection of each compound without the need for their chromatographic separation (Fig. 2). This can be seen in the analysis of isoflavone metabolites in dog serum where equol was the predominant metabolite.

The isoflavones in soy are O-linked β-glucosides and are hydrolyzed prior to being absorbed. They are converted to their β-glucuronides, their predominant form in the blood and urine. The molecular ions of both of these glycosidic forms undergo

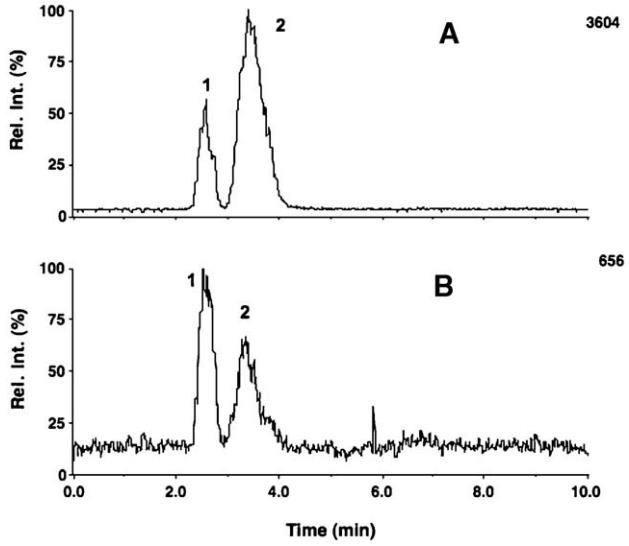

Fig. 9. Analysis of *m*- and *p*-synephrine by isocratic LC-ESI-MRM-MS with mass transitions of m/z 168/150 and 168/135. The ion chromatogram in A demonstrates the elution of *p*-synephrine (1) and *m*-synephrine (2) standards. The ion chromatogram in B was obtained from a commercial dietary supplement marketed to increase muscle fuel burning.

fragmentation to form aglycone daughter ions (Barnes et al., 1994; Sfakianos et al., 1997). In contrast, the isoflavones in kuzdu root dietary supplement are C-linked β-glucosides (Prasain et al., 2003). When subjected to collision-induced dissociation, the C-glycoside puerarin forms daughter ions containing fragments of the glycoside moiety (Fig. 3) (Prasain et al., 2004). This stability of the carbon–carbon bond is also observed physiologically — puerarin administered orally is absorbed without undergoing hydrolysis or metabolism (Prasain et al., 2004). Puerarin is the sole urinary metabolite detected on days 1 and 2 (Fig. 4). Only on the third day are puerarin metabolites observed. These analyses were carried using the MRM method.

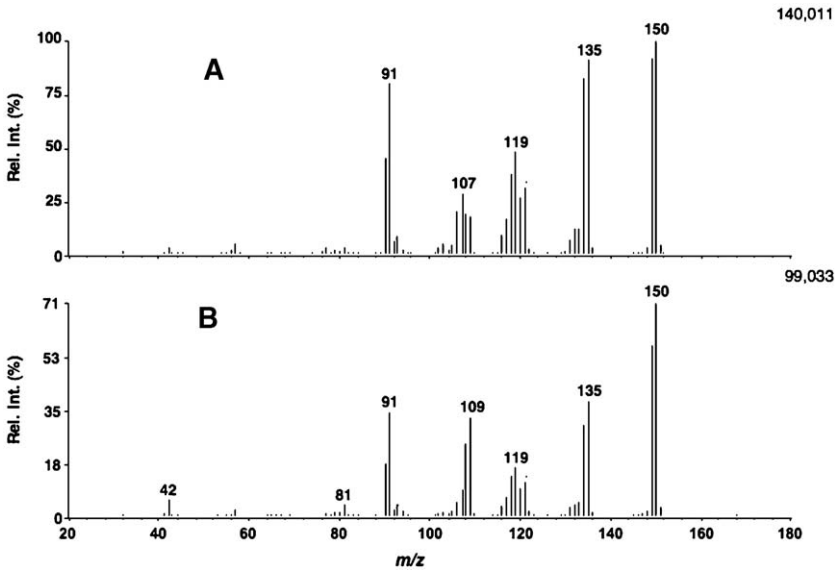

Fig. 8. Product ion spectra of protonated m/z 168 molecular ions of *p*-synephrine (A) and *m*-synephrine (B).

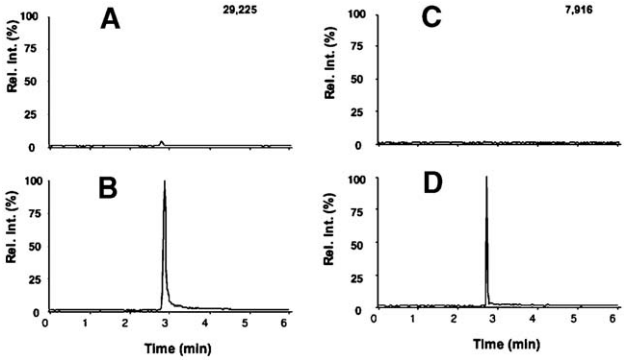

Fig. 10. Microanalysis of isoflavones in aqueous humor using LC-ESI-MRM-MS. Aqueous humor was obtained from animals on an isoflavone-free diet (A and C) and a diet containing 250 mg genistein/kg (B and D). The data in A and B are for unconjugated genistein; whereas those in C and D are for its β-glucuronide. The ion intensity for each pair is shown in the top right corner for each chromatogram.

These approaches can also be applied to psychoactive drugs. Salvinorin A is the bioactive component of *Salvia* that has hallucinogenic effects when its smoke is inhaled. Salvinorin A undergoes collision-induced dissociation to produce m/z 295 and m/z 373 [M-60] as the major daughter ions (Fig. 5). Combination of the molecular ion and these two daughter ions enables the development of an excellent MRM-based quantitative analysis method (Fig. 6). To demonstrate the specificity of the method, unspiked serum and serum (1 ml) spiked with 0.1 nmol of salvinorin A were analyzed by LC-ESI-MRM-MS (Fig. 7). No salvinorin A signal was observed for the unspiked sample, whereas the spiked sample contained a strong signal.

The *m*- and *p*-isomers of synephrine represent a more difficult challenge. They have the same molecular ion and almost identical product ion spectra (Fig. 8). In this case, although they can be differentiated from other organic compounds, they have to be chromatographically separated in order to be individually and specifically measured (Fig. 9).

Studying the penetration of psychoactive and/or dietary supplement bioactives into the brain requires investigation of fluids outside the blood compartment. One of these of current interest is the aqueous humor of the eye. While this fluid can be collected quite easily in microliter quantities, it contains a high salt concentration that interferes with the electrospray ionization process. The use of a micro pre-concentrator allows for the capture of the organic bioactives and the removal of the salts without dilution of the original sample. Using this system, it was found that rats fed the isoflavone genistein in their diet (250 mg/kg) had substantial accumulation of unconjugated genistein in aqueous humor (Fig. 10). Genistein β-glucuronide, the principal blood metabolite, was also present, but in 4–5 times lower concentration.

Discussion

This paper demonstrates the power of LC-ESI tandem mass spectrometry methods for the investigation of the uptake, distribution, metabolism and excretion of both dietary supplements and psychoactive agents. Unlike LC procedures based on the detection of eluted substances by absorbance, fluorescence or electrochemical methods, mass spectrometry is not only a universal detector, but also gives great specificity (Wang et al., 2002). This comes from its ability to detect the molecular ion of each compound, thus confirming its identification.

Tandem mass spectrometry resulting from the fragmentation of the molecular ion provides another level of confirmation of identity. It also allows the selection of parent ion/daughter ion combinations that permit the detection and quantification of the compound in question in complex matrices. Where there are several compounds to be analyzed with unique parent ion/daughter ion combinations, chromatographic separation is unnecessary. Analyses in this case are short and often can be carried out isocratically. This is not the case where the compounds are positional isomers and have the same parent ion/daughter ion combination. They have to be chromatographically separated.

Although LC-MS methods minimize the work up necessary for analysis of dietary supplements, psychoactives and their metabolites in drug formulations and physiological materials, it is important to remove the electrolytes that are present prior to analysis. This can usually be achieved in part by extraction into solvents, particularly those that are immiscible with water (ether or ethyl acetate). Further separation from the electrolytes occurs on the LC column so long as the dietary supplements and psychoactive compounds are not eluted too quickly. However, this can also be accomplished off-line using Zip-tips (columns containing microliter amounts of reverse-phase materials), or by using membrane micro pre-concentrators.

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