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Analytical Methods

A validated GC–MS method for the detection of tropane alkaloids in buckwheat (*Fagopyron esculentum* L.) fruits, flours and commercial foods

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ABSTRACT

A novel analytical method was developed and validated for the rapid and simultaneous detection of toxic tropane alkaloids (scopolamine, atropine) in commercial buckwheat (*Fagopyron esculentum* Moench.) samples and related food products, using gas-chromatography–mass-spectrometry in single-ion mode. A suitable and tailored protocol for extraction, sample clean-up and derivatization was set up in order to maximise recoveries and detection limits. The limits of detection for atropine and scopolamine were found to be 0.3 and 1 μg/kg, respectively, while limits of quantitation were obtained at 1 and 6 μg/kg, respectively, corresponding approximately to less than one *Datura stramonium* seed per million of buckwheat fruits, a ratio accepted by the European law on animal nutrition. The established method is considered suitable for the routine determination of traces of tropane alkaloids in flour or other buckwheat products for food and feed purpose and was applied to a variety of commercial samples and buckwheat-derived food products (pasta, porridge, crackers, and flakes). The protocol may be enforceable to other potential food and feed contaminants from the *Datura* genus (*D. innoxia*, *D. ferox*).

1. Introduction

Tropane alkaloids have a long history of food-related toxicological issues intertwined with an established hallucinogenic background, as mentioned in most traditional pharmacopoeias (Christen, 2000; Huxtable, 1992). While scopolamine and hyoscyamine are mostly biosynthesised by members of the Solanaceae family, atropine is spontaneously produced by hyoscyamine racemisation during extraction. The interest for the determination of these substances in feeds and foods stems not only from their presence in specific developmental stages of certain plants (e.g. *Solanum tuberosa* L.) or for their detection in nonedible parts of otherwise eatable species (e.g. *Lycopersicon* spp.), but also for the occurrence of food poisoning due to the accidental or mistaken presence of tropane alkaloid-rich plant material, most often represented by *Datura stramonium* L. (jimsonweed, thorn apple) seeds (Fretz et al., 2007; Kitano, Chatani, Ogawa, & Takemae, 2003; Van Raamsdonik, Vancutsem, & Jorgensen, 2009). In most temperate climates, annual *D. stramonium* and co-generic *D. innoxia* and *D. ferox* can easily thrive as infestants in a large number of crops and wherever weed management, post-harvest handling or controls are not adequately performed, some seeds may go undetected to subsequent stages of the food chain (EFSA, 2008). Albeit the whole plant is known to contain toxic alkaloids, seeds are usually deemed to be the most frequent responsible of contamination, due to their small size and to the simultaneous maturation of *Datura* and *Atropa* species with crops like grains, cereals, legumes and pseudocereals sharing the same cultural cycle (Friedman & Levin, 1989; Lawrence, Chandler, & Buchanan, 1994; Miraldi, Masti, Ferri, & Barni Comparini, 2001). As a consequence of the high sensitivity of mammalians to these anticholinergic alkaloids, both in their racemic or enantiomeric pure forms, even extremely low amounts of atropine derivatives may trigger severe poisoning symptoms (Müller & Wanke, 1998). Thus, given the sensible amount of tropane alkaloids (about 2 g/kg) in *Datura* and *Atropa* species, the presence of seeds must be as low as possible, that is less than one jimsonweed seed per million of crop seeds/fruits, according to European law. Following the promulgation and enforcement of Directives 2002/32/EC and 882/2004/EC on undesirable substances of plant origin in feeds and foods and their control, several episodes of contamination with jimsonweed seeds were reported as a consequence of microscopical observations involving wheat, maize, soybean, millet, linseed, sunflower, and rapeseed, with values near or slightly above the legal limit of 1 mg/kg. For red millet and buckwheat flour instead, sensible amounts of tropane alkaloids were detected after quantitative analysis, albeit no legal limit for these substances has been estab-

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lished at present time (Van Raamsdonik et al., 2009). Few years ago an outbreak of food intoxication involving more than 60 people and related to *Fagopyrum esculentum* Moench. (=*Polygonum fagopyrum* L.) flour contaminated with scopolamine and atropine was reported in Slovenia and a subsequent food security alert was issued through the European Community (Perharic, 2005). This contingency is of particular relevance for cultivations in which weed control is less strict and entrusted to laborious, manual operations like in organic agriculture.

Buckwheat derived raw materials (dehulled fruits, crushed seeds, flour) and byproducts are traditional food staples in Central Europe and Japan due to the good adaptation of *F. esculentum* to cold, continental climates, where it has represented for centuries an effective ersatz for cereals (Pomeranz, 1983). Its well-balanced nutritional properties, low-allergenic profile, the absence of gluten, a good protein and fibre content and the noticeable antioxidant properties, make it an attractive ingredient for niche products like baby food, infant formulas aimed at the reduction of infantile colics or gluten-free food for coeliacs (Catellier & Cherewyk, 2002; Li & Zhang, 2001; Zhang, 2005). The emergence of positive nutraceutical evidences of buckwheat enhanced flours may also enlarge the consumers' interest for baked buckwheat products, improving the market and increasing the need for a careful quality control. (Lin, Liu, Yu, Lin, & Mau, 2009).

A consistent number of analytical methods is available for the detection of tropane alkaloids in biological fluids, in order to spot *ex post* the potential causes of human and animal poisoning (Balikova, 2002; Cherkaoui, Mateus, Christen, & Veuthey, 1997; Kintz, Villain, Barguil, Charlot, & Cirimele, 2006; Namera et al., 2002; Steenkamp, Harding, van Heerden, & van Wyk, 2004). However, with the sole exception of the report of an HPLC-based method, at present time no validated, low-cost, accurate and easy-to-perform protocol is available for routine control of tropane alkaloids contamination in buckwheat derived products aimed at human or animal consumption (Rancic & Spasic, 2009). This consistently lowers the possibility to monitor and prevent this kind of food poisoning. Thus, our study was aimed at optimising and validating a simple and reliable gas-chromatography–mass-spectrometry method in selected-ion mode (SIM) for the analysis of tropane alkaloids in *F. esculentum*. In order to provide a method enforceable to quality control of both plant material and derived products, a specific goal was the achievement of a limit of detection as near as possible to 1 ppb, in accordance with the only regulation currently in force (Van Raamsdonik et al., 2009).

2. Materials and methods

2.1. Chemicals and solvents

Standards of nicotine, racemic atropine and scopolamine were purchased from Sigma–Aldrich (Milan, Italy); Hexamethyldisilazane was purchased from Fluka, KOH from Prolabo (Milan, Italy) and all solvents from Carlo Erba Reagenti (Milan, Italy).

2.2. Plant and food material

D. stramonium dried seeds and *F. esculentum* dried fruits utilised for method setup were collected from cultivated plants kindly provided by Giardino delle Erbe Officinali, Casola Valsenio (Ravenna, Italy). Fruits were dehulled by hand, separating the husk from the seed. Sixteen commercial products containing different amounts of buckwheat, namely entire fruits, flours, crackers, flakes, porridge and pasta were also acquired from local groceries. Both plant and food material were finely ground to pass a 0.2 mm mesh and subsequently kept at −20 °C and in the dark until analysis.

2.3. Preparation of contaminated samples

In order to obtain artificially contaminated samples of buckwheat flour, *D. stramonium* seeds were mixed with buckwheat fruits and then ground. Different levels of contamination were tested: 10, 5, 1, and 0.1 g/kg. These samples were used to setup the extraction method.

2.4. Standard solutions for quantification

Appropriate amounts of nicotine (internal standard), scopolamine and atropine were weighed and added to HPLC-grade methanol (10 ml) in order to yield final stock solution concentrations of about 1000 mg/l. Further dilutions were obtained adding adequate amounts of HPLC-grade methanol for each individual stock solution in order to obtain working solutions at 10000, 1000, 100, 50, 10, and 1 μg/l. These solutions were used alone or pooled to prepare samples used in method validation. Stock and working solutions were kept refrigerated (4 °C) when not in use and replaced on an as-needed basis.

2.5. Preparation of spiked samples

One gram of buckwheat flour was spiked with the appropriate amount of atropine and scopolamine solutions in order to obtain a final concentration of alkaloids of 1000, 500, 100, 50, 10, and 1 μg/kg. These samples were utilised to perform the optimisation and validation of the method.

2.6. Extraction and derivatization method

Five grams of buckwheat samples were defatted twice with 60 ml of hexane in an automatic Soxhlet apparatus for 60 min. One gram of defatted plant or food material was then added to 1 ml of a 1 μg/l standard solution of nicotine as internal standard, alkalinized with 0.5 ml of KOH (5% in methanol), then subjected to static extraction for 24 h, with 7 ml of CH_2Cl_2. The extract was then filtered and taken to dryness under vacuum, washed with 2 ml of hexane, dissolved in 2 ml of CH_2Cl_2, transferred in a vial, dried under nitrogen flow, added to 0.1 ml of hexamethyldisilazane and then kept at 80 °C for 15 min. The hexane fraction was checked in order to verify a possible loss of analytes, with negative results.

2.7. Chromatographic apparatus and conditions

GC–MS analysis was performed on a 6890 N gas chromatograph coupled to an 5973 N mass selective detector (Agilent technologies, Santa Clara, CA) with a DB-5MS-UI 30 m × 0.25 mm × 0.25 μm (Agilent J&W) capillary column (temperature: 60 °C for 3 min, 10 °C/min until 160 °C, 5 °C/min until 280 °C, 280 °C for 5 min). Solvent delay: 6 min. Injection mode: splitless, 0.2 min. Injector temperature: 280 °C. Injected volume: 1 μl. Acquisition mode: scan (*m/z* 41–500), SIM at 84, 162 *m/z* up to 13.5 min for nicotine, then at 124, 361 *m/z* for atropine and 138, 375 *m/z* for scopolamine. Under these conditions the retention times of atropine and scopolamine were, respectively, 28.31 and 30.22 min.

2.8. Linearity, limit of detection and quantification

Linearity of the method was checked at a range between 1 (detection limit) and 1000 μg/kg for each tropane alkaloid and six concentration levels (1, 10, 50, 100, 500, 1000 μg/kg) were considered. Three replicates were performed for each concentration. Each solution was added to a 'blank' buckwheat sample, together with