

# QuEChERS Application to the Determination of Pyrrolizidine Alkaloids

Subjects: [Food Science & Technology](#)

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The identification of concerning high levels of pyrrolizidine alkaloids (PAs) in a wide variety of food products has raised the occurrence of these natural toxins as one of the main current issues of the food safety field. Consequently, a regulation with maximum concentration levels of these alkaloids has been published to monitor their occurrence in several foodstuffs. According to legislation, the analytical methodologies developed for their determination must include multiresidue extractions with high selectivity and sensitivity, as a set of 21 + 14 PAs should be simultaneously monitored. However, the multiresidue extraction of these alkaloids is a difficult task due to the high complexity of food and feed samples. Accordingly, although solid-phase extraction is still the technique most widely used for sample preparation, the QuEChERS method can be a suitable alternative for the simultaneous determination of multiple analytes, providing green extraction and clean-up of samples in a quick and cost-effective way.

QuEChERS

sample preparation

food safety

Pyrrolizidine alkaloids

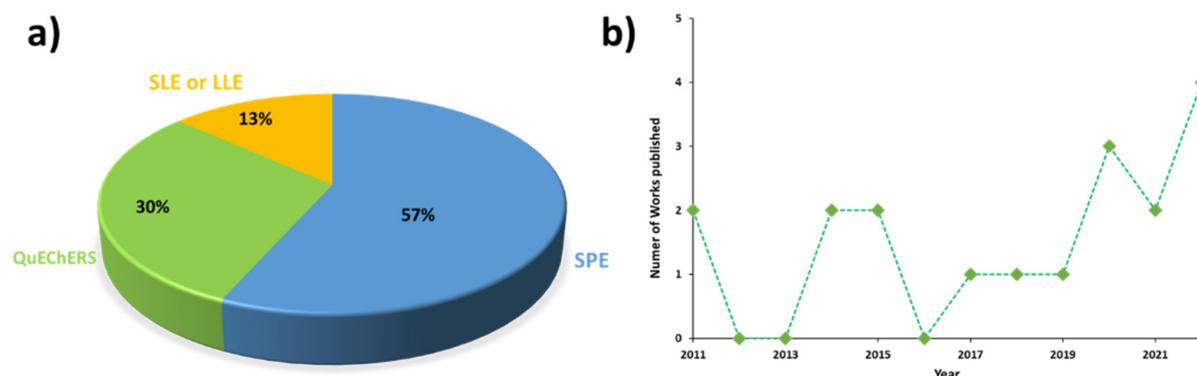
## 1. Introduction

In recent years, the awareness about natural toxins of plant origin in food and feed, such as pyrrolizidine alkaloids (PAs), has risen as one of the main issues of food safety. PAs are probably the most widely extended natural plant toxins, as more than 600 different chemical structures of these alkaloids have been identified from over more than 6000 plant species, being the main PA-producing plants belonging to the families Asteraceae, Fabaceae, Boraginaceae, Orchidaceae and Apocynaceae <sup>[1]</sup>. In this sense, these alkaloids can be introduced into the food chain from different vegetables and botanical sources. In some cases, these PA-producing plants are directly consumed by animals (through forage) and humans (e.g., borage, salads, food supplements, teas and herbal infusions made of PAs producing plants, such as chamomile or rooibos, etc.). However, currently, the major sources of PA consumption in animal and humans seem to be feed and plant-derived products contaminated with PA-producing plants, as many of these plants grow in fields as weeds, leading to the contamination of food crops <sup>[2]</sup> <sup>[3]</sup>. Accordingly, several different contamination paths have been reported, such as cross-contamination during harvesting processes, natural horizontal transfer through soil, as well as food fraud and adulteration <sup>[2]</sup> <sup>[3]</sup> <sup>[4]</sup>. As a consequence, many food alerts have notified in the last years high levels of these alkaloids in a wide variety of food products: spices and aromatic herbs (57% of the food alerts notified), teas and herbal teas (15% of the food alerts notified), food supplements (13% of the food alerts notified), herbs (8% of the food alerts notified), pollen (7% of the food alerts notified) and honey (1% of the food alerts notified) <sup>[2]</sup> <sup>[5]</sup>. Nonetheless, these alkaloids have also been

detected in other plant-derived products, such as cereals, flours and salads, as well as in some animal-derived products such as milk and dairy products, eggs, meat and meat products [2][6][7][8][9]. However, contamination of PAs in products of animal origin is less frequent, and the concentration levels found of these alkaloids are often low in this type of foodstuff. The intake of PAs represents a potential health risk, as they are known to produce both acute and chronic effects. In this sense, the ingestion of these alkaloids is mainly associated with liver damage (hepatic veno-occlusive disease (HVOD), liver cirrhosis and liver failure), but they can also produce genotoxic and carcinogenic effects at long-term exposure. Some of them (monocrotaline, riddelliine, and lasiocarpine) have been classified as potential carcinogens to humans (Group 2B) by the International Agency for Research on Cancer (IARC) [10][11][12][13]. Therefore, due to the wide spread of these alkaloids in a large variety of food products and the potential health risk that their frequent intake may entail for consumers, the analytical control of these alkaloids in food and feed is of utmost importance and constitutes a matter of interest. For this reason, in December 2020, the European Commission published a regulation amending Regulation (EC) No. 1881/2006 to monitor the occurrence of PAs in some food products [14]. This regulation sets maximum total concentration levels of PAs (ranging from 1.0 to 1000 µg/kg) in different foodstuffs, including: tea and herbal infusions, herbal food supplements, pollen-based food supplements, pollen, pollen products, dried herbs and cumin seeds [14]. Moreover, according to this legislation, every analytical methodology used to monitor these contaminants in food or feed must include the analysis of 21 PAs (including their *N*-oxide forms, PANOs). At the same time, 14 additional PAs can be considered if the chromatographic method employed enables the individual separation and identification of them without coelution problems, as they are isomers of one or more of the previous mentioned 21 PAs that are known to co-elute with some of them [14]. The coelution of these isomers is one of the main issues in the analysis of PAs. Consequently, powerful and efficient methods are required to perform the determination of PAs, which must include multiresidue extraction with high selectivity and sensitivity, as well as being quick and environmentally friendly procedures. However, the multiresidue extraction of these natural toxins is a difficult task, as they can be subjected to multiple matrix interferences that hamper their extraction and identification due to the high complexity of food and feed samples. In this sense, the QuEChERS (acronym of quick, easy, cheap, effective, rugged and safe) strategy can be a suitable approach for the determination of multiple analytes at the same time, as it enables the simultaneous green extraction and clean-up of samples before their instrumental analysis by gas chromatography (GC) or liquid chromatography (LC) [15][16].

The QuEChERS method was first proposed by Anastassiades et al. (2003) [17] and afterward validated by Lehotay et al. (2005) [18] for the simultaneous multiresidue extraction of a wide variety of pesticides (covering a broad range from non-polar to polar compounds) from fruit and vegetable samples. Since then, this method has gained great popularity, expanding its application range to other matrices and analytes due to its inherent advantages (quick, cheap, simple and user-friendly) and high throughput extraction efficiency [15][16]. However, despite its multiple advantages and compliance with the green analytical chemistry (GAC) principles, this method has scarcely been applied in the determination of PAs in contrast with other extraction and purification conventional methods, such as solid-phase extraction (SPE) [2][13]. In this sense, despite that conventional SPE requires more time and reagents to be performed, 58% of the published works that carry out the determination of PAs in food and feed samples used this technique, followed by 29% of works that apply the QuEChERS strategy, whereas only 13% of the

articles only perform solid–liquid or liquid–liquid extraction (depending on the sample) without clean-up or purification steps (**Figure 1a**). Nevertheless, a slight increase in the number of works published using QuEChERS for the determination of PAs has been observed in recent years (**Figure 1b**). Although the general trend shows great fluctuations over the last 11 years in its application (**Figure 1b**), it is expected that its increase will continue in the coming years.



**Figure 1.** (a) Overview of the main extraction methods used for pyrrolizidine alkaloids from food and feed, and (b) evolution of the number of published articles using QuEChERS for the determination of pyrrolizidine alkaloids in food and feed samples over the last 11 years (2011–2022). Data obtained from Scopus, Web of Science and Google search engines up to March 2022. LLE: liquid–liquid extraction; SLE: solid–liquid extraction; SPE: solid–phase extraction.

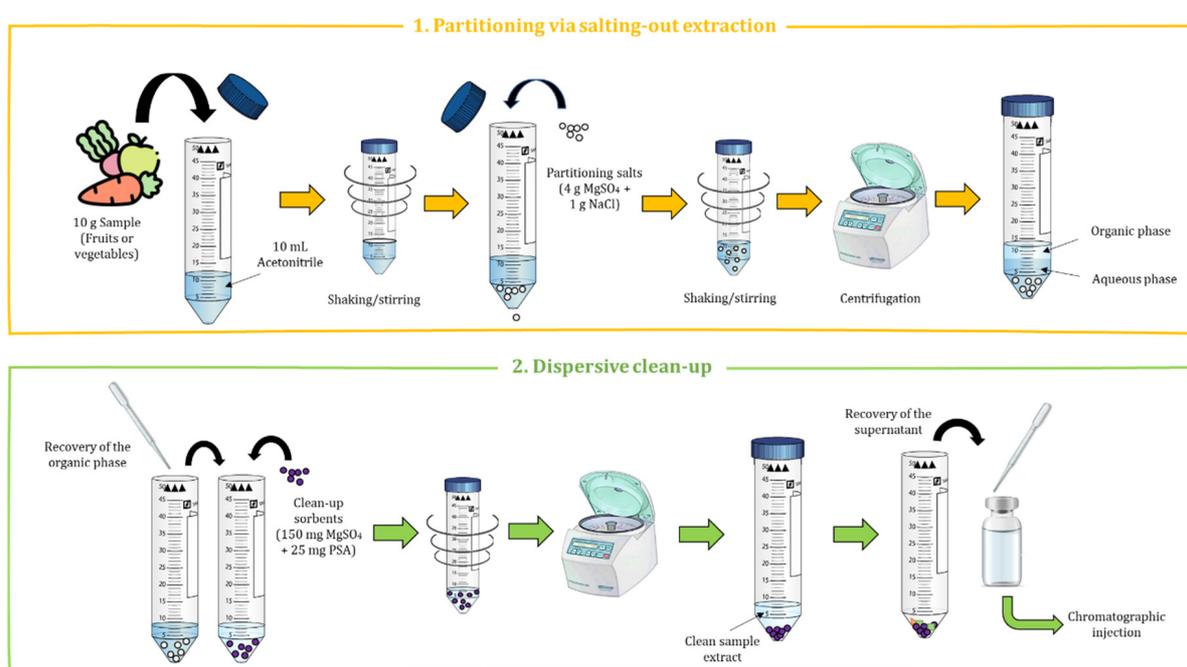
## 2. Basis of the QuEChERS Method

The QuEChERS was designed as a multiresidue approach for the determination of multiple analytes (more than 200 pesticides) at the same time, involving simultaneous extraction and clean-up of samples (particularly, fruit and vegetables) [17]. This method is based on the dispersion of salts (salting-out effect) to extract and isolate a wide variety of analytes from complex matrices in addition to the subsequent clean-up of the sample extract obtained. In this sense, there are two clear steps in this approach:

- (i) Extraction step based on partitioning via salting-out extraction, achieving an equilibrium between an aqueous and an organic phase.
- (ii) Clean-up step carried out by dispersive solid-phase extraction (dSPE) using different sorbent materials and salts to remove matrix interferences.

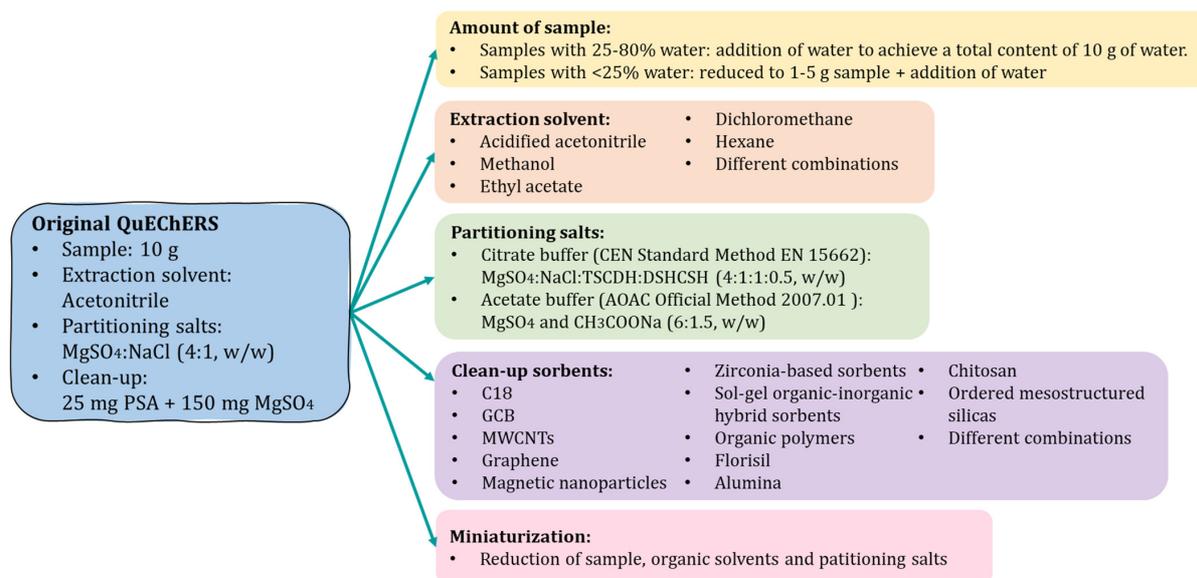
The original QuEChERS procedure involves using 10 g of sample, 10 mL of organic solvent (acetonitrile, ACN), 5 g of partitioning salts (4 g  $\text{MgSO}_4$  and 1 g NaCl) and 175 mg of clean-up sorbents (25 mg primary secondary amine (PSA) and 150 mg  $\text{MgSO}_4$ ) [17]. Accordingly, the sample is first subjected to a solid–liquid extraction (SLE) with ACN carried out by manual shaking and followed by the salt partitioning step with  $\text{MgSO}_4$  and NaCl to promote water partition from the organic layer and its dehydration. The addition of these salts enables a decrease in the

solubility of polar compounds in the aqueous phase and removal of water from the organic phase. To achieve efficient and homogeneous interaction among the salts, the organic solvent and the sample, a stirring process followed by centrifugation is carried out, which allows for the separation of both phases. Afterward, an aliquot of the supernatant corresponding to the organic phase is recovered for the subsequent dSPE clean-up step. PSA is a weak anion exchange sorbent; thus, it can interact strongly with the acid matrix interferences that may have been co-extracted during the process, such as sugars, fatty acids and organic acids, promoting their elimination from the ACN phase. Conversely, the addition of  $MgSO_4$  in the clean-up step removes the residual water content in the extract and improves the interaction of the above-mentioned matrix interferences on the PSA sorbent, leading to a final extract with less polarity due to the precipitation of the polar interferences which improves their retention in the PSA sorbent. After a brief shaking and centrifugation, the supernatant is recovered and can be directly analyzed by GC or LC [15][16][17][18]. **Figure 2** shows the schematic layout of the original QuEChERS procedure.



**Figure 2.** Schematic layout of the original QuEChERS procedure with its two differentiated steps.

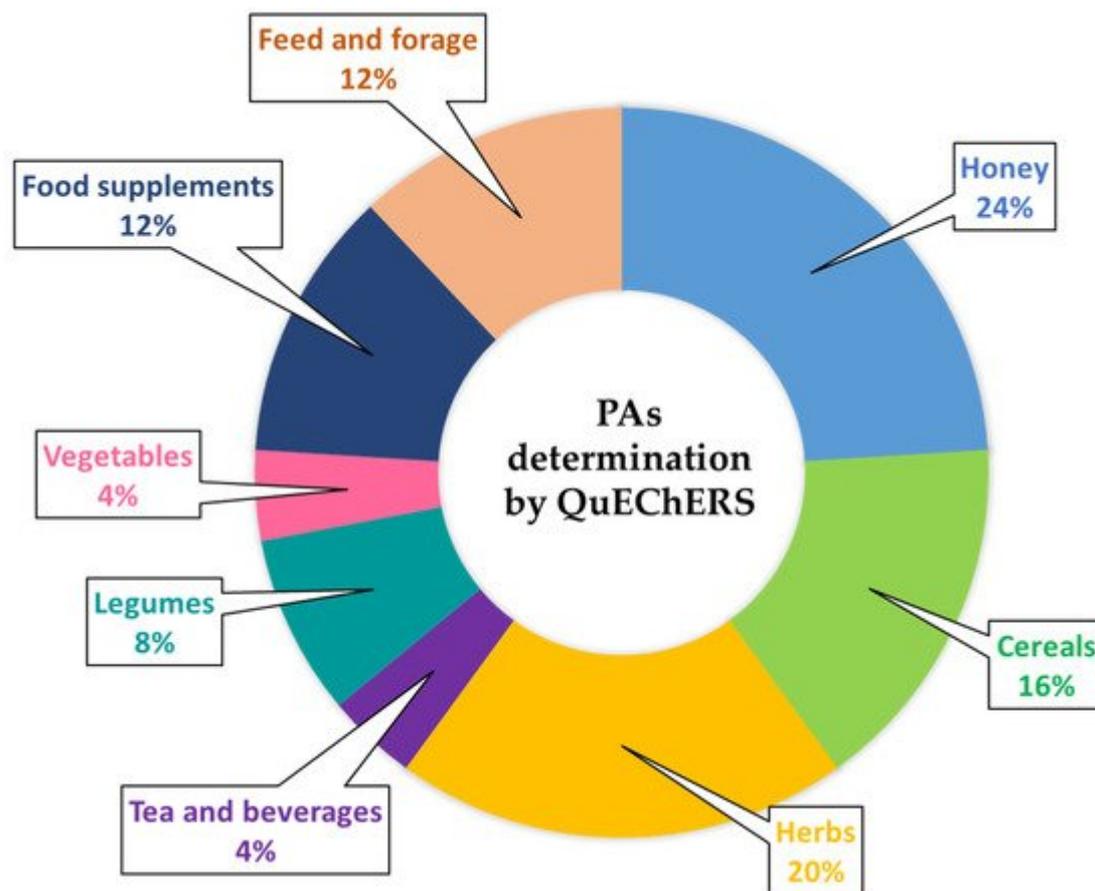
Nevertheless, since its origin, despite its high efficiency, the QuEChERS concept has evolved to be adapted to other analytes and matrices. In this sense, due to its flexibility, several modifications have been successfully introduced in its key parameters in order to improve its extraction efficiency, to spread its potential application to a wide range of matrices and to achieve simultaneous extraction of multiple compounds belonging to different chemical families [15][16]. Some of the most notable modifications in the QuEChERS procedure are depicted in **Figure 3**.



**Figure 3.** Most notable modifications of the original QuEChERS procedure. PSA: primary secondary amine; TSCDH: trisodium citrate dihydrate; DSHCSH: disodium hydrogen citrate sesquihydrate; C18: octadecylsilane, GCB: graphitized carbon black; MWCNTs: multiwalled carbon nanotubes.

### 3. Evolution of the Original QuEChERS Method and Its Application to the Determination of Pyrrolizidine Alkaloids in Food and Feed Samples

**Table 1** summarizes the different QuEChERS strategies carried out within the last 11 years for the extraction and analysis of PAs and PANOs in different food and feed products. As it can be observed, this strategy has been mainly applied to the determination of PAs in honey samples, followed by the analysis of herb samples (highlighting oregano) and (herbal) teas (including both the dry product and the beverage) (**Figure 4** and **Table 1**), likewise, its application to cereal samples, such as wheat, sorghum and quinoa. In contrast, to a lesser extent, it has been applied to the analysis of legumes (pea and soy) and vegetables (leek) (**Table 1** and **Figure 4**). It has also been used in products employed as food supplements, such as pollen, and in feed and forage samples (**Table 1** and **Figure 4**).



**Figure 4.** Overview of the different food and feed matrices used as samples for the determination of pyrrolizidine alkaloids by modified QuEChERS method over the last 11 years (2011–2022). Data obtained from Scopus, Web of Science and Google search engines up to March 2022.

**Table 1.** Application of modified QuEChERS to the determination of pyrrolizidine alkaloids in food and feed samples.

Sample (Amount)	Analytes	QuEChERS			Analysis	Recovery (%)	LOQ	Ref.
		Extraction Solvent	Partition Salts	Clean-Up				
Honey (1.5 g)	9 PAs	LLE with 10 mL H <sub>2</sub> SO <sub>4</sub> (0.1 M), addition of zinc dust. Supernatant with 10 mL ACN	4 g MgSO <sub>4</sub> 1 g TSCDH 0.5 g DSHCSH 1 g NaCl	150 mg PSA 900 mg MgSO <sub>4</sub>	HPLC-Q-Orbitrap-MS/MS ESI positive ion mode HRMS mode Column: C8 at 35 °C	92–115	0.1–0.7 µg/Kg	[19]
Honey (2.5 g)	9 PAs	LLE with 10 mL H <sub>2</sub> SO <sub>4</sub> (0.05 M), addition of zinc dust.	4 g MgSO <sub>4</sub> 1 g TSCDH 0.5 g DSHCSH 1 g NaCl	150 mg PSA 45 mg C18	UHPLC-Q-MS ESI positive ion mode SIM mode	67–122	0.081–4.35 µg/Kg	[20]

Sample (Amount)	Analytes	QuEChERS			Analysis	Recovery (%)	LOQ	Ref.
		Extraction Solvent	Partition Salts	Clean-Up				
		Supernatant with 10 mL ACN		900 mg MgSO <sub>4</sub>	Column: C8 at 34 °C			
Honey (1 g)	16 PAs/PANOs	Dilution with 4 mL water, followed by 4 mL ACN	0.8 g MgSO <sub>4</sub> 0.2 g TSCDH 0.1 g DSHCSH 0.2 g NaCl	dSPE (500 mg MgSO <sub>4</sub> )	HPLC-TQ-MS/MS ESI positive ion mode Column: C18	97–105	1–50 µg/Kg	[21]
Honey (5 g)	7 PAs/PANOs	10 mL water followed by 10 mL ACN	4 g MgSO <sub>4</sub> 1 g NaCl	-	HPLC-QTRAP-MS/MS ESI Positive ion mode and MRM mode Column: C18	50–100	-	[22]
Honey and herbal beverage (1 g)	7 PAs	1 mL water followed by 5 mL ACN	1 g NaCl	50 mg PSA	UPLC-IM-QTOF-MS/MS ESI Positive ion mode HDMS <sup>E</sup> mode Column: C18 at 50 °C	61–120	1–20 µg/Kg	[23]
Bottled tea (4 mL) and tea leaves (2 g)	27 PAs/PANOs	<i>Bottle tea:</i> 4 mL of ACN with 1% formic acid <i>Tea leaves:</i> 12.5 mL water followed by 10 mL of ACN with 1% formic acid	<i>Bottle tea:</i> approximately 2.2 g MgSO <sub>4</sub> , 0.6 g TSCDH, 0.2 g DSHCSH, 0.6 g NaCl <i>Tea leaves:</i> 4 g MgSO <sub>4</sub> , 1 g TSCDH, 0.5 g DSHCSH, 1 g NaCl	<i>Bottle tea:</i> - <i>Tea leaves:</i> 150 mg PSA, 45 mg C18, 900 mg MgSO <sub>4</sub>	UHPLC-TQ-MS/MS ESI Positive ion mode MRM mode Column: C18 at 40 °C	88–107	2–88 ng/L 0.10–1.61 µg/Kg	[24]
Teas and herbs (1 g)	28 PAs/PANOs	30 mL ACN:water (75:25, v/v) with 0.5% formic acid	6 g MgSO <sub>4</sub> 1.5 g CH <sub>3</sub> COONa	400 mg PSA 400 mg C18 400 mg GCB 1200 mg MgSO <sub>4</sub>	HPLC-Q-Orbitrap-MS/MS ESI positive ion mode HRMS mode Column: C18 at 40 °C	87–111	5 µg/Kg	[25]

Sample (Amount)	Analytes	QuEChERS			Analysis	Recovery (%)	LOQ	Ref.
		Extraction Solvent	Partition Salts	Clean-Up				
Herbs (5 g)	30 PAs/PANOs	Addition of 10 mL water, followed by 10 mL ACN with 1% formic acid	4 g MgSO <sub>4</sub> 1 g TSCDH 0.5 g DSHCSH 1 g NaCl	200 mg graphene	HPLC-QTRAP-MS/MS ESI Positive ion mode and MRM mode Column: C18 at 40 °C	61–128	1 µg/Kg	[26]
Oregano (0.2 g)	21 PAs/PANOs	1 mL water followed by 1 mL ACN	0.4 g MgSO <sub>4</sub> 0.1 g TSCDH 0.05 g DSHCSH 0.1 g NaCl	25 mg PSA 150 mg MgSO <sub>4</sub>	UHPLC-IT-MS/MS ESI positive ion mode MRM mode Column: Polar C18 at 25 °C	77–96	0.5– 25.0 µg/Kg	[27]
Aromatic herbs (0.2 g)	21 PAs/PANOs	1 mL water followed by 1 mL ACN. Re-extraction with 0.5 mL of ACN prior to clean-up step	0.4 g MgSO <sub>4</sub> 0.1 g TSCDH 0.05 g DSHCSH 0.1 g NaCl	25 mg LP-MS-NH <sub>2</sub> 150 mg MgSO <sub>4</sub>	UHPLC-IT-MS/MS ESI positive ion mode MRM mode Column: Polar C18 at 25 °C	73–105	1.2– 9.9 µg/Kg	[28]
Herbal dietary supplements (1 g tablets, capsules and softgels, 10 g for liquids)	11 PAs/PANOs	<i>Tablets and capsules:</i> 10 mL deionized water with 2% formic acid, afterward 10 mL ACN. <i>Softgels:</i> defatted with 4 mL hexane, addition of 10 mL deionized water with 2% formic acid, afterward 10 mL ACN. Liquids: 10 mL ACN with 2% formic acid	4 g MgSO <sub>4</sub> 1 g NaCl	Softgels: 100 mg C18 and 300 mg MgSO <sub>4</sub>	UHPLC-Q-Orbitrap-MS/MS ESI positive ion mode HRMS mode Column: HSS T3 at 40 °C	70–120	≤50– 2500 µg/Kg	[29]
Food supplements, feed and honey (2.5 g)	14 PAs/PANOs	10 mL water followed by 10 mL ACN with 1% acetic acid	4 g MgSO <sub>4</sub> 1 g CH <sub>3</sub> COONa	-	HPLC-Orbitrap-MS ESI both positive and negative ion	-	-	[30]

Sample (Amount)	Analytes	QuEChERS			Analysis	Recovery (%)	LOQ	Ref.
		Extraction Solvent	Partition Salts	Clean-Up				
					mode Column: C18 at 35 °C			
Leek, wheat, and tea (10 g, 2 g and 1 g, respectively)	11 PAs/PANOs	Acidification with 10 mL Milli-Q water with 0.2% formic acid, followed by 10 mL ACN	4 g MgSO <sub>4</sub> 1 g NaCl	100 mg C18 300 mg MgSO <sub>4</sub>	HPLC-Q-Orbitrap-MS/MS ESI both positive and negative ion mode HRMS mode Column: polar-reversed phase at 25 °C	71–93	≤1–100 µg/Kg	[31]
Pea, soy, wheat flour and quinoa (1 g)	56 PAs/PANOs	10 mL water, followed by 10 mL ACN	4 g MgSO <sub>4</sub> 1 g TSCDH 0.5 g DSHCSH 1 g NaCl	-	UHPLC-Q-Orbitrap-MS/MS ESI both positive and negative ion mode HRMS mode Column: C18 at 50 °C	31–132	1 µg/Kg	[32]
Sorghum, oregano, and mixed herbal tea (1 g) <a href="#">[19]</a> <a href="#">[23]</a> <a href="#">[29]</a> <a href="#">[30]</a> <a href="#">[31]</a> <a href="#">[32]</a> <a href="#">[33]</a> <a href="#">[34]</a> <a href="#">[35]</a>	33 PAs/PANOs	10 mL MeOH:water:formic acid (60:39.6:0.4, v/v/v)	-	100 mg PSA C18 or zirconia-coated silica	UHPLC-QTRAP-MS/MS ESI Positive ion mode MRM mode Column: C18 at 50 °C	78–117	0.5–10 µg/Kg	[33]
Pollen (1 g)	20 PAs	SLE with 10 mL H <sub>2</sub> SO <sub>4</sub> (0.1 M), addition of zinc dust. Supernatant with 10 mL ACN	4 g MgSO <sub>4</sub> 1 g TSCDH 0.5 g DSHCSH 1 g NaCl	150 mg PSA 900 mg MgSO <sub>4</sub>	UHPLC-TQ-MS/MS ESI positive ion mode MRM mode Column: RP-MS at 40 °C	73–106	4.0–9.0 µg/Kg <a href="#">[19]</a>	[34]
Forage grass (1 g)	5 PAs	10 mL of ACN/ammonium carbonate (200	-	50 mg PSA	HPLC-TQ-MS/MS ESI positive	63–98	10 µg/Kg	[35]

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indicated for the analysis of PAs by GC, as PANOs cannot be analyzed with this technique because they are unstable at the temperatures needed for volatilization [\[2\]](#)[\[37\]](#)[\[38\]](#). Therefore, as PAs and PANOs are both toxicologically important and both forms need to be included in the analytical determination of these alkaloids [\[1\]](#)[\[14\]](#), this reduction procedure can be carried out to ensure the extraction and determination of both types. However, in all the works for the determination of PAs by QuEChERS extraction, none of them used GC for the instrumental analysis of the sample extracts obtained after the QuEChERS procedure (**Table 1**). In contrast, all of them used LC

Sample (Amount)	Analytes	QuEChERS			Analysis	Recovery (%)	LOQ	Ref.
		Extraction Solvent	Partition Salts	Clean-up				
		mg/L), 84:16 (v/v, pH 8.5) solution.			ion mode MRM mode Column: C8 at 30 °C			
Feed (2.5 g)	5 PAs	10 mL ACN followed by 10 mL 0.1% formic acid in water	4 g MgSO <sub>4</sub> ·1 g NaCl	-	UHPLC-TQ-MS/MS ESI Positive ion mode MRM mode Column: C18 at 40 °C	72–98	5 µg/Kg	[2], [36], [20]

Nonetheless, some works also indicated the simultaneous determination in both positive and negative ion mode [30][31][32]. However, the reason is that in these works, in addition to PAs, other compounds are also included in the chromatographic analysis, as previously indicated (e.g., plant toxins, mycotoxins, pesticides, phytoestrogens, etc.). Thus, the ionization in negative ion mode is employed for the identification of these other analytes. Additionally, most of the works used multiple reaction monitoring (MRM) as detection mode (Table 1), as it is common for all types of analytes when MS/MS is performed. In a lesser extent, other authors have used high resolution mass spectrometry (HRMS) as a detection mode (Table 1), since they used a Q-Orbitrap as mass spectrometry analyzer, which is suitable for this type of detection. Moreover, in general, other compounds different from PAs are also simultaneously co-analyzed in the chromatographic method of these works. Therefore, the HRMS mode is suitable for this purpose, as it detects a high number of compounds with tentative analysis. Conversely, only one work carried out the analysis with selected ion monitoring (SIM) as detection mode, since a single quadrupole (Q) was used as a mass spectrometry analyzer for the detection [20].

Regarding the QuEChERS method, it can be observed that none of the works published in the literature for the determination of PAs in food and feed have carried out the original QuEChERS procedure, since they all present one or more modifications (Table 1). Nonetheless, despite the modifications made, good extraction efficiency has been achieved for the extraction of PAs, as in general satisfactory recovery values have been achieved with adequate sensitivity (Table 1). Some of the most notable modifications in the QuEChERS procedure and their application to the determination of PAs are described below.

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