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An Overview About Recent Advances in Sample Preparation Techniques for Pesticide Residues Analysis in Cereals and Feedstuffs

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Additional information is available at the end of the chapter

1. Introduction

Nowadays, more than 1100 pesticides are possibly used in various combinations and at different stages of cultivation and during postharvest storage to protect crops against a range of pests and fungi and/or to provide quality preservation. Pesticide residues in cereals samples, which might pose a potential risk for human health due to their sub acute and chronic toxicity, could possibly end up in the final products of crops. Contaminants of animal feed can cause harmful health effects in the animals and may be harmful to people through secondary exposure of consumers to products deriving from these animals. Contamination of feedstuffs may include both naturally occurring and synthetic toxic compounds [1].

International regulatory agencies have placed emphasis on the control of pesticides such that shall not contain residues of individual pesticides at levels exceeding regulatory maximum residue limits (MRLs), for example, 10 µg kg^{-1}. To analyzed a large number of pesticides in various food commodities consistently remain a challenge for analytical chemists [2].

Pesticides can be analyzed by gas chromatography (GC) with electron capture detection, flame ionization detection, or nitrogen-phosphorus detection and/or liquid chromatography (LC) with ultraviolet, diode array, fluorescence, or electrochemical detection. However, these techniques may lack the selectivity and/or sensitivity required to meet the requirements for analysis of residues due to the complexity of food matrices. These techniques have been largely replaced by GC and LC coupled to mass spectrometric techniques, especially they using tandem mass spectrometry [3].

The aim of this chapter is to present an overview about recent advances in sample preparation techniques that were developed for the determination of pesticide residues in cereals and feedstuffs by gas or liquid chromatographic methods. The different extraction and cleanup procedures were pointed out in this chapter with several applications related to the analysis of cereals and feedstuffs.

1.1. Pesticides residues in cereals and feedstuffs

During cultivation cereals are attacked by a great variety of pests, diseases and weeds. A key challenge to the protection of current production is the emergence of new pests and diseases, in addition to the spread of current diseases. Crop protection through pesticides has made a significant contribution to growth the cereals productivity since the 1950s. However, losses due to pests globally are still high. The extensions of these losses vary between countries and crops, but one estimate suggests an overall loss of around 40 per cent. Another more recent assessment suggests losses of 26 to 29% for soybean and wheat, and 30 to 40% for maize and rice. The same study suggests that losses for wheat could be as high as 50 per cent without effective plant protection, and even higher for other crops. Improved crop protection in the face of new pests and diseases, as well as resistant strains of current diseases, will rely on a variety of approaches. The well-managed use of different classes of pesticides (herbicides, fungicides, insecticides, etc) must continue to play a key role. In face of this, particular attention should be addressed to pesticide residues due to the common use of these compounds in agriculture [4].

Plant protection products may be ingested or absorbed by livestock in three ways: (1) following direct application of the product to the animal, (2) through residues in feeding stuff, (3) as a result of treatment of their accommodation. The usual source of residues is through the legitimate use of pesticides (herbicides, insecticides and fungicides) in the production of crops used in preparation of feeds. The need for information relevant to the conduct of risk profiles or for management of residues will always remain. Published data about pesticides residues on feed are very scattered and not easy to find. The results are not necessarily published and a compilation of feed monitoring data is still in the early stages [1]. The analysis of undesirable contaminants in various food and feed samples is nowadays a problem of primary concern for quality control laboratories due to human and animal health risks associated with the accumulation of these substances. Contaminants in animal feeding stuff can cause harmful health effects in the animals and may be harmful to humans through secondary exposure of consumers to contaminants deriving from these animals. In the European Union and also in several countries, feeding stuffs are subject to legislation covering their composition, manufacture, storage, transport and usage.

1.2. Aspects of analytical methods

In the last years, one of the current trends in analytical chemistry is the method development for optimized many tools used in classical methods. Fast analysis, consumption of small amounts of samples and reagents, high sensitivity and automation are

some of the most important goals desired to be achieved. For many years a large number of research laboratories and analytical instrument manufacturing companies have been investing their efforts in this field, which includes new sample preparation methods and rapid analysis. Nowadays, improved pesticides multiresidue analysis methodologies with high sensitivity and expanded scopes, which include as many compounds and commodities as possible in a single method, are always required for checking compliance with MRLs and/or for risk assessment of consumer exposure to pesticides. Otherwise, the multiresidue method development is difficult due to the fact that compounds of different polarity, solubility and volatility have to be extracted and analyzed simultaneously [5]. In practice, multiresidue methods consist of the following basic steps [6]:

i.   isolation of residues from a representative sample (extraction);
ii.   separation of co-extracted matrix components (cleanup);
iii. identification and quantification of target analytes (quantitative step), and if the need is important enough, this is followed by next step;
iv.   confirmation of results by an additional analysis.

The choice of sample treatment applied depends heavily on the complexity of the matrix. Water, in general, represents a less complicated matrix than air, sediment, soil or food samples. This choice is also related to the detection method. The more sensitive and detection method is used, the less stages of sample treatment will be required. Modern analytical strategies tend towards automatization and integration of sample pretreatment in the chromatographic systems as far as possible. Development of solventless (or at least with low solvent consumption) sample preparation techniques constitute a pillar of green analytical chemistry and have taken a rapid development during last year's. The great interest in this approach is due to toxicological, environmental and economical aspects [7].

For extraction, although different organic solvents, and mixtures of organic solvents, have been used to extract a wide range of compounds with different physico-chemical properties from food, the use of acetone, ethyl acetate, and acetonitrile has predominated in multiresidue methods. These solvents provide high pesticide recoveries over a wide polarity range; however, at the same time a lot of matrix components are co-extracted. To achieve required performance characteristics, cleanup techniques, are commonly employed for their removing. These procedures lead to increasing overall cost of the method, extending analysis time and requiring additional labor [6].

The difficulties of pesticide residue analysis in cereals and animal feed samples are caused by the needed of the elimination of chemically non-related main matrix components (e.g., organic matter, lipids, proteins) and, then, if required, by removal of other chemically related analytes that could interfere in the instrumental determination of the investigated compounds [8]. Feedstuffs are also burdened with large quantities of other components after extraction as animal feeds can be complex mixtures that include constituents such as grains, milling by products, added vitamins, minerals, fats, and other nutritional and energy sources. Even simpler cereal matrices contain much more co-extractants than typical matrices of high water content such as fruits and vegetables [1]. This fosters the

development of strategies to isolate/extract the pesticide fraction from the whole fatty matrix. In fact, it is very difficult to avoid the co-extraction of fatty material, even more, taking into account that some of the pesticides which are usually targeted are fat-soluble non-polar compounds (e.g. organochlorine), and tends to concentrate and remain in the fat. Since, high recoveries of most multiclass pesticides must be obtained in an ideally fat-free extract, an additional clean-up step is usually included prior to subsequent steps in the analytical process. Additionally, the exact composition of the sample is often unknown to the testing laboratory [9].

2. Sample preparation

Despite advances in the sensitivity of analytical instrumentation for the end-point determination of analytes in food samples, a pre-treatment is usually required to extract and isolate the target analytes from the food matrix, thus facilitating their determination [9]. Extraction of pesticides from food depends on their polarity and the type of matrix. Generally, it comprises homogenization of the sample with an organic solvent alone or mixed with water or pH adjusted, using an ultrasonic bath, a blender or a homogenizer [10]. In most cases, although the analytes of interest are isolated from the bulk matrix, several contaminants may also be co-extracted, as well as part of the matrix, which could interfere in the determination step of the analysis. After the extraction process, generally a clean-up procedure is carried out in order to remove the co-extracted compounds that may act as interferences during chromatographic analysis, causing problems in detection and quantitation of the analytes [11]. The clean-up step aims at the isolation of the target analytes from potential interfering co-extractives as well as discarding the extraction solvent and preparing the target analytes in an appropriate chemical form for its characterization and quantification. Therefore, pesticide residue analysis protocols involve two main stages: the isolation of the pesticides from the matrix (sample treatment) and the analytical method for the determination. Sample treatment, which involves both the extraction of the pesticides and the purification of the sample extract obtained, still remains as the bottleneck of the entire procedure, despite much progress on automation has been accomplished [9]. In food analysis, traditional methods for sample preparation are laborious, time consuming and usually involve large amounts of solvents, which are expensive, generate considerable waste, contaminate the sample and can enrich it for analytes. In addition, usually more than one clean-up stage prior to detection is required [12]. As a result, modern sample preparation procedures have been developed or improved to overcome the drawbacks of the traditional approaches. Growing concern over food safety necessitates more rapid and automated procedures to take into account the constant increase in the number of samples to be tested, so interest in procedures that are fast, accurate, precise, solventless, inexpensive and amenable to automation for on-line treatment is ongoing. Today special attention is paid to such analytical sample preparation procedures which ensure reduction of the amount of liquid solvents used or their complete elimination in the course of the analytical procedure. A great increase in interest in the so-called solventless method is the result of

both ecotoxicology (dumping residual solvents, usually highly toxic, into the environment) and economics (high purity solvents are expensive) [12].

Different studies have been described in the literature about the sample preparation and chromatographic determination of pesticide residues in food and feedstuffs and these results are described in this chapter.

2.1. Solid-liquid extraction

The first step in the pesticide residues analysis from semisolid and solid samples is usually the exhaustive extraction of the target compounds from the matrix in which they are entrapped. The essentially non-selective character of this initial treatment makes mandatory the subsequent purification of the obtained extract, first by elimination of matrix [8]. In the last decades, one of the most applied pesticide extraction technique from cereals was solid–liquid extraction (SLE). Before the SLE, solid samples are transformed into fine and homogeneous particles by mechanical grinding, mixing, rolling, agitating, chopping, crushing, macerating, mincing, pressing, or pulverizing. The homogenized solid samples are repeatedly extracted with an immiscible organic solvent, and the extracts are then centrifuged, concentrated and/or purified before the final analysis [10]. An important step in the preparation of food samples prior to final analysis is isolation and/or enrichment. The procedures consist of the transfer of analytes from the primary matrix into the secondary one with a concurrent purging of interfering substances (isolation) and increasing the analytes concentrations to a level above the detection limit for a given analytical technique (enrichment). In the case of organic contaminants, such as pesticides, in cereals samples, it is necessary to replace the solid matrix with a liquid one. For this purpose, an appropriate extraction method should be used. Conventional extraction of organic analytes from food samples usually begins with a homogenization step, followed by solvent extraction aided by shaking is based on the partitioning of analytes between liquid and solid phases [9]. When considering this technique, there are many inherent disadvantages, e.g., it is laborious and time-consuming, expensive and apt to form emulsion, it requires the evaporation of large volumes of solvents and the disposal of toxic and flammable chemicals. Moreover, a relatively large amount of matrix is required. Smaller sample sizes become important when dealing with real life problems, such as consumer complaints and alleged chemical contamination. Recent regulations pertaining to the use of organic solvents have made classical SLE unacceptable because of very large amounts of solvents used in this technique. For these reasons (to reduce the usage of solvents), many innovations can be found in analytical processes that can be applied to food preparation for extraction [13, 14]. This has resulted in the recognition that SLE can now be replaced with faster and less expensive techniques. These new approaches in pesticides residues extraction from cereals and feedstuffs samples were showed in the next reviewed sections.

2.2. QuEChERS

The QuEChERS (quick, easy, cheap, effective, rugged and safe) method was introduced by Anastassiades *et al.* [15] as a new approach to extract a wide range of pesticides from

different food matrices with high water content. This basic procedure is based on a liquid partitioning with acetonitrile followed by a dispersive solid phase extraction (d-SPE) cleanup with primary secondary amine (PSA). This procedure has been applied with success in several nonfatty (<2%) and low-fat (2–20%) food matrixes. In this method anhydrous magnesium sulphate is use to reduce water in the sample, along with either sodium chloride [16].

Since 2003, modifications to the original method to ensure efficient extraction of pH dependent compounds (by using different buffers solutions, e.g. acetate or citrate) [17, 18] or addition of water to dry samples (e.g. cereals) in order to obtain the necessary moisture have been introduced [19]. To remove matrix components in the clean-up step, modifications of the original d-SPE step by using graphitized carbon black (GCB) and C18 sorbent, SPE in cartridge or Florisil cartridges have been used. The QuEChERS method is particularly popular for determination of polar, middle polar and non-polar pesticide residues in various food matrices because of its simplicity, inexpensiveness, amenability to high throughput, and relatively high efficiency results with a minimal number of steps, enabling a laboratory to process significantly larger number of samples in a given time as compared to the earlier methods, e.g. liquid-liquid extraction (LLE) methods [7, 20]. QuEChERS offers several advantages over most conventional techniques because it does not require glassware or auxiliary equipment (e.g. vacuum manifolds), uses low volumes of solvent, generates little solvent waste and provides high recovery of analytes [21]. When compared with other sample preparation methods, it is clear that it is extremely fast and inexpensive. It has already received world wide acceptance because of its simplicity and high through put enabling a laboratory to process a high number of samples in a short period of time [20].

The Table 1 summarizes the results described below and another applications based on QuEChERS extraction method for pesticides residues determination in cereals and feedstuffs.

Walorczyk [22] employed a buffered QuEChERS method to prepare samples of cereals grain and some dry feedstuffs prior to the determination of 122 pesticides by GC-MS/MS. A 5 g finely ground sample was placed in centrifuge tube and 10 mL water were added. Later, 15 mL acetonitrile were added and the mixture shaken vigorously. Further, 0.5 g disodium hydrogen citrate sesquihydrate, 1 g trisodium citrate dihydrate, 4 g anhydrous magnesium sulphate, and 1 g sodium chloride were added, and the mixture was hand-shaken, then centrifuged. Afterwards, a 7.5 mL aliquot of the supernatant was transferred to a centrifuge tube (15 mL) containing 0.75 g anhydrous magnesium sulphate, 0.5 g C18 and 0.125 g PSA. The tube was vortexed and centrifuged. A 3 mL aliquot of the supernatant was transferred into a glass test tube, the extract was evaporated to nearly dryness under a stream of nitrogen and the residue was re-dissolved in 1.5 mL toluene prior to its injection into the GC-MS/MS system. Despite the fact that the method was found to be useful for the purpose of multiresidue screening as it permitted detection at low levels (0.01 mg kg^{-1}) for approximately 68% of the target pesticides, many recoveries and RSD values were not as good as required for validation in compliance with the criteria established by the European Union.

Analytes	Sample	Analytical technique	LOD (µg kg⁻¹)	Recovery (%) (RSD %)	References
140 multiclass pesticides	wheat, bran, feedstuffs	GC-MS/MS	-	70.0-120.0 (≤ 20)	[1]
24 multiclass pesticides	wheat, bran, flour	GC-MS	2.5	70.0-120.0 (≤ 20)	[18]
122 multiclass pesticides	wheat, bran, feedstuffs	GC-MS/MS	≤ 10.0	73.0-129.0 (1.0-29.0)	[22]
80 multiclass pesticides	wheat flour	GC-MS/MS and LC-MS/MS	-	70.0-110.0 (≤ 10)	[23]
171 multiclass pesticides	wheat flour	LC-MS/MS	-	70.0-120.0 (≤ 20)	[24]
simeconazole enantiomers	wheat and rice	GC-MS/MS	0.4-0.9	101.1-93.7 (2.4-5.7)	[25]
13 phenoxyacid pesticides	rice	LC-MS/MS	≤ 13.3	45.0-104.0 (≤ 13.3)	[26]
180 multiclass pesticides	corn, oat, rice, wheat	GC-TOF-MS and UHPLC-MS/MS	-	70.0-120.0 (≤ 20)	[27]
41 multiclass pesticides	maize	GC-MS	8.0-55.0	70.0-120.0 (≤ 20)	[28]
90 multiclass pesticides	wheat	LC-MS/MS	-	70.0-120.0 (≤ 20)	[29]
42 multiclass pesticides	polish rice	LC-MS/MS	0.07-4.0	70.0-120.0 (≤ 20)	[30]
151 multiclass pesticides	barley, basmati rice, rice flour, popcorn, wheat, seven grains, and buckwheat	UHPLC-MS/MS	-	81.0-110.0 (≤ 20)	[31]
23 multiclass pesticides	cereal based infant foods	LC-MS/MS	-	60.4-125.4 (≤ 29.7)	[32]
9 organophosphate and 1 pyrethroid	maize and soy	GC-MS	0.2-6.0	105.0-52.0 (4.6-8.2)	[33]
7 neonicotinoid pesticides	brown rice, millet, oat, maize	HPLC-DAD	2.0-5.0	76.0-123.0 (0.9-12.6)	[34]

Table 1. Different methods applied for determination of pesticide residues in cereals and feedstuffs based on QuEChERS extraction method.

In a new work Walorczyk [1] improved his previous method. The most important aspects considered during the present work were (i) extension of the scope of the previous method to include as many as 144 target analytes and (ii) re-design of the GC-MS/MS acquisition method. The linearity of the calibration curves was excellent in matrix-matched standards, and yielded the coefficients of determination $r^2 \geq 0.99$ for approximately 96% of the target analytes. Average recoveries of the pesticides spiked at 0.01 mg kg^{-1} into a feed mixture and wheat grain were in the range 70–120% with associated RSD values ≤ 20% for approximately 60 and 67% of the compounds, respectively. Base on these results, the proposed approach has been proven to be highly efficient and suitable for routine determinations of multiclass pesticides in a range of cereal and related matrices. In this study, 145 samples of matrices of differing complexity including cereals grain, bran, whole ears, straw, hay, feed mixtures and other samples such as malt, starch and dry vegetables have been analyzed. A total of 15 different compounds have been detected, among which pirimiphos methyl, deltamethrin, tolylfluanid, dichlofluanid and tebuconazole were the most frequently encountered ones.

Kolberg *et al*. [18] developed and validated a multiresidue approach also based on QuEChERS method for the determination of 24 pesticides in wheat, white flour and bran using triplequadrupole GC-MS in negative chemical ionization mode. The method was validated evaluating the following parameters: linearity, limit of detection, limit of quantification, matrix effect as well as precision and accuracy, evaluating the percentage of recovery at four different spike levels. The linear range used in the calibration curves was from 1.0 to 100 µg L^{-1} for wheat and 2.0 to 200 µg L^{-1} for flour and bran, both with values of $r^2 \geq 0.99$. The recoveries had been considered satisfactory presenting values between 70 and 120% with RSD < 20% for the majority of compounds.

Koesukwiwat *et al*. [26] modified the original QuEChERS method for the analysis of phenoxy acid herbicide residues in rice samples. The new approach was based on the extraction with 5% (v/v) formic acid in acetonitrile and inclusion of citrate buffer for helped partitioning of all the analytes into the acetonitrile phase. The extract was then cleaned up by d-SPE using C18 and alumina neutral as selective sorbents. Further optimization of sample preparation and determination allowed recoveries between 45 and 104% for all 13 phenoxy acid herbicides with RSD ≤ 13.3% at 5.0 µg kg^{-1} concentration level. Limit of detections (LODs) of 0.5 µg kg^{-1} or below were attained for all 13 phenoxy acids. Quantitative analysis was done by UHPLC-MS/MS in the multiple-reaction monitoring (MRM) mode using two combinations of selected precursor ion and product ion transition for each compound. This developed method when compared with original QuEChERS method produced relatively higher recoveries of the acid herbicides with a smaller range of variation and less susceptibility to matrix effects.

Otherwise, the main disadvantage of the QuEChERS method compared to other common methods is that the 1 g mL^{-1} final extract concentration is lower than the 2–5 g mL^{-1} concentrated extract of most traditional methods. If matrix is not the limiting source of noise in the analysis, this leads to a higher LOQ for the same injection volume in the QuEChERS method [3]. This method is adaptable and can be easily tailored to cope with new matrices

through the selection of alternative sorbents. In fact the initial extract can be divided across tubes containing different sorbents to cater for problem analytes. Work in progress indicates that the developed extraction conditions will recover the majority of food contaminants [35]. Although QuEChERS has mainly been used for the determination of pesticides, some other compounds, such as pharmaceuticals, or veterinary drugs have been determined using QuEChERS in other food and environmental samples [7, 36].

2.3. Pressurized-liquid extraction (PLE)

Also known as accelerated solvent extraction (ASE), pressurized fluid extraction (PFE), pressurized hot solvent extraction (PHSE), subcritical solvent extraction (SSE) and hot water extraction (HWE), the PLE utilizes solvents that are raised to the near supercritical region, where they show better extraction properties [37]. This effect is due the decreasing of surface tension and increasing of solubility and diffusion rate into the sample showed by the solvent at high temperatures. Pressure keeps the solvent below its boiling point and, in solid samples, like cereals, forces its penetration into the pores of the sample [12].

The faster extraction process (5-10 min) is obtained by the use of high pressures (500-3000 psi) and temperatures (50-200 °C). This technique requires smaller amounts of solvent compared with traditional extraction, this is a very positive advantage because reduces the dilution of the sample [38]. The main factor that's affects de extraction time and efficiency of extraction is the temperature meanwhile the sample mass [12]. There are three ways to perform PLE: static, dynamic (flow-through) and mixed mode. In static mode, the sample is enclosed in a stainless steel vessel filled with an extraction solvent, and following extraction the remaining solvent is purged with N_2 into a collection vial. Flow-through systems continuously pump solvent through the sample, but this has the disadvantage of using larger volumes of solvent and of diluting the extract. A desiccant, such as sodium sulphate, diatomaceous earth or cellulose can be added directly to the extraction cell or sorbent materials can be used to provide in situ cleanup [39, 40]. When compared with LLE, the PLE show as main advantage the low solvent consumption, but requires highly expensive and specialized equipment. The limitation of PLE is the presence of large amounts of co-extracted lipids when working with fatty matrices, which means that post-cleanup of the extract is required to carry out lipid elimination [12].

The effect of sample matrix depends on sample composition. Food samples can differ significantly in their physical-chemical properties, type of compounds present, or granulation (particle diameter). The factor that mainly affects the extraction of trace compounds in the presence of extractable major sample components are lipids, that offers special problems in the subsequent determination by gas or liquid chromatography [41].

Carabias-Martinez *et al*. [37] developed a method based on pressurized liquid extraction and liquid chromatography–electrospray ionization mass spectrometry (LC-ESI-MS) for the determination in cereal samples of seven endocrine-disrupting compounds: bisphenol A (BPA), 4-tert-butylbenzoic acid (BBA), 4-nonylphenol (NP), 4-tert-butylphenol (t-BP), 2,4-

dichlorophenol (DCP), 2,4,5-trichlorophenol (TCP) and pentachlorophenol (PCP). Methanol was selected as the extraction solvent. The recoveries achieved for the all seven compounds were in the 81 to 104% range, with relative standard deviations of 4 to 9%. The detection limits achieved in corn breakfast cereals were in the 0.003 to 0.043 $\mu g\ g^{-1}$ range.

A method based on pressurized liquid extraction and LC-MS/MS has been developed for determining nine benzoylureas in fruit, vegetable, cereals and animal products. Samples (5 g) were homogenized with diatomaceous earth and extracted in a 22 mL cell with 22 mL of ethyl acetate at 80 °C and 1500 psi. Method LOQs were between 0.002 and 0.01 mg kg^{-1}. Excellent linearity was achieved over a range of concentrations from 0.01 to 1 mg kg^{-1}. Validation of the method was performed in seven different commodities (milk, eggs, meat, rice, lettuce, avocado, and lemon). The recoveries ranged from 58 to 97% and the RSDs from 5 to 19% [42].

In order to extract eight acetanilide herbicides from cereal crops, Zhang *et al*. [43] evaluated the effect of four parameters for PLE efficiency (temperature, static time, static cycles and solvent). The results of final method, based on accelerated solvent extraction (ASE) and solid-phase extraction (SPE) for cleanup using graphitized carbon black/primary secondary amine (GCB/PSA), GCB, Florisil and alumina-N were compared with shake-flask extraction method. At 0.05 mg kg^{-1} spiked level, recoveries and precision values for rice, wheat and maize were from 82.3 to 115.8% and from 1.1 to 13.6%, respectively. For all the herbicides, LOD and LOQ ranged from 0.8 to 1.7 $\mu g\ kg^{-1}$ and from 2.4 to 5.3 $\mu g\ kg^{-1}$, respectively.

2.4. Supercritical-fluid extraction (SFE)

From 1960, when applications of supercritical fluid extraction have been extensively examined until 90 years, few researchers investigated the use of supercritical fluids in analytical, non-chromatographic applications. After that, commercial supercritical fluid extraction (SFE) instruments have been developed and the studies began to be published using SFE as extraction technique in combination with chromatographic techniques for analytical applications for analysis of pesticide residues [44, 45].

The unique properties exhibited by supercritical fluids have been applied for the analysis of pesticide residues in solid samples SFE is selective and Less solvent consuming, thus it is environmental friendly. The critical step in the off-line SFE methods is evaporation of solvent at the end of extraction to acquire high pre-concentration factor. This procedure is time-consuming and can contaminate the environment. Another negative factor is loses or degradation of collected analytes [46].

Carbon dioxide (critical conditions = 30.9 ºC and 73.8 bar) is the most used supercritical solvent, because is cheap, environmentally friendly and safe. Supercritical CO$_2$ (SCCO$_2$) had high diffusivity and easily tuneable solvent strength. Another advantage is that CO$_2$ is gaseous at room temperature and pressure, which makes analyte recovery very simple and provides solvent-free analytes. Also, important for food and natural products sample preparation, is the ability of SFE using CO$_2$ to be operated at low temperatures using a non-oxidant medium,

which allows the extraction of thermally labile or easily oxidized compounds. The main drawback of SCCO$_2$ is its low polarity, and some modifiers as required to change the polarity of supercritical fluid and increase de solvent power. The modifiers can also reduce the analyte–matrix interactions improving their quantitative extraction [45].

In a typical commercial equipment, the fluid is pumped, at a pressure above its critical point, with the sample placed in an inert extraction cell. The temperature of the cell is increased to overcome the critical point of the fluid. After depressurization, the analytes are collected in a small volume of organic solvent or on a solid-phase filled cartridge (solid adsorbent trap). Extraction can be performed in three ways: static, dynamic or recirculating (mixed) mode: in the static extraction mode, the cell containing the sample is filled with the supercritical fluid, pressurized and allowed to equilibrate; using the dynamic mode, the supercritical fluid is passed through the extraction cell continuously; finally in the recirculating mode the same fluid is repeatedly pumped through the sample and, after the required number of cycles, it is pumped out to the collection system [47].

SFE is usually an efficient extraction method, primarily applicable to solid samples. However, as well as its numerous advantages (efficacy, selectivity, short extraction times, low solvent volumes) it also has serious drawbacks: difficult optimisation, high apparatus and maintenance cost, high blank and noise levels, co-extraction of lipidic content of samples [48].

With commercially equipments available SFE has been used not only for analytical applications in samples preparation, but mostly for food science, pharmaceutical and environmental science in lab or pilot scale [45]. Recently, only a few applications describing sample preparation for determination of pesticides in cereals were published.

Aguilera et al. [49] used the SFE method followed by a cleanup step with aminopropyl SPE for the analysis of 22 pesticides in white and wild rice. The authors tested different experimental conditions for extraction with CO2: temperature, volume and pressure in order to optimize the extraction method. The authors noticed that fat extraction from rice was appreciable and the use of higher pressures might also increase extraction of fat and other non-fat material in this matrix. For the final procedure validated pesticide mean recoveries obtained from rice samples, at fortification levels around 0.5 mg kg^{-1}, ranged between 74 and 98%, except for captafol and dimethoate for which mean recoveries lower than 21% were determined.

2.5. Solid-phase extraction (SPE)

Solid-phase based extraction techniques are widely applied to many matrices, like foods and include: matrix solid-phase dispersion (MSPD), solid-phase extraction (SPE), solid-phase microextraction (SPME) and stir-bar sorptive extraction (SBSE). In this technique a sorbent will retain and concentrate some target analytes from the sample solution due strong affinity between sorbent and analytes [12]. Solid-phase extraction involves the use of disposable cartridges and disks to trap analytes. As the sample solution passes through the activated sorbent bed, analytes concentrate on its surface, while the other sample

components pass through the bed or vice versa, if necessary for cleanup [50]. When compared with LLE, the advantages of SPE are: simultaneous removal of interfering substances and concentrations of analytes, multiple samples can be treated in parallel and the use of a relatively small quantity of solvent [12]. Before SPE can be applied to a solid matrix (soil, vegetables and fruits), a separate homogenization step and, often, filtration, sonication, centrifugation, and liquid/liquid cleanup are required. However, the presence of interfering substances, such as salts, humic acids, and other humic substances in water or proteins, lipids and carbohydrates in food makes the determination of polar or early-eluted pesticides, difficult or almost impossible [51].

An analytical method for the determination imazaquin residues in soybeans was proposed by Guo *et al*. [52] based on liquid/liquid partition strong anion exchange solid-phase extraction. This technique was used, in order to achieve an effective cleanup, removing the greatest number of sample matrix interferences. In this procedure, the combination between optimized chromatographic conditions and detection by ultraviolet the procedure was showed to be sensitive and reliable for determining the imazaquin residues in soybean samples. This method is characterized by recovery >88.4%, precision 6.7% RSD, and sensitivity of 0.005 mg kg^{-1}. The proposed method was successfully applied to the analysis of imazaquin residues in soybean samples grown in an experimental field after treatments of imazaquin formulation.

A sensitive and simple method for simultaneous analysis of acetochlor and propisochlor in corn and soil has been developed by Hu *et al*. [53]. The extraction of pesticides from soil and corn matrices was performed with methanol/water and acetone, respectively, followed by solid phase extraction (SPE) to remove co-extractives, prior to analysis by gas chromatography with electron capture detection (GC-ECD). Primary secondary amine (PSA) SPE cartridges (500 mg, 3 mL) were used for sample preparation. The elution was made with 5 mL petroleum ether-acetic ether (95/5, v/v) and 3 mL petroleum ether-acetic ether (95/5, v/v), respectively. The recoveries of two pesticides ranged from 73.8% to 115.5% with relative standard deviations (RSD) less than 11.1% and sensitivity of 0.01 mg kg^{-1}. The method was successfully applied to determine acetochlor and propisochlor in real corn and soil samples. The authors related that residues of acetochlor and propisochlor residues were detected (<0.01 mg kg^{-1}) in corn at harvest time with holding period of 2.5 months after treatments of the pesticides.

The use of SPE in combination with HPLC-DAD was employed to determine bispyribac-sodium residues in rice. The liquid–liquid partition and anion exchange solid phase procedures that were developed provide effective extraction and cleanup methods for analysis feasibility, with recoveries between 83.98 to 98.51% with a RSD from 0.56 to 6.36% and sensitivity of 0.01 mg kg^{-1}, with main advantages of high precision, accuracy and good selectivity. Another favorable feature is the reduction of sample processing time [54].

2.6. Matrix solid-phase dispersion (MSPD)

In 1989, Barker *et al*. [55] introduced the MSPD, as a sample preparation technique for solid or semi-solid samples. It provides both a porous structure to enable the solvent to penetrate

the matrix and extract the analytes, but also has some functionality which can retain the fat/lipids [56]. MSPD can combine the procedures of homogenization, disruption, extraction and cleanup into one simple process. In fact, it is a sample preparation strategy that consists of a manual blending of samples with a bulk dispersing agent, to produce complete disruption of the original matrix structure, thus providing an enhanced surface area for subsequent sample extraction. Usually, the blended material is then transferred and packed into a column to perform sequential extraction and eventual cleanup with an appropriate solvent or a sequence of solvents [57].

Most applications have utilized 5–10 mL of solvent to perform analyte extraction. Evidence from some studies indicates that most target analytes are eluted in the first 4 mL of extractant, provided that 0.5 g of the sample is mixed with 2 g of the solid support [9]. In general, the choice of the adsorbent used depends on the polarity of the analyte and on the possible coextracted components of the matrix. The most usual adsorbents are Florisil and C18; nevertheless, there are others (e.g., diatomaceous earth, alumina, silica and C8) that have been used for the analysis of pesticides in food [11].

The main advantages of MSPD procedure compared to other extraction techniques are as follows: (1) the analytical protocol is simplified and shortened; (2) the possibility of emulsion formation is eliminated; (3) solvent consumption is substantially reduced; and (4) the extraction efficiency of the analytes is enhanced as the entire sample is exposed to the extractant. An interesting feature of the MSPD technique is that it can be used for extracting analytes from both solid and liquid foods. The main disadvantage is the lack of automation of the procedure [9].

Tsochatzis *et al*. [58] development and validation of such a method for the determination of eight rice pesticides penoxsulam, tricyclazole, propanil, azoxystrobin, molinate, profoxydim, cyhalofop-butyl and deltamethrin) and 3,4-dichloroaniline, the main metabolite of propanil. Pesticide extraction and cleanup was performed by an optimized MSPD protocol on neutral alumina (5 g) using acetonitrile as the elution solvent. Samples were analyzed by high-performance liquid chromatography with diode array detection (HPLC-DAD). Method validation was performed by means of linearity, intra-day accuracy, inter-day precision and sensitivity. Linear regression coefficients (r^2) were always above 0.9948. Limits of detection (LOD) and quantification (LOQ) varied from 0.002 to 0.200 mg kg^{-1} and 0.006 to 0.600 mg kg^{-1}, respectively. Recoveries were investigated at three fortification levels and were found to be acceptable (74–127%) with relative standard deviations (RSD) below 12%. Application of the method for the analysis of five commercial rice grain samples showed that the pesticide levels were below the LOD.

Michel and Buszewski [58] proposed MSPD for extraction of carbendazim residue from wheat grain and determination by column-switching HPLC-DAD. For extraction, a representative portion (200 g) of sample grain was transferred into a mill, and sample material disintegrated by high speed blending. A subsample of 5 g was weighed into a mortar of ca. 10 cm of diameter, 10 g of acidic silica gel was added and ground to obtain a homogeneous mixture. The extraction column was fitted with polyethylene frit, the

powdery sample was transferred through a wide mouth polypropylene funnel (10 cm top i.d.). Mortar and pestle were rinsed with 20 mL of methanol-dichloromethane (1:5, v/v), and the rinsings were carefully poured into the column. The carbendazim residues were extracted with total volume of 120 mL eluent and collected in round-bottomed flasks. The mean recovery rate for fortified samples was 87.3% with a RSD of 2.9%. The achieved method LOQ was 0.04 µg g^{-1}. The method was applied to the determination of carbendazim residues in wheat grain samples from a treated field.

MSPD method have been published also for determination of other residues and contaminants e.g. mycotoxins in cereal samples. Rubert *et al*. [60] reported a reliable and rapid method for the determination of 14 mycotoxins in flour samples (with different cereals composition). MSPD was performed weighing 200 g which were prepared using a food processor and mixed thoroughly. Portions of 1 g were weighed and placed into a glass mortar (50 mL) and were gently blended with 1 g of C18 for 5 min using a pestle, to obtain homogeneous mixture. The homogeneous mixture was introduced into a 100×9 mm i.d. glass column, and eluted dropwise with 20 ml of acetonitrile/methanol (50/50, v/v) 1 mM ammonium formiate by applying a slight vacuum. In a total of 49 samples investigated, 9 mycotoxins were identified. Nivalenol and beauvericin were the mycotoxins found most frequently in the studied samples. The samples that presented major contamination were wheat flours and bakery preparations.

Juan *et al*. [61] determinate ochratoxin A (OTA) in organic and non-organic cereals and cereal products from Spain and Portugal. A method based on extraction with matrix solid phase dispersion (MSPD) using octylsilica (C8) followed by liquid chromatography coupled with fluorescence detection (LC–FD) was used to determine OTA from the selected samples. Recoveries of OTA from the studied samples spiked at 10 ng g^{-1} level ranged from 78 to 89% with a RSD of 3.7%. The limits of detection and quantification of this method were 0.05 and 0.19 ng g^{-1}, respectively. Furthermore, LC–FD after OTA methylation was used to confirm the identity of OTA in all positive samples. This procedure was applied to 83 organic and non-organic samples including rice, wheat, barley, rye, oats and maize from Spain and Portugal.

2.7. Gel-permeation chromatography (GPC)

In the last years polystyrene divinylbenzene type gel with mixture of elution solvents as ethyl acetate and cyclohexane has been used in pesticide residue analysis of cereals samples taking advantage of GPC to isolate pesticides from co-extractants with higher molecular weight (600–1500 g mol^{-1}). GPC is able to separate the pesticides from lipids, waxes and other low volatile larger non-polar co-extractives. Generally, in pesticide residue analysis in fatty food, addition of sulfuric acid or additional SPE cleaning (avoiding decomposition of several pesticides occurring in the former case) are desirable to offer satisfactory protection for the gas chromatographic capillary column [3].

Regarding analysis of pesticide residues in cereals and feedstuffs, Lee *et al*. [62] developed a method for the analysis of 106 compounds in cereal-based animal feed applying ethyl

acetate extraction, GPC and d-SPE cleanup steps, additionally determination by comprehensive two-dimensional gas chromatography (GC×GC) with TOF-MS detection in the full scan mode. The method was demonstrated to give recoveries between 70 and 110% and RSDs below 20% at two levels of 0.01 and 0.1 mg kg^{-1} for the majority of the studied compounds. Zhang *et al*. [63] used GPC for the determination of pesticides in unpolished rice. GPC provides ideal cleanup for high molecular weight components. Otherwise, low molecular weight components may result in serious interferences that can affect the final result.

GPC is highly effective as a post-extraction cleanup procedure in removing interferences with high molecular weight (e.g. lipids, proteins and pigments) prior to the analysis by GC or LC. GPC also increases analytical precision and accuracy. It also ex-tends the column life and lifetime of the instrument. This technique can provide very good results. However, GPC, use large columns, need long analysis times and large amounts of solvents. Some pesticides with high molecular mass (e.g. pyrethroids) need not be sufficiently separated from wide elution band of co-extractants that can result in lower recoveries [64].

3. Instrumental identification and quantification techniques

The two main analytical techniques used in food analysis are gas chromatography (GC) and liquid chromatography (LC), which allow identification and quantitation of pesticide residues in complex matrices.

GC is combined with different kinds of detection methods, mainly depending on the class of pesticides to be quantified [48]. Electron capture detection (ECD), nitrogen-phosphorus detection (NPD), flame-ionization detection (FID), flame-photometric detection (FPD) and mass-selective detection (MSD) have been employed for pesticide residue determination in cereal samples. GC-MS is also frequently used for determination pesticide residues in cereal samples and can be done by electron impact (EI), and Positive or negative chemical ionization (PCI, NCI). The Table 2 summarizes details of several methods developed for the determination of pesticide and others analytes in different cereal samples.

LC has been used for the analysis of polar and/or non-volatile and/or thermally labile pesticides for which GC conditions were not suitable [65]. Most used detectors are UV, diode array detector (DAD) and MSD, as we can see the Table 2. Traditional UV detectors and DADs are often less selective and less sensitive than GC instruments but, in recent years, the commercial availability of atmospheric pressure ionization (API), in combination with MS instruments, has increased the sensitivity by several orders of magnitude [66]. LC-MS/MS applications reported for the analysis of pesticides residues in cereal samples are performed with two different ionization techniques: electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI). Although ESI is the more often used when compared with APCI source.

Analytes	Sample	Extraction Procedure	Cleanup	Analytical technique	LOQ (µg kg⁻¹)	Recovery (%) (RSD %)	References
bisphenol A (BPA), 4-tert-butylbenzoic acid (BBA), 4-nonylphenol (NP), 4-tert-butylphenol (t-BP), 2,4-dichlorophenol (DCP), 2,4,5-trichlorophenol (TCP) and pentachlorophenol (PCP)	breakfast cereals	PLE	-	LC–ESI-MS	3.0 – 43.0	81 - 104 (4 - 9)	37
benzoylureas	fruit, vegetable, cereals and animal products	PLE	-	LC-MS/MS	2.0 – 10.0	58 - 97 (5 - 19)	42
acetanilide herbicide	rice, wheat and maize	PLE	SPE	GC-ECD	2.4 - 5.3	82.3 - 115.8 (1.1 - 13.6)	43
22 pesticides	white and wild rice	SFE	SPE	GC-ECD GC-TSD	-	74 98	49
acetochlor and propisochlor	Soil and corn	methanol/water/SPE		GC-ECD	10.0	73.8 - 115.5 (< 11.1)	53
Bispyribac sodium	rice	liquid–liquid partition and anion exchange solid phase		HPLC-DAD	10.0	83.98 - 98.51 (0.56 - 6.36)	54
Penoxsulan, tricyclazole, propanil, azoxystrobin, molinate, profoxydim, cyhalofop-butyl, deltamethrin and 3,4-dichloroaniline (the main metabolite of propanil)	rice	MSPD		HPLC-DAD	6.0 - 600.0	74 – 127 (< 12)	58
carbendazim	wheat grain	MSPD		HPLC-DAD	40.0	87.3 (3.3)	59

Table 2. Different methods applied to the determination of pesticide and others analytes in different cereal samples.

4. Conclusions

Although detection equipment's are becoming more specific and sensitive, there is still a need for efficient sample preparation methods for pesticides residues analysis in cereals and feedstuffs, which are compatible with modern analytical techniques. Despite the advances in separation and detection of the chromatographic systems, cleanup remains important for obtaining reliable data. There is still a need for effective, environmentally friendly and fast methods for sample treatment and determination of pesticide residues in fatty food matrices e.g. cereals and feedstuffs. Modern sample preparation techniques should be not only simple, reliable, cheap and take into account chemical laboratory waste problems, but also

must be similar to common analytical techniques, in order to minimize errors. For these reasons, modern trends in analytical chemistry are towards the simplification and miniaturization of sample preparation, and the minimization of sample size and organic solvent used. The development of such procedures combined with modern chromatographic-mass spectrometric techniques will enable analysis at the low levels now required by legislation for many pesticides, but more importantly, result in methods which produce more reliable data to support food safety monitoring programs. This is the trend although it is impossible to develop a unique protocol covering such a wide range of compounds. Finally, multiclass multiresidue methods covering large number of pesticides are highly desirable, although the different nature, classes and physico-chemical properties of pesticides hamper the development of such methods.

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