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Review

An Overview of Methods for L-Dopa Extraction and Analytical Determination in Plant Matrices

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Abstract: L-dopa is a precursor of dopamine used as the most effective symptomatic drug treatment for Parkinson's disease. Most of the L-dopa isolated is either synthesized chemically or from natural sources, but only some plants belonging to the Fabaceae family contain significant amounts of L-dopa. Due to its low stability, the unambiguous determination of L-dopa in plant-matrices requires appropriate technologies. Several analytical methods have been developed for the determination of L-dopa in different plants. The most used for quantification of L-dopa are mainly based on capillary electrophoresis or chromatographic methods, i.e., high-performance liquid chromatography (HPLC), coupled to ultraviolet-visible or mass spectrometric detection. HPLC is most often used. This paper aims to give information on the latest developments in the chemical study of L-dopa, emphasizing the extraction, separation and characterization of this compound by chromatographic, electrochemical and spectral techniques. This study can help select the best possible strategy for determining L-dopa in plant matrices using advanced analytical methods.

Keywords: levodopa; plant matrices; extraction; review; chromatographic methods

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1. Introduction

L-Dopa or levodopa (LD) is an amino acid analogue belonging to the class of catecholamine compounds. It is a precursor of dopamine (DP) and norepinephrine that act as neurotransmitters in brain areas related to psychomotor and emotional functions. LD is currently considered the most effective oral dopaminergic treatment for the main motor symptoms of Parkinson's disease (PD). This latter is the most widespread neurodegenerative movement disorder in the world: only Europe has a prevalence rate of around 108–257/100,000 and an incidence rate of 11–19/100,000 per year [1,2]. PD arises when the substantia nigra neuronal cells die and cannot biosynthesize dopamine (DA), a fundamental neurotransmitter, as it plays an essential role in physiological motor control. The symptoms of PD can be kept under control with strategies to replace or improve dopamine [1]. The LD pharmacological treatment is based on its replacement for DP to increase its bioavailability at the peripheral synaptic level, where the LD is decarboxylated to DP because of the amino acid aromatic decarboxylase (AADC) enzyme [3]. The pharmacological efficacy decreases after a certain period of intake; serious side effects such as motor fluctuations (commonly called on-off phenomenon), orthostatic hypotension, hallucinations and dyskinesias occur after a half-life time t1/2 of 50 to 90 min. These reasons led to the development of extended-release LD formulations, combined with other drugs, to extend the half-life and bioavailability and reduce side effects [2,4–8].

The LD drug is chemically synthesized through a process that requires a costly metal catalyst and advanced technologies [9]. There are also natural sources, and the production of LD from different plants has advantages compared to chemical methods, such as a pure

enantiomerically compound and low-cost approach. LD from natural sources also reduces the secondary effects and helps slow the disease's progression. Some plants belonging to the Fabaceae family naturally contain significant amounts of LD [10]. Among these, the genus *Mucuna* includes the highest concentration of LD, which explains its widespread use in the management of Parkinson disease. The *Mucuna pruriens* is the most considered, containing up to 10% of LD in its seeds [11,12]. However, the seeds are covered by stinging hairs, and the beans contain elevated levels of tryptamines which may cause hallucinations in humans, so other plant-matrices as a natural source of LD are also investigated. The control of crucial human body functions can be affected by a lack or excess of LD and its metabolites. Consequently, it is necessary to monitor the concentration of LD in all plant-matrices destined for human consumption.

LD's low molecular weight and polar nature generally make its determination by reversed phase liquid chromatography challenging. A possible solution is to use an ion pair reagent to increase retention time. In general, it is necessary to work below the pK_a of the compound, where it will be protonated and not charged, and to decrease the organic content of the mobile phase [13]. In addition, LD aqueous solutions are unstable and degrade naturally over time, so the extraction procedure also requires special attention [13]. This review summarizes several analytical techniques developed in the last twenty years to analyze and quantify LD in plants consumed by animals and humans. It offers an entry into the extensive analytical literature on this compound, emphasizing the advantages and drawbacks of the proposed extraction, separation and characterization approaches. The described methods should make it possible to measure the content and changes in the concentrations of this biologically active plant compound in different plant matrices to evaluate their nutritional and toxicology aspects following consumption by animals and humans. Before introducing the main analytical techniques employed for LD extraction and detection in plant matrices, a brief description of its chemical and physical properties and its metabolic pathway in plants is provided, highlighting the main compounds involved during the biosynthetic routes both as LD precursors and as its conversion products. Among all, those characterized by chemical–physical properties more similar to LD are likely to be found in the extracts at an appreciable content and therefore must be taken into due consideration during its detection.

1.1. Chemical and Physical Properties

LD structure is characterized by the catechol moiety bonded to the amino acid functionality (-CH_2NH_2COOH) in -meta and -para positions to the hydroxyl groups in positions 3 and 4, respectively (Figure 1). The main chemical and physical properties are summarized in Figure 1.

Chemical Properties
Formula $C_9H_{11}NO_4$
Exact Mass: 197.069
Mol. Wt.: 197.188
Boiling Point: 537.89 [°C]
Melting Point: 396.26 [°C]
Log P: 0.05
Critical Temp: 588.32 [°C]
Critical Pres: 59.26 [Bar]
Critical Vol: 510.5 [cm^3/mol]
Gibbs Energy: -451.83 [kJ/mol]
Heat of Form: -668.94 [kJ/mol]

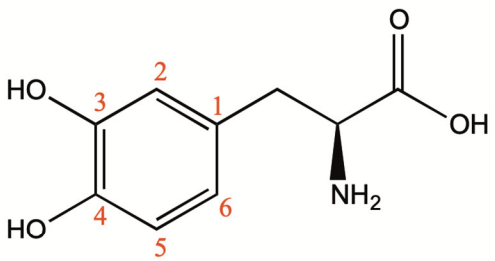

Figure 1. L-Dopa, (3,4-dihydroxyphenyl)-L-alanine, structure and its chemical and physical properties.

Furthermore, LD has three ionizable groups (Figure 2). When the pH value is on average between pK_{a1} = 2.3 and pK_{a2} = 8.11, LD is present as zwitterion that forms a network of intermolecular bonds where the protonated amine groups and the deprotonated carboxylic acid groups are linked. For this reason, LD is not very soluble in this pH range (LD solubility in water is 3.3 g/L), and acids are required to prepare aqueous solutions. This point is especially crucial regarding LD pharmacological bioavailability along the gastrointestinal lumen as well [14,15].

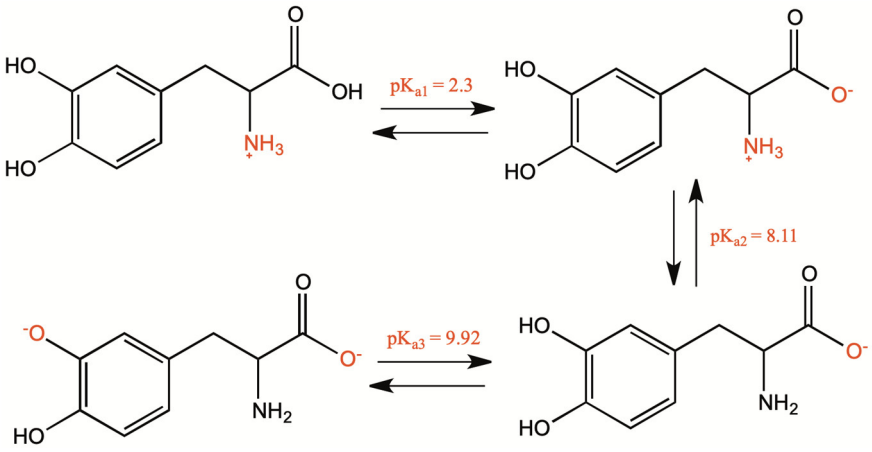

Figure 2. Ionization of L-Dopa at various pH values.

1.2. Biosynthesis and Conversion Routes of Levodopa in Plants

Plants produce hundreds of non-protein amino acids, among which LD, a secondary metabolite belonging to the class of catecholamines. Metabolism refers to the whole regulatory aspects implied in the biosynthesis of functional compounds, generally called metabolites. Metabolism in plants can be primary or secondary: primary metabolism generates all the essential compounds for the organism's growth (primary metabolites); secondary metabolism produces all compounds that are considered not essential for the organism's growth (secondary metabolites) but are equally important since directly involved in the interaction with the external environment [16,17]. Among catecholamines, norepinephrine (NE), epinephrine (EP), dopamine (DP) and normetanephrine (NMP) are other secondary metabolites whose structures are shown in Figure 3.

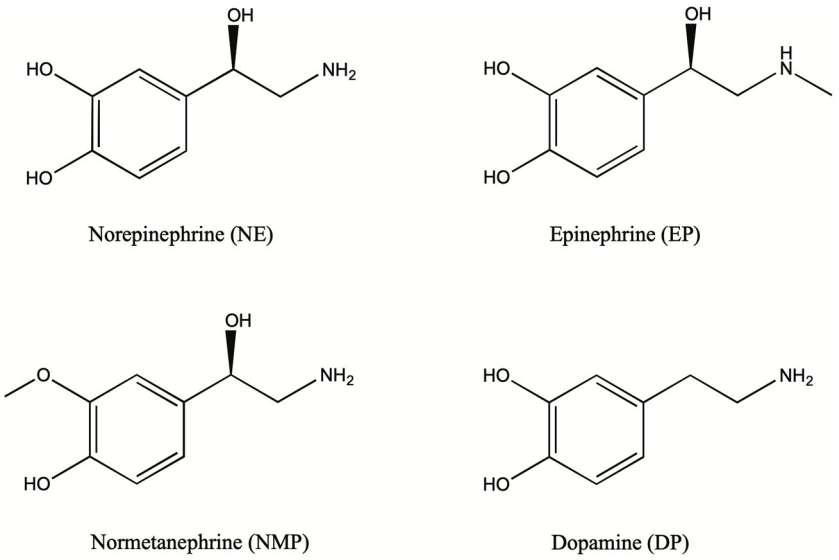

Figure 3. Norepinephrine (NE), epinephrine (EP), dopamine (DP) and normetanephrine (NMP) structures.

Shikimic acid pathway is the most important metabolic pathway of secondary metabolites in plants, and it represents the plant's biosynthetic key for L-tyrosine [16,18]. Like in mammals, L-tyrosine is involved in plants as a precursor for the catecholamines' biosynthetic pathway.

L-tyrosine can be converted into LD by tyrosine hydroxylase, or in tyramine upon decarboxylation of the same substrate. DP can derive both from tyramine hydroxylation and L-dopa decarboxylation (Figure 4) [16,19]. This last synthetic route has been reported in plants such as *Cytisus scoparius* Scottish broom, *Monostroma fuscum* marine alga, *Lophophora williamsii* peyote cactus and *Portulaca* callus [19,20].

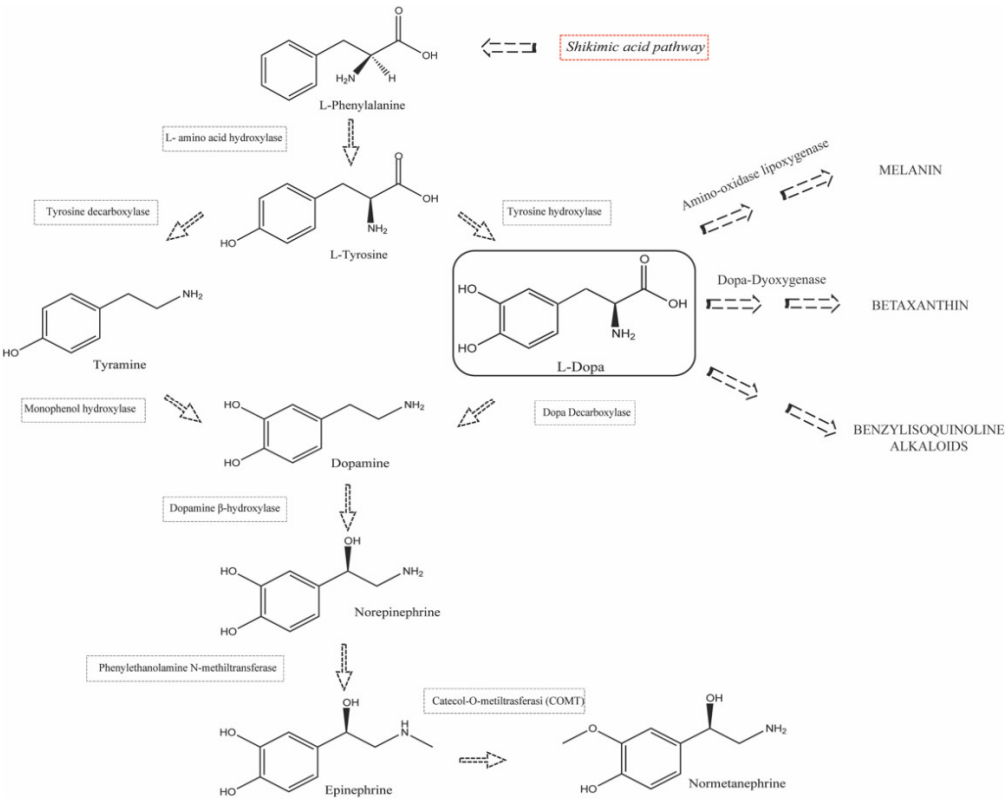

Figure 4. Plant catecholamine synthesis pathway.

In plant organisms, LD plays an important role as a precursor for other classes of compounds. LD can be converted by 4,5-DOPA dioxygenase into betalamic acid, which is a key compound in the biosynthesis of betalaines, red-purple and yellow pigments found in plants of the order *Caryophyllales* and two genera of fungi: *Amanita* and *Hygrocybe* [21–23]. Furthermore, it can be oxidized toward melanin: at first, LD is oxidized to dopaquinone by the enzyme polyphenol oxidase (PPO), and then it is metabolized to melanin by the plant lipoxygenase [18,19,24]. LD also represents a key precursor in the biosynthesis of benzylisoquinoline alkaloids, involved in specific plants (like basal eudicots in the order *Ranunculales*) for defense against herbivores and pathogens [21]. As far as the defense is concerned, in some legumes (e.g., *Mucuna pruriens*), LD plays an important role as an allelopathic compound that is exuded from the roots in order to inhibit the growth of surrounding plants [18,21,24,25]. The catecholamines catabolism in some plant species also involves their methylation: this is the case of the peyote cactus, *Lophophora williamsii*, in which LD is decarboxylated to dopamine and subsequently leads to the biosynthesis of mescaline, a hallucinogenic alkaloid, through the key compound 4-hydroxy-3-methoxyphenethylamine [26].

2. Levodopa Extraction Techniques

As it was previously outlined, extraction from natural products is nowadays considered the method of choice for providing LD over chemical synthesis which is time consuming, requires expensive and harmful chemicals and generates a racemic mixture of LD. This justifies the growing interest in developing an extraction protocol to ensure LD recoveries are as high as possible, to remove interfering endogenous compounds and to be quick, easy and cheap.

In a general workflow, analytes are extracted directly from the plant matrices after undergoing simple pre-treatment steps consisting of homogenization and freeze drying. Homogenization, in particular, was found to be effective in increasing LD concentration in extracts from *Mucuna pruriens* seeds with respect to the extract obtained without any pre-conditioning (151.5 ± 5.1 μg/g dw vs. 146.0 ± 4.5 μg/g dw) [15].

Typical steps within food sample preparation after pre-treatment and extraction generally include clean-up and concentration. To this regard, literature data show that in the case of plant matrices, LD pre-concentration and clean-up steps are rarely provided. In contrast, for biological samples (e.g., plasma, blood, animal tissues or urine), a sample preconcentration step or solid phase extraction (SPE) is always required. Such a distinction in sample preparation may be ascribed to the different content of LD in biological and plant samples. For samples containing low levels of LD, like biological ones, pre-concentration and SPE are essential in order to guarantee the minimum levels for analyte detection and quantification [27–34]. On the other hand, plant samples mainly involved in LD extraction studies, e.g., different varieties of *Mucuna pruriens* seeds and *Vicia faba* broad beans, are rich in this analyte. To get an idea of the LD content in plants, an average concentration of 4.96 and 4.39 g/100 g were estimated, respectively, in white and black variety of *Mucuna pruriens* seeds [35], whereas an average concentration of 7.68 mg/g dw was found in *Vicia faba* seeds [36]).

The extraction techniques used for LD, whose specifications are reported in Table 1, range from the traditional liquid–solid extraction (LSE), Soxhlet extraction, maceration extraction and reflux extraction to the latest and less used microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE). The last two techniques involve substantial improvements in terms of automation and solvent consumption so to be referred as green techniques.

Regardless of the particular extraction technique used, in all cases the transferring of LD from the solid plant matrix into the extracting liquid phase revealed to be highly dependent on the pH: the extracting solutions used are all acidic in order to inhibit the LD oxidation process and avoid the formation of its zwitterionic form, which is poorly soluble. Controversies arise about the appropriateness of using solutions slightly acidified

with acid acetic/formic acid or strongly acidified with hydrochloric/perchloric acid. The use of mineral or concentrated organic acids for L-dopa extraction is surely efficient but it is limited by the requirement of costly and energy-demanding downstream processes. The potential degradation of L-dopa in a strongly acidic environment is also to be considered. Acids of moderate strength meet the need to find more sustainable solvents even if they could be less efficient in preserving LD stability towards oxidation or formation of aggregated structures.

While the pH strongly influences the extraction yield, on the other hand, the sample ionic strength does not seem to affect the extraction process. IUPAC defines the salting-out effect as "the addition of particular electrolytes to an aqueous phase in order to increase the distribution ratio of a particular solute" [37]. It is usually exploited to improve the extraction efficiency (as it generally occurs for solid-phase microextraction SPME), but in the case of LD extraction from plant matrices it seemed to play no role.

Table 1. Overview of the methods used for LD extraction occurring in plant samples.

Extraction Technique	Matrix	Variety	Solvents and Optimized Conditions	Recovery Percent (Mean Value Percent)	References
Solid–liquid extraction (LSE)	*Mucuna pruriens* dehulled and whole seed	*White and black var. utilis*	HCl 0.1 M; solvent: sample ratio 100:1 (*v*/*w*), extraction time 2 × (30 sec under homogenization and 1 h under stirring); extraction temperature 22 °C	101.8%	[35]
	Broad bean, cocoa and beans	//	$HClO_4$ 0.2 M, solvent: sample ratio 5:1 (*v*/*w*), extraction time 24 h under shaking time for time; extraction temperature 25 °C	Within-day 84.4–96.0% Between-day 84.0–83.1%	[38]
	Vicia faba seeds (cotyledons and embryo axis)	*var. Alameda var. Brocal*	$HClO_4$ 0.83 mol/kg; solvent: sample ratio 100:1 (*v*/*w*); extraction time 1 min under homogenization; extraction temperature 4 °C	//	[25]
	Vicia faba broad beans	*Iambola, San Francesco, FV5, Cegliese, Extra-early purple and Aguadulce supersimonia*	5% *w*/*v* $HClO_4$ solution; solvent: sample ratio 10:1 (*v*/*w*); extraction time 5 min under homogenization; extraction temperature 4 °C	//	[39]
	Vicia Faba roots, sprouts and seeds	//	Formic acid:ethanol (1:1 *v*/*v*); solvent: sample ratio (10–40):1 (*v*/*w*); extraction time 5 × 120 min at 120 rpm; extraction temperature 4 °C	94.1–116.6%	[40]
	Mucuna pruriens seed cooked and raw	//	Water; solvent: sample ratio 400:1 (*v*/*w*); extraction time 20 min under stirring; extraction temperature 25 °C	//	[41]
	Avena sativa seeds	*GK Iringo, GK Kormorán* and *GK Zalán*	Aqueous solution of 0.1% (*m*/*v*) ascorbic acid and 1% (*v*/*v*) MeOH; solvent: sample ratio 6:1 (*v*/*w*); extraction time 5 h under shaking; extraction temperature 25 °C	95.2–99.6%	[42]
	Vicia Faba roots, sprouts, leaf, seedling, pod, flower, stem	//	Ethanol solution 95% (*v*/*v*); solvent: sample ratio //; extraction time 72 h in freezer; extraction temperature −18 °C	//	[43]

	Vicia Faba sprouts	//	Ethanol solution 95% (*v*/*v*); solvent: sample ratio //; extraction time 48–72 h; extraction temperature −18 °C	//	[44]
	Mucuna and Stizolobium pruriens seed	*M. sempervirens, M. birdwoodiana, M. macrocarpa, M. interrupta, M. paohwashanica, Stizolobium pruriens* var. *pruriens, S. pruriens* var. *utilis*	HCl 0.1 M; solvent: sample ratio 20:1 (*v*/*w*); extraction time 2 × 5–10 min; extraction temperature 100 °C with a steam bath.	//	[45]
	Mucuna pruriens seed	//	0.1 M phosphate-buffered solution (pH = 7.0); solvent: sample ratio 5000:1(*v*/*w*); extraction time 5 h; extraction temperature 25 °C under stirring.	99.35%	[46]
	Mucuna pruriens leaves	//	0.1 M phosphate-buffered solution (pH = 7.0); solvent: sample ratio 500:1 (*v*/*w*) ; extraction time 5 h; extraction temperature 25 °C under stirring	98.30%	
	Mucuna pruriens seed	//	Citric acid 58% (wt%); solvent: sample ratio 7:1; extraction time 90 min; extraction temperature 60 °C	80–84%	[47]
Ultrasound-assisted solvent extraction (UASE)	*Mucuna pruriens* seed	*Arka Dhanwantri*	Water acidified with 0.1 M HCl (pH: 2.6); solvent: sample ratio 10:1 (*v*/*w*); frequency 35 kHz; extraction time 5, 10, 15 min; extraction temperature 25 °C.	(5 min) 30.7% (10 min) 25.6% (15 min) 31.5%	[48]
		Arka Ashwini		(5 min) 29.0% (10 min) 27.7% (15 min) 26.8%	
		White		(5 min) 29.3% (10 min) 31.4% (15 min) 30.8%	
		Brown		(5 min) 23.9% (10 min) 28.7% (15 min) 30.6%	
	Vicia faba sprouts and seeds	//	MeOH and water mixture (80:20); solvent: sample ratio 1:5 (*v*/*w*); frequency //; extraction time 30 min; extraction temperature 25 °C.	//	[49]
	Vicia faba flowers, fruits and leaves	//	Water boiling deionized; solvent: sample ratio 50:1 (*v*/*w*); frequency //; extraction time 15 min; extraction temperature 100 °C.	100.32%	[36]
	Vicia Faba seeds	//	HCl 10 mM 5 mL; solvent: sample ratio // (*v*/*w*); frequency //; extraction time 2 × 60 min; extraction temperature 25 °C.	99.8%	[50]
	Lens culinaris seeds			105.0%	

	Vicia Faba sprouts, leaves, flowers, pods, roots	//	Aqueous MeOH 50% (*v*/*v*); solvent: sample ratio 200:1 (*v*/*w*); frequency //; extraction time 30 min; extraction temperature below 40 °C.	//	[51]
	Vicia faba seeds	*Bachus, Bolero White, Windsor Bonus, Rambo Amigo, Olga Granit, Albus Fernando, Amulet*	Aqueous CH_3COOH 0.2% (*v*/*v*); solvent: sample ratio 25:1 (*v*/*w*); frequency 40 kHz; extraction time 2 × 20 min; extraction temperature 25 °C.	//	[15]
	Wild type legume grain	*Acacia nilotica, Bauhinia purpurea, Canavalia ensiformis, Cassia hirsuta, Caesalpinia bonducella, Erythrina indica, Mucuna gigantea, Pongamia pinnata, Sebania sesban, Xylia xylocarpa*	HCl 0.1 M; solvent: sample ratio 10:1 (*v*/*w*); frequency // kHz; extraction time 30 min and stirring for 1 h. extraction temperature 25 °C.	//	[52]
	Mucuna sanjappae seed	//	HCl 0.1 M; solvent: sample ratio 300:1 (*v*/*w*); frequency // kHz; extraction time 20 min; extraction temperature 25 °C.	//	[53]
	M. pruriens seeds	*Macrocarpa*	HCl 0.1 M; solvent: sample ratio 300:1 (*v*/*w*); frequency // kHz; extraction time 20 min; extraction temperature 25 °C.	//	[54]
Microwave-assisted solvent extraction (MASE)	*Mucuna pruriens* seed	*Arka Dhanwantri*	Water acidified with 0.1 M HCl (pH = 2.6); solvent: sample ratio 10:1 (*v*/*w*); MW power 400 W; irradiation time 5, 10, 15 min; extraction temperature 60 °C.	(5 min) 53.5% (10 min) 58.7% (15 min) 58.4%	[48]
		Arka Ashwini		(5 min) 50.6% (10 min) 59.6% (15 min) 54.0%	
		White		(5 min) 50.5% (10 min) 49.6% (15 min) 58.5%	
		Brown		(5 min) 56.1% (10 min) 54.9% (15 min) 54.8%	
Reflux extraction	*Mucuna pruriens* seed	*Arka Dhanwantri*	Water acidified with 0.1 M HCl (pH = 2.6); solvent: sample ratio 10:1 (*v*/*w*); extraction time 300 min, extraction temperature 100 °C	60.2%	[48]
		Arka Ashwini		65.7%	
		White		57.2%	
		Brown		59.8%	
	Mucuna pruriens powder and extracts	//	MeOH and 0.1 M HCl mixture (70:30); solvent: sample ratio 100:1 (*v*/*w*); extraction time 30 min; extraction temperature 25 °C	98.83%	[55]
	Mucuna pruriens seed	*Preta Kaunch*	HCl 0.1 M; solvent: sample ratio 2:1 (*v*/*w*);	98.1–106.7%	[56]

			extraction time 180 min; extraction temperature 25 °C		
Maceration	*Mucuna pruriens* powder formulation	//	Water and EtOH mixture (30:70); solvent: sample ratio //; extraction time 7 days; extraction temperature cold	94.5%	[57]
	Phaseolus vulgaris dried seed, seeding and callus	//	HCl 0.1 M and EtOH mixture (1:1); solvent: sample ratio 1:10; extraction time 5 days; extraction temperature 25 °C	99.55–100.27%	[58]
Soxhlet extraction	*Mucuna utilis* seed	//	MeOH; solvent: sample ratio //; extraction Soxhlet time //; extract obtained sonication for 60 min with 100 mL HCl 0.1 M; extraction temperature 25 °C	98.67–100.4%	[59]

// This indicates that values were not reported.

Starting from these general considerations, the main extraction techniques listed above will be described in detail and critically discussed with reference to the most significant applications reported in the literature.

2.1. Liquid–Solid Extraction (LSE)

Liquid–solid extraction (LSE) is one of the most widely used extraction methods. At first, it involves the penetration of the extracting solution into the solid sample with subsequent analytes dissolution into the solution, followed by analytes diffusion out of the solid sample and, finally, their collection. The physical-chemical properties of the extraction solvent, the solid sample granulometry, the solvent–solid ratio, the temperature and the extraction time are parameters that must be taken into consideration in order to optimize the efficiency of the extraction process [60]. As it was previously discussed, all liquid–solid extraction methods include a pre-treatment step of the finely grounded solid sample and, sometimes, they also require a possible homogenization of the solid with the extracting solution in order to increase the surface contact area and facilitate the liquid extraction, improving the passage of LD in the solution [25,35,39].

Solvent selection is generally based on the law of similarity and inter-miscibility so that solvents with polarity values similar to the solute are likely to perform better. Alcohols, such as ethanol and methanol, are universal solvents for phytochemical investigation [60]; despite this, LD appears to be insoluble in ethanol and methanol as well as not very soluble in water for the reasons previously explained [15]. Accordingly, the choice of 95% ethanol [43,44] or water as extracting solutions [41] is doubtful, especially if neither sample homogenization nor agitation is foreseen in the extraction procedure.

The solid–solvent ratios used are quite different, strongly depending on the concentration of the analyte of interest in the plant sample. Large ratios are needed for matrices rich in LD. As an example, for *Mucuna pruriens* extraction, ratios values of 5000:1 *v*/*w* for seeds or 500:1 *v*/*w* for fresh leaves both in 0.1 M phosphate-buffered solution pH = 7.0 have been reported [46]. Intermediate ratios are used for matrices with a medium content of LD (e.g., for *Vicia faba* the ratio 10:1 *v*/*w* in 5% *w*/*v* $HClO_4$ solution was used [39]) and, finally, small ratios suggest minor LD quantities (e.g., for *Avena sativa* a ratio of 6:1 *v*/*w* in aqueous solution of 0.1% ascorbic acid and 1% methanol was reported [42], for cacao and broad beans the ratio used was 5:1 *v*/*w* in $HClO_4$ 0.2 M [38]).

Regarding the extraction temperature, stability studies conducted by Zhou et al. [14] have shown that the oxidation kinetics of LD is favored at high temperatures and in a neutral pH environment: despite the acidic environment (pH = 2), at 80 °C the analyte already starts showing degradation after less than 100 h of treatment. For this reason,

although high temperatures increase diffusion and solubility, in the specific case of LD it is advisable to proceed at low extraction temperatures or at least at room temperatures, as mostly reported, with some exceptions such as the work of Yang et al. [45], where a surprisingly high temperature of 100 °C was adopted for LD extraction. A prolonged extraction time is another parameter that might negatively influence LD stability and therefore its content in the final extract.

The values of the LSE percentage recoveries are not always reported. A high percentage recovery of 101.8% was achieved for LD extraction from *Mucuna pruriens* dehulled and whole seed [35]. Appreciable values of 94.1 to 116.6% were also reported for the extractions from *Vicia faba* roots, sprouts and seeds [40].

An extraction efficiency of 9.2 ± 0.1 wt% of LD was achieved from *Mucuna pruriens* seeds by employing citric acid as solvent extraction. In order to find more sustainable solvents, the authors demonstrated that organic acids, such as citric acid, have hydrotropic properties: in acidic environments, they interact with protonated compounds characterized by low solubility. Thanks to the intermolecular interactions between the solvent and LD, a high extraction selectivity was assured, with relative purities of levodopa higher than 90%.

2.2. Ultrasound-Assisted Solvent Extraction (UAE)

The ultrasound-assisted extraction (UAE) is based on the ultrasonic energy waves that generate acoustic cavitation in the liquid medium and allow the cells' disruption: this method improves analyte dissolution and its mass-transfer to the solution [60,61].

Based on the literature, the UAE method for the extraction of LD from plant matrices is more commonly used compared to reflux, maceration or microwave-assisted extraction (MAE) extraction methods. The extracting solutions used are mostly acidified solutions such as water acidified with 0.1 M HCl (pH = 2.6), HCl 0.1 M, HCl 10 mM, aqueous CH_3COOH 0.2% (*v*/*v*) [48,50,52–54]. The extraction times are usually in a range between 15 and 30 min, with the exception of the work of Chen et al. [50], where a UASE extraction of LD from *Lens culinaris* and *Vicia faba* seeds matrices was applied in duplicate for 60 min by using a 10 mM HCl extracting solution. The percentage recovery values obtained from the two matrices are 99.8% for *Vicia faba* and 105.0% for *Lens culinaris*: these values suggest that the methodology used allows high recoveries and therefore shows high extraction efficiency.

Generally, a long-term exposure of the extraction matrix to ultrasounds is discouraged. Palonowska et al. [15] conducted a study on the optimization of LD extraction from *Vicia faba* and pointed out that a prolonged exposure of the plant matrix to ultrasounds has a negative effect. They demonstrated that the content of LD extracted from dry beans of *Vicia faba* var. major Bachus decreased from 74.8 ± 1.4 μg/g (dw) to 71.9 ± 0.7 μg/g (dw), by switching ultrasonic extraction time from 10 to 60 min, respectively. Initially, a positive effect of ultrasounds employment was observed up to 10 min of treatment. A further increase in the sonication time decreased the LD content in the extract. A possible explanation for these results might be that ultrasounds cause overheating, thus promoting the thermal degradation of LD. It is therefore advisable to shorten the sonication time as much as possible or at least to take precautions aimed at reducing overheating, such as water circulation in the ultrasonic bath [48].

2.3. Microwave-Assisted Extraction (MAE)

Microwave-assisted extraction (MAE) is an extraction technique that involves the use of microwaves in order to heat the sample–solvent mixture and thus facilitate the extraction of the analyte. The microwave heat source has the advantage of acting on the entire volume by interacting with polar compounds (e.g., water and some organic components) present in the plant matrix based on ionic conduction and dipole rotation mechanisms [60,62].

In the literature, there is only one work by Dahnani et al. [48] where MAE was used for LD extraction from *Mucuna pruriens* seeds. In this work, the performances of the conventional extraction method of refluxing were compared with those of two green methods, namely ultrasound and microwave-assisted solvent extraction (MASE). The MASE recovery percentages obtained on four *Mucuna pruriens* seeds (Arka Dhanwantri, Arka Ashwini, White, Brown) fall within the range of 54.0–58.5% for 15 min of extraction. These values are encouraging when compared with the percentages recorded on the same matrices by using UAE extraction treatment, which were comprised in the range of 26.8–31.5%. Better recoveries of 57.2 to 65.7% were obtained with a reflux extraction. The higher extraction yield obtained using refluxing in comparison to UAE and MAE was justified by the authors in terms of the exhaustive extraction by refluxing. It is worth noting that extraction times of the order of 5 to 15 min were employed for UAE and MAE against the 5 h employed for reflux. In general, higher extract yields were obtained with UAE and MAE by increasing the extraction time, even if a definite trend was not derived. The authors concluded their study pointing out that, among the three extraction methods explored, the microwave-assisted extraction provided overall the best results in terms of yield and quality of *Mucuna pruriens* extract by employing shorter times and with minimal solvent consumption.

2.4. Reflux Extraction

Reflux extraction is a common solid–liquid extraction process that takes place at a constant temperature and is based on repeated cycles of evaporation and subsequent condensation of the solvent over a given period of time. This process is efficient, simple, economical and very popular at an industrial scale [63]. As reported in Table 1, there are few works where a reflux extraction protocol has been performed, although notable recovery percentages ranging in the interval of 98.1–106.7% were achieved for LD extraction from *Mucuna pruriens* seeds var. Preta Kaunch [56]. For this extraction, the following conditions have been used: HCl 0.1 M; solvent: sample ratio 2:1 (*v*/*w*); extraction time 180 min; extraction temperature: 25 °C. The long extraction time adopted is to be noticed, for it constitutes a disadvantage of this technique (extraction times generally range from a minimum of 30 min [55] to a maximum of 300 min [48]) along with the higher solvent volumes needed compared to UAE, MASE or LSE extractions.

2.5. Maceration and Soxhlet Extraction

Maceration is a simple and low-cost extraction technique which has some drawbacks such as low extraction yield and need of large amounts of solvents. The extraction starts by grinding the plant sample into smaller particles to increase the surface area and improve the mixing with solvent. Then, the material–solvent mixture plant is kept for a long time, agitated at different intervals and finally filtered through a filtration medium.

The Soxhlet extraction is an automatic continuous extraction method which makes it possible to achieve a high extraction efficiency. A small amount of dry sample is placed in a thimble-holder, located in a distillation flask containing the extraction solvent. When an overflow level is reached, the solution of the thimble is aspirated by a siphon and is delivered back into the distillation flask, carrying the extracted solutes into the bulk liquid. The solute remains in the distillation flask, whereas the solvent comes back to the solid sample. The process is repeated until complete extraction takes place.

Due to the need for larger amounts of solvents and longer extraction times compared to UAE, MAE or LSE techniques, there are only a few works in the literature where maceration and Soxhlet extractions are used, as it is possible to see from Table 1. In particular, maceration has been reported for LD extraction from *Mucuna pruriens* powder formulation with a recovery percentage of 94.5% [57] and from *Phaseolus vulgaris* dried seed, seeding and callus with recoveries in the range of 99.55–100.27% [58]. In both cases, the high recovery percentages were achieved at the expense of the extraction times that were extended up to 7 days and 5 days, respectively. Singh at al. used the Soxhlet method for LD extraction from *Mucuna pruriens* seeds, obtaining recovery percentage values in the range of 98.67–100.4% [59].

3. Levodopa Detection Methods

In the last 20 years, many attractive papers have been published showing the optimized techniques used to identify and quantify the LD in plant matrices (Table 2). Most of these require a preliminary separation step, and the HPLC is most often used; others allow a direct detection of the analyte.

Table 2. Analytical methods and operation conditions employed in the last twenty years for the LD detection with their main strengths and drawbacks.

Methods	Sample Source	LOD Range	Stationary Phase	Mobile Phase	Detection Mode	Strengths	Drawbacks	References
HPLC-UV	Broad bean, cocoa and beans	10 ng/mL– 15 μg/mL	RP-C18 (mean particle diameter 5 μm, 125 × 3 mm I.D.)	Solvent (A): acetate buffer, pH = 4.66; solvent (B): methanol	Photodiode array detector (DAD)	It is highly reproducible, rapid and efficient	Sensitivity is rather limited so it is suitable for plant matrices with medium and high concentrations of LD. Selectivity is also limited since it does not allow the unambiguous identification of structurally similar molecules.	[38]
	Mucuna pruriens dehulled and whole seed			Solvent (A): water/methanol/phosphoric acid 975.5:19.5:1 (*v*/*v*/*v*), pH = 2.0; solvent (B): 70% methanol.				[35]
	Vicia faba seeds (cotyledons and embryo axis)			Ammonium phosphate buffer (0.05 mol/kg, pH = 2.0)				[25]
	Vicia faba broad beans			Water (H_2O) and acetonitrile (ACN) both containing 0.1% (*v*/*v*%) acid formic				[39]
	Vicia faba roots, sprouts, seedling, leaf, flower, pod, stem			Solvent (A): 0.1% acetic (98%); Solvent (B): methanol (2%).				[43]
	Vicia faba sprouts			Solvent (A): 82% buffer solution (32 mM citric acid, 54.3 mM sodium acetate, 0.074 mM Na_2EDTA, 0.215 mM octyl sulphate pH = 4); Solvent (B): 18% methanol				[44]

Mucuna and *Stizolobium pruriens* seed		Solvent (A): 0.1 N acetic acid (90%); Solvent (B): methanol (10%)	[45]
Mucuna pruriens seed		Solvent (A): 0.1% formic acid (98%); Solvent (B): methanol (2%)	[48]
Vicia faba flowers, fruits and leaves		Solution of 50 mM potassium dihydrogen phosphate (pH = 2.3)	[36]
Vicia faba sprouts, leaves, flowers, pods, roots		Solvent (A): water with 0.3% formic acid; Solvent (B): acetonitrile with 0.3% formic acid	[51]
Vicia faba seeds		Solvent (A): 97% *v*/*v* of an aqueous solution of 0.2% *v*/*v* acetic acid; Solvent (B): 3% *v*/*v* methanol	[15]
M. pruriens seeds		Water, methanol and acetonitrile (5:3:2) containing 0.2% triethylamine, pH = 3.3	[54]
Mucuna pruriens powder formulation		Solvent (A): water 80% *v*/*v*; Solvent (B): methanol 20% *v*/*v*	[57]
Mucuna pruriens powder and extracts	RP-C18 (mean particle diameter 5 µm)	Water: Methanol: Acetonitrile (100:60:40) containing 0.2% Triethylamine, pH = 3.3	[55]
Mucuna sanjappae Seed	RP-C18 (250 × 4.6 mm I.D.)	Methanol	[53]

	Mucuna utilis seed		RP-C18 (250 × 4.0 mm I.D.)	Solvent (A):0.5% *v*/*v* of acetic acid 30%; Solvent (B): methanol 70%				[59]
LC-MS	*Vicia faba* roots, sprouts and seeds	18 µg/Kg	RP-C18 (mean particle diameter 2.6 µm, 100 × 4.6 mm I.D.)	Solvent (A): ultrapure water with 0.5% (*v*/*v*) formic acid 50%; Solvent (B): methanol 50%	Photo diode array detector (DAD) and triple quadrupole (TQ) mass spectrometer	Robust analytical technique that provides higher sensitivity and selectivity than LC-UV methods. It allows to unambiguously identify the compounds under analysis, through the possibility of fragmentation.	It is a technique susceptible to matrix effects: co-eluting compounds could interfere with the ionization of the analyte under examination. Detection in MRM mode is to be preferred.	[40]
		0.01 µg/mL	Not reported	Not reported	Photo diode array detector (DAD) and quadrupole-time-of-flight (QTOF)-mass spectrometer			[49]
	Avena sativa seeds		RP-C18 (mean particle diameter 4 µm, 250 × 2 mm I.D.)	Solvent (A): solution 0.1% (*v*/*v*) of formic acid (97%); Solvent (B): ACN/MeOH 75/25 containing 0.1% (*v*/*v*) formic acid (3%)	Ion Trap mass spectrometer			[42]
HPTLC	*M. pruriens* seeds	Not reported	Silica-coated aluminum sheet (10 cm × 10 cm with 0.2 mm thickness)	n-butanol, acetic acid and water were used as mobile phase at 4:1:1	UV-Vis thin layer scanner	It makes it possible to obtain a preliminary separation of the analytes in a fast, efficient, easy and low cost analysis	It is generally employed only for qualitative analysis. It is poorly reproducible, as it works in an open system, whose environmental conditions could alter the results.	[54]
CE-UV	*Vicia faba* seeds / *Lens culinaris* seeds	LOD value 0.7 µg/mL.	47 cm (40 cm from inlet to the detector) × 75 µm i.d. fused-silica capillary	35 mM NaH_2PO_4, pH = 4.55, 17.5 kV and 30 °C.	Photo diode array detector (DAD)	It allows faster analysis and higher efficiency than LC-UV	It is less sensitive than HPLC-UV	[50]

Electrochemical methods	*Mucuna pruriens* seed, leaves	LOD value 1.54 µM	Working electrode: gold modified pencil graphite	Supporting electrolyte: 0.1 M phosphate-buffered solution (pH = 7.0)	Differential pulse voltammetry (DPV)	It makes it possible to identify and quantify the analyte in a fast and economical way, through the use of conventional or modified nanostructured electrodes, which permits a better selectivity and sensitivity of analysis.	The technique still shows limitations especially related to the problem of electrode poisoning and oxidizable interfering compounds in the same range of anode potential.	[46]
	Mucuna pruriens seed cooked and raw	LOD value 5.12 ng/mL	RP-C18 (mean particle diameter 3.5 µm, 150 × 2.1 mm I.D.) Working electrode: Glassy carbon	Eluent/supporting electrolyte: 103 mM sodium acetate, 0.88 mM citric acid, 2.14 mM 1-octanesulphonic acid sodium salt with pH adjusted to 2.38 by orthophosphoric acid	Amperometric detection at a potential of +0.7 V after micro-high performance liquid chromatography separation			[41]
	Sunflower seed, sesame seed, pumpkin seed and fava bean seed	LOD value 14.3 nmol/L	Working electrode: glassy carbon modified by graphene quantum dots decorated with Fe_3O_4 nanoparticles/functionalized multiwalled carbon nanotubes	Supporting electrolyte: 0.1 mol/L PBS at pH = 5.5	Differential Pulse Voltammetry (DPV)			[64]
	Sweet potato	17 nM	Working electrode: nitrogen-doped graphene supported with nickel oxide nanocomposite	Supporting electrolyte: 0.05 M PBS at pH = 7	Differential pulse voltammetry (DPV)			[65]
Spectrophotometry	Wild type legume grain	LOD 1.12 µg/mL	//	//	//	It is an easy to use and low-cost technique that allows both	It is generally not preceded by a separation step. This implies that the sample can	[52]

UV-Vis	*Phaseolus vulgaris* dried seed, seeding and callus				qualitative and quantitative evaluation	contain interfering compounds causing potential false positives.	[58]
NMR	*Mucuna pruriens* seed	LOD value 0.0175 mg/g	//	//	//	It is a highly reproducible technique. It makes it possible to get structural details of the compounds under examination.	Requires expensive equipment and provides low sensitivity compared to LC-MS. It is hardly used for quantification, due to the chemical noise and signal overlapping.	[56]

// This indicates that values were not reported.

3.1. High Performance Liquid Chromatography Coupled to UV–Vis (HPLC-UV)

The most widely used analytical method to detect LD from plant matrices is high performance liquid chromatography (HPLC) coupled to diode array detection (DAD) set at 280 nm. Generally, HPLC-UV requires an analyte concentration in the sample greater than that needed for LC-MS, as the sensitivity is limited. Similar molecules could absorb at the same wavelength value too (especially metabolites deriving from the same synthesis pathways or degradation products of a precursor metabolite); therefore, the HPLC-UV selectivity is lower than that of LC-MS which can instead provide a unique and unambiguous identification.

As far as the plant matrices reported in the references are concerned, LD is structurally similar to tyrosine, dopaquinone or dopamine, which at a wavelength of 280 nm can still give absorption and generate chromatographic peaks whose absorption spectrum is not unambiguously identifying the analyte. In this case, the use of standards and the comparison of retention times are more helpful than the only analysis of UV absorption spectra to validate the separation method and confirm the identity of the compounds.

Despite these disadvantages, HPLC-UV remains the most widely used technique since it is highly reproducible, rapid (in all methods proposed, LD peak can be observed up to 10 min into the chromatogram), efficient, very robust and has better sensitivity compared to UV–Visible spectrophotometry as well as being less expensive than LC-MS.

Regarding the chromatography separation conditions, all the works report the employment of columns with a classic C-18 stationary phase (although the most suitable chromatographic columns for the separation of small and polar molecules, such as LD, are different, e.g., the ZORBAX Eclipse Plus Phenyl-Hexyl [13]); instead, the most used mobile phases are acidified aqueous solutions with a pH range between 2 and 4.6 mixed with methanol or acetonitrile. Figure 5 shows an example of chromatographic separation conducted by Duan et al. [51], where LD was detected in faba tissues at different growth stages and separated from vicine and convicine ((A) 8-day-old sprout; (B) New leaf at vegetative stage; (C) Old leaf at vegetative stage; (D) Flower bud; (E) Pod hull at S4 stage; (F) Bean at S4 stage; (G) Stem at ripening; (H) Root at ripening stage; (I) Standard peaks of vicine and L-dopa, see Table 2 for operation conditions).

The limit of detection (LOD) reported for the different methods developed goes from 10 ng/mL [38] up to 15 μg/mL [48]. The best LOD/LOQ values are obtained by Baranowska et al. [38], respectively, 10/30 ng/mL on broad bean, cocoa and bean samples. The HPLC analyses were performed using gradient elution with acetate buffer (pH = 4.66) and methanol, with a DAD detector and fluorescence detector as well, which provides lower limits of quantification and detection because they are more sensitive and selective than the first detector. This is the only work that uses the fluorescent detector coupled to DAD. In fact, all other works report higher LOD/LOQ values because only DAD detectors are employed, e.g., LOD/LOQ 0.115/0.348 μg/mL by Kasture et al. [57] are better than the others that only use the DAD detector, by employing a RP-C18 (250 mm × 4.6 mm × 5 μm) column and water–methanol 80:20 as mobile phase.

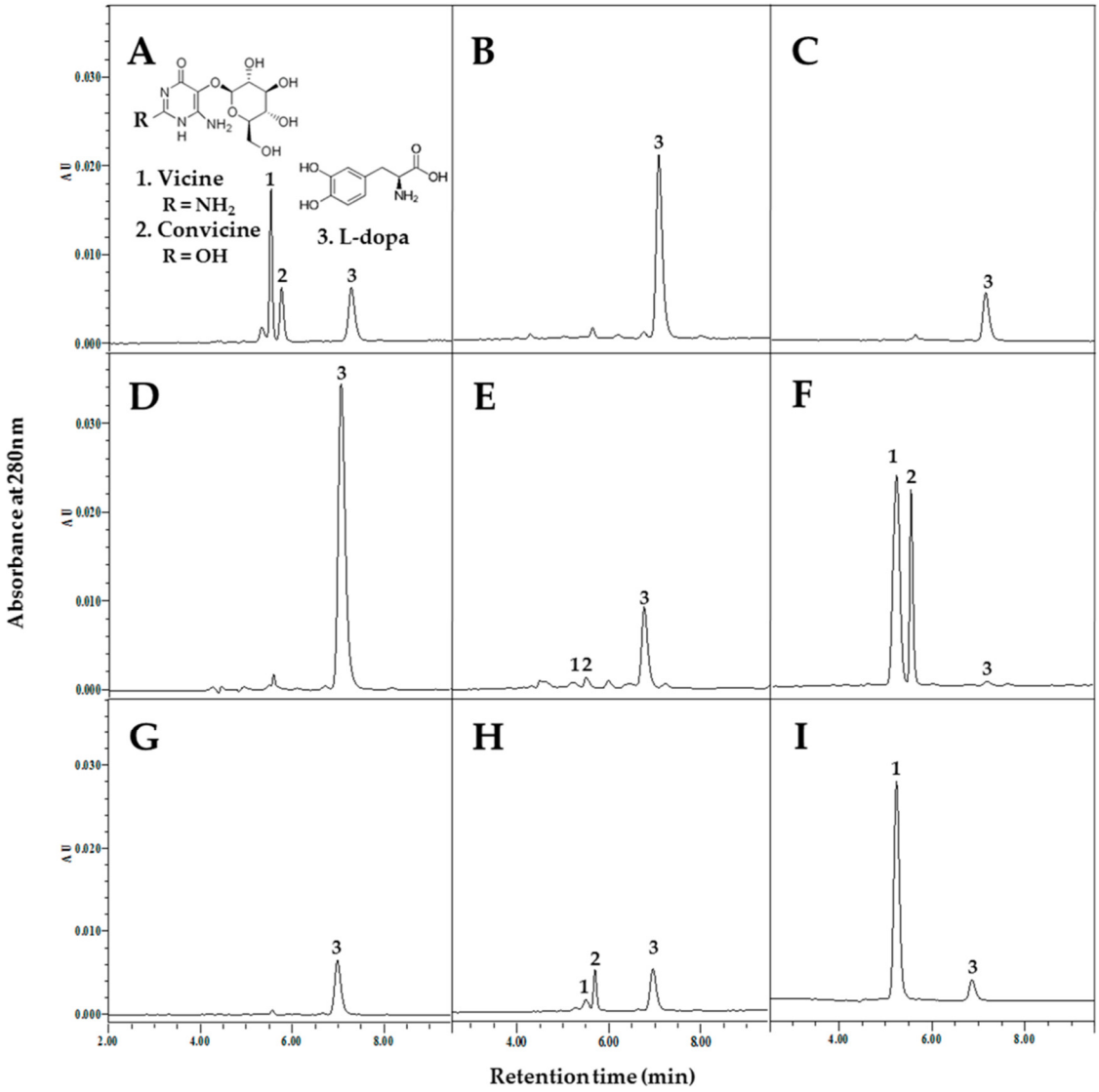

Figure 5. HPLC chromatograms of extracts from different faba bean tissues. (**A**) 8-day-old sprout; (**B**) New leaf at vegetative stage; (**C**) Old leaf at vegetative stage; (**D**) Flower bud; (E) Pod hull at S4 stage; (**F**) Bean at S4 stage; (**G**) Stem at ripening; (**H**) Root at ripening stage; (**I**) Standard peaks of vicine and L-dopa. Reprinted with permission from Ref. [51].

Kasture et al. [57] and Baronowska et al. [38] showed the most suitable values of LOD/LOQ, so they theoretically provide methods that allow a more selective and sensitive quantification and separation of LD in plant matrices (in the specific case of Kasture et al. [57], on *Mucuna pruriens* samples).

Nevertheless, despite the good LOD/LOQ values, the choice of the water and methanol mix as mobile phase by Kasture et al. [57] is in contradiction with most of the works reported here, where acidified mobile phase are largely applicated. Among all, three of them used a phosphate buffer as mobile phase for LD separation (e.g., Goyoaga et al. [25] proposed a separation method with only ammonium phosphate buffer 0.05 mol/kg, pH = 2.0), although it is not the best choice for instrument maintenance because it can reduce the column life, due to precipitation of phosphate salts. For this latter reason, acidified aqueous solutions (e.g., water: acetonitrile both containing 0.1% (*v*/*v*%) acid formic by Renna et al. [39], 0.1% formic acid: methanol 98:2 by Duan et al. [51]) are preferred, because they allow a more efficient LD separation (LD chromatographic peaks appreciably resolved in the chromatograms of the *Vicia faba* samples reported by Renna et al. [39] and Duan et al. [51] in comparison to Kasture et al. [57] who do not show any chromatogram)

and a more efficient LD quantification than the only use of organic phase (e.g., Patil et al. [53] reported the LD separation from *Mucuna sanjappae* seeds by using only methanol on a RP-C18, 250 × 4.6 mm I.D. column.) or only water use as aqueous phase [57]. The reason for the use acidic solutions mobile phases could be the ion pair formation between analyte and counter-ion of the acid used, which provides better analyte stabilization and better separation than other matrix compounds. This is especially helpful for the analysis of plant matrices with a medium and low content of LD, where a best resolution and selectivity of chromatographic peaks are required.

In addition, the lack of analytical method validation for all reported papers (Rathod et al. [55], Singh et al. [59], Kasture et al. [57] are the only ones who validated the method performed by following the International Council for Harmonization ICH guidelines) represents a disadvantage for those who approach the choice of an appropriate HPLC-UV LD analysis method, which is consistent and satisfying the analytical requirements for different plant matrices.

3.2. Ultra-High-Performance Liquid Chromatography Coupled to Mass Spectrometry (UHPLC-MS)

Ultra-high-performance liquid chromatography coupled to mass spectrometry (UHPLC-MS) is a valid method to determine the presence of specific compounds in a matrix. So far, only a few examples have been published in the literature where LC-MS has been used to determine LD presence in plant matrices. Varga et al. [42] determined LD in extracts from *Avena sativa* seeds by using a UHPLC coupled with an electrospray ionization ion trap mass spectrometer (ESI- ITMS). Separation was performed under gradient elution by using Eluent A consisting of 0.1% (*v*/*v*) formic acid and 3% (*v*/*v*) of Eluent B, containing ACN/MeOH at a volume ratio of 75/25 containing 0.1% (*v*/*v*) formic acid on a Synergi Hidro-RP250 × 2 mm I.D., mean particle diameter 4 μm analytical column. They reported the most abundant fragmentation of protonated precursor ion $[(M+H)]^+$ at *m*/*z* 198: $[(M+H)\text{-}NH_3]^+$ at *m*/*z* 181 and $[(M+H)\text{-}H_2O\text{-}CO]^+$ at *m*/*z* 152. For MS analysis, they optimized ESI conditions. They found that a good peak intensity is reached with a gas pressure of 60 psi and a drying gas pressure of 25 psi at a nebulizing gas temperature of 350 °C. For this validated method, RSD% was reported as better than 4%. Furthermore, the high specificity allowed to obtain lower LODs is 18 μg/kg. In this way, Varga et al. [42] have been able to detect LD in different species of oat, where LD concentration is three time lower than in *M. pruriens.*

Pavòn-Perèz et al. [40] determined the presence of LD from *Vicia faba* roots, sprouts and seeds. In this case, separation was carried out on a Phenomenex (Torrance, CA, USA) Kinetex XB Core-Shell C_{18} column (100 mm × 4.6 mm, 2.6 μm), thermostated at 35 °C, using a mobile phase composed of 50% ultrapure water with 0.5% (*v*/*v*) formic acid and 50% methanol. LD detection was carried out by using an SPD-M20A diode array detector (DAD) and an LCMS-8030 triple quadrupole mass spectrometer. The separation method was validated following the ICH guidelines. Repeatability showed an RSD value of 1.40%, while recovery yield values were recorded from 94.14 to 116.62%, with RSD values ≤ 5.66%. For the MS analysis, full spectra were acquired in a 50–1200 *m*/*z* range. They applied a multiple reaction monitoring (MRM) method for quantification using the transition *m*/*z* 198 → *m*/*z* 152. The method developed by Pavòn-Perèz et al. [40] shows detection and quantification limits of 0.01 and 0.05 μg/mL: these values are much lower than those reported by Kasture et al. [57] for HPLC/UV method (LOD/LOQ 0.115/0.348 μg/mL). Compared to HPLC-UV alone, the opportunity to couple an HPLC system with the mass spectrometry makes it possible to identify compounds under investigation, based not only on their retention time but also on the mass. Furthermore, the possibility of applying fragmentation is helpful for characterization, in particular for the natural extract analyses, where the presence of isomers with similar retention times is possible.

3.3. High Performance Thin Layer Chromatography (HPTLC)

High performance thin layer chromatography (HPTLC) is a simple, robust, rapid and efficient analytical technique in quantitative analysis. HPTLC allows for a better separation than TLC, and the separation can be further improved with bidimensional HPTLC. Aware et al. [54] used HPTLC to determine LD presence on extracts from *M. macrocarpa* beans. The sample was spotted on a silica-coated aluminum sheet (10 cm × 10 cm with 0.2 mm thickness), and LD was separated with a mixture of n-butanol: acetic acid: water (4:1:1). Although HPTLC provides a better resolution and a visual result, it can only be used as a preliminary step as a complementary method for quantitative analysis is still required.

3.4. Capillary Electrophoresis Coupled to UV–Vis (CE-UV)

Chen et al. [50] reported a simple method to determine LD from broad beans and lentils. Experiments were performed by using a Beckman P/ACE system 5510 coupled to a photodiode array (PDA) detector, set at 210 nm. For this separation, a 47 cm × 75 μm i.d. fused-silica capillary was employed.

The authors stressed the influence of pH on the LD charge state and then on its stability and solubility. In this regard, capillary electrophoresis revealed a powerful tool to determine the pK_a values of LD (2.30, 8.11 and 9.92) along with its isoelectric point (5.20). The appearance of pseudo-peaks in electropherograms registered at neutral and basic pH confirmed LD instability in these conditions and the requirement for an acidic environment for its determination, in accordance with the pK_a values derived. The separation conditions were optimized by using as running buffer 35 mM NaH_2PO_4, pH = 4.55. Column was kept at 30 °C and a voltage of 17.5 kV was applied for the separation. For this method, a LOD of 0.7 μg/mL was calculated. The method was validated for the quantification of LD in beans.

Generally, CE allows for faster analysis, higher efficiency and lower sensitivity than LC-UV even if in the present case comparable performances were observed considering that a LOD of 0.7 μg/mL was achieved and that migration times of about 10 min were observed and then of the same order of the elution times reported in Table 2 for HPLC methods. The use of an aqueous buffer without organic solvent and the reduced cost of reagents and capillaries compared with that of HPLC solvents and columns are undoubted advantages of the proposed electrophoretic method.

3.5. UV-Vis Spectrophotometry

UV-Vis spectrophotometry is an analytical technique that is easy to use and low cost. Generally, it is used as a direct method without a preliminary separation step. This implies the risk of occurring in false results or inaccurate measurements, as the technique cannot really ascertain the compound identity and purity.

Vadivel et al. [52] determined the LD content in wild type legume grains collected from South India by measuring the ultra-violet light absorption at 282 nm. The LD content varied in the range of 1.34–5.45 g/100 g of dry matter. The samples of *Mucuna gigantea* showed the maximum content of LD. The spectrophotometric method adopted was not discussed at all, and there are no data concerning selectivity, accuracy and precision. The authors focused their interest on studying the effect of processing techniques used by Indian tribal groups on the level of LD. As an example, a drastic reduction of LD (29–46%) was observed during soaking and cooking in an alkaline solution due to the enhanced seed coat permeability increasing the leaching out of LD. Furthermore, alkaline conditions promoted the chemical conversion of LD into melanin pigments. Sprouting and oil-frying treatment also caused a significant decrease in LD content (34–48%), suggesting that enzymes able to metabolize LD, such as polyphenol oxidase, could be synthesized upon germination of seeds.

Rahami-Nezhad et al. [58] developed a UV-spectrophotometric method based on a nitrosation reaction of LD in an acidic medium and then a treatment with NaOH to form a stable red compound which absorbs at a wavelength of 470 nm. The method revealed to be highly selective for the detection of LD comparing with phenolic compounds such as resorcinol, pyrogallol, phenol and tyrosine. The authors stressed the selectivity towards LD by showing that the formation of the deep red color in the reaction solution was not observed for the other compounds investigated. Indeed, tyrosine gave no coloration whereas resorcinol, pyrogallol and phenol gave less intense coloration. In order to exclude that the method can generate a false positive in the determination of LD, besides the visual inspection of the reaction solutions, it would have been desirable to demonstrate the lack of absorbance from the phenolic compounds upon reaction at the detection wavelength.

The method has been fully validated showing good performances. Percentage RSD values of intra- and inter-day were found between 0.24 and 0.36% as well as 0.08 and 0.36%, respectively, indicating satisfactory precision. It was successfully applied to the quantification of LD in 33 biotypes of *P. vulgaris* seeds. No significant differences were observed by comparing the results obtained by the proposed method with those deriving from a reference HPLC method. Among the tested biotypes, black seeds possessed higher amounts of LD in comparison to yellow and brown types. LD content was determined also in seed dark germination and callus culture of *Phaseolus vulgaris* in different conditions. The results obtained show that tyrosine significantly increased the concentration of LD and thus could be exploited for the large-scale production of LD.

3.6. Electrochemical Methods

Electrochemical methods are of considerable interest for the analysis of biological compounds since they offer important advantages compared to classical methods such as fast response time, simple equipment, low cost, high sensitivity and even selectivity without samples pretreatment. The modification of conventional electrodes by nanomaterials such as metal nanoparticles, graphene oxide and carbon nanotubes, makes it possible to achieve detection limits at the nanomolar level, high precision and accuracy by substantially improving the response selectivity.

To date, considering the ease of LD to oxidize, there are a plethora of electrochemical sensors for LD anodic determination in areas such as the pharmaceutical or clinical, whereas there are few applications to the analysis of food samples. Amperometric detection was firstly apply to LD detection in raw and cooked *Mucuna* bean seeds by Mwatseteza et al. [41] by applying a constant potential of +0.70 V vs. Ag/AgCl at a glassy carbon electrode. It is worth noting that the employment of an unmodified electrode was possible thanks to a preliminary micro-high performance liquid chromatography separation that nullifies the feasibility of electrochemical techniques for direct sample analysis. Detection of low concentration of L-dopa up to 5.12 ng/mL was achieved even if no data are reported on the method's accuracy.

A gold modified pencil graphite electrode was employed for LD detection by differential pulse voltammetry (DPV) in *Mucuna pruriens* leaves and seeds [46]. The detection limit for L-dopa was 1.54 μM. The appreciable separation of the LD current peak from that of ascorbic acid, along with the good recoveries evaluated in all the real samples, indicated the successful applicability of the method for determinations in complex matrices.

A glassy carbon electrode modified with a new nanocomposite consisting of graphene quantum dots decorated with magnetic nanoparticles and carboxylated multiwalled carbon nanotubes was employed as a new sensing platform for the electrochemical determination of L-dopa by DPV in sunflower seed, sesame seed, pumpkin seed and fava bean seed [64]. Good sensitivity and selectivity with low overpotential for the determination of LD and a detection limit of 14.3 nmol/L were obtained. The influence of some coexistent interfering substances was examined, setting their tolerance limit as the amount of foreign ion causing ±10% error in the determination of LD. The lowest value was

obtained for dopamine, as it would be expected considering its similar structure with LD. The results obtained on LD quantification in the real samples were in good agreement with those obtained by a reference spectrophotometric method.

Finally, a nitrogen-doped graphene oxide incorporated nickel oxide modified electrode was applied for the sensitive determination of LD by DPV in sweet potato with a limit of detection of 17 nM. The selective determination of LD was achieved in the presence of common interferents, displaying a minor response from uric acid, acetaminophen and L-cysteine. The recovery percentages from sweet potato (Ipomoea batatas) samples were in the range of 97.8–101.5% [65].

3.7. Nuclear Magnetic Resonance Spectroscopy (NMR)

Nuclear magnetic resonance spectroscopy (NMR) has been largely used to determine the chemical structure of compounds isolated from different food matrices. It is a simple, robust, rapid and not disruptive method, although it requires very expensive instrumentation. Furthermore, it is barely used for quantification analysis as an internal standard need to be added to the sample's solution. In this way, the sample cannot be directly recovered after analysis. For the LD identification, there is only one publication where they used this technique. Fernandez-Pastor et al. [56] were able to quantify the presence of LD from different *Mucuna pruriens* seeds. NMR analyses were performed by using 600 MHz Varian Direct Drive NMR spectrometer. Samples were dissolved in a mixture of 0.1 M $HCl{\cdot}H_2O$:D_2O:DMSO-d_6 (8:1:1) and syringic acid was added as internal standard. In this work, they were able to set values of 0.0175 mg/g for LOD and 0.0578 mg/g for LOQ.

4. Conclusions and Outlook

In the last twenty years, some progress has been made in developing sensitive and selective extraction and detection methods for the unambiguous identification of LD in plant matrices, mainly *Vicia faba* and *Mucuna pruriens* seeds and green parts. However, one major difficulty in LD determination is quantitatively extracting the compound of interest. Using efficient extraction methods can improve the use of natural products with high levels of this bioactive compound in the Parkinson's disease.

LSE, UASE, MASE techniques are the most widely used, although the different procedures proposed still show some contradictions concerning extracting solutions, time and temperature values. Extraction efficiency typically increases by applying more acidic extraction conditions. Chromatographic separations based on a single separation step can show limited selectivity in complex matrices. In the future, the application of multidimensional techniques could considerably reduce the matrix effects and thus significantly improve the analytical performances. Regarding detection techniques, the DAD is typically the method of choice for medium and high content of LD matrices due to its high versatility and diffusion.

Nevertheless, tandem mass spectrometry offers some advantages since characteristic fragments can be generated, thus providing the structural information necessary for LD characterization. A step forward could be done by the validation of LC-MS/MS method for LD quantification in different plant matrices since this analytical technique remains the most sensitive, with LOD and LOQ values lower than those from other analytical techniques reported in the literature. In conclusion, advances have been achieved in LD analysis; however, further efforts are required to establish analytical protocols that can be applied for routine determinations of this compound.

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