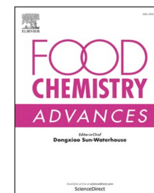
Food Chemistry Advances 6 (2025) 100921

Contents lists available at ScienceDirect

Food Chemistry Advances

journal homepage: www.elsevier.com/locate/focha



# Novel protocol significantly improving spermidine content in extracts from wheat germ: Aqueous conversion of polyamines into neutral-base form, solubility in ethanol and acid precipitation as phosphate salts

Eros D'Amen *, Francesca Truzzi, Anne Whittaker, Giovanni Dinelli

*Department of Agricultural and Food Sciences, Alma Mater Studiorum—University of Bologna, Bologna 40127, Italy*

ARTICLE INFO

*Keywords:*
Spermidine
Polyamine extraction protocols
Polyamine detection
HPLC-ESI-MS
Spermidine supplements

ABSTRACT

There is an increased demand for natural spermidine (SPD) supplements, necessitating improved extraction protocols to overcome current limitations in polyamine (PA) extraction protocols from plant-based sources. The present work describes the development and underlying rationale for a five-phase laboratory-scale extraction protocol that exploits the chemical properties of SPD using a novel approach that differs from all previous water-acid methods. Phase 1: Wheat germ (WG) milling, phase 2: alkaline reaction of PAs with $Ca(OH)_2$, phase 3: solubilization of PAs in ethanol, phase 4: ethanol-insoluble bio-compound separation by filtration, and phase 5: precipitation of PA salts using $H_3PO_4$. This protocol takes advantage of the high ethanol solubility of SPD in base form, compared to the ethanol-insoluble protonated form (SPDH+). The precipitation step permits the separation and purification of SPD from other components extracted in the natural matrix. In so doing, it was possible to attain a high SPD content extract. The average SPD content of the extract, performed on 10 separate WG 100 g batches, was 7.6 ± 1.9 %, significantly exceeding previous methods. The industrial scalability of the protocol using 50 kg WG was implemented with minimal adjustments, yielding 9.6 % SPD/extract. The proposed workable extraction, with no requirement for complex or specialized equipment, has potential towards developing SPD supplements.

## 1. Introduction

Polyamines (PAs) are low-molecular weight aliphatic hydrocarbons containing multiple terminal (primary) and internal chain (secondary) amino groups. PAs, principally composed of putrescine (PUT), spermidine (SPD) and spermine (SPE), are essential cellular components ubiquitous to all living organisms (Sagar et al., 2021). In particular, SPD plays a critical role in the activation of eukaryotic translation initiation, exerting key roles in translation, signaling, and post-translational modifications involved in multiple regulatory pathways (Hirano et al., 2021; Park & Wolff, 2018). The ever-increasing interest in SPD is based on widespread studies underlining the relationship between aging, SPD, and autophagy, indicating that SPD is an important and specific inducer of autophagy, a fundamental mechanism involved in immune and cell renewal functions which decline with aging (Eisenberg et al., 2009; Madeo et al., 2018, 2019; Truzzi et al., 2022; Zou et al., 2022). Additional cellular roles attributable to SPD include DNA stabilization, inflammation suppression, oxygen stress suppression, enhancement of mitochondrial metabolic function and respiration, as well as improved proteostasis and chaperone activity (Hirano et al., 2021; Madeo et al., 2018, 2019; Sagar et al., 2021; Truzzi et al., 2023). Consequently, SPD has been reported to extend lifespan, provide cardio-protection and ameliorate cognitive function (including memory-related neurological diseases), intestinal barrier function, obesity and metabolic syndrome (Hirano et al., 2021; Madeo et al., 2018, 2019; Sagar et al., 2021; Zou et al., 2022). Based on the aforementioned benefits, SPD, has become a hot topic in the field of nutrition and food processing (Zou et al., 2022).

Intracellular SPD in the human body, ranging in millimolar levels (Petrarca et al., 2017), can be derived from three sources: oral intake through diet (exogenous), *de novo* biosynthesis in cells (endogenous), and intestinal microbiota, respectively (Hirano et al., 2021; Sagar et al., 2021). Dietary intake is considered the predominant source of PAs, with increased uptake reputed to be associated with the Mediterranean and Japanese diets (Soda et al., 2021). However, in instances where PA consumption is low, due to the overall low amounts of PAs in local foods, there is a need for exogenous supplementation (Allison, 2024; Soda,

* Corresponding author.
*E-mail address:* eros.damen2@unibo.it (E. D'Amen).

https://doi.org/10.1016/j.focha.2025.100921
Received 22 October 2024; Received in revised form 27 January 2025; Accepted 10 February 2025
Available online 11 February 2025
2772-753X/© 2025 The Authors. Published by Elsevier Ltd. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

2021). This need can also be extended to individuals with SPD deficiency, such as the elderly with decreased PA biosynthetic ability (Schwarz et al., 2018). Overall, promising research on the benefits of SPD specifically have aroused widespread interest, with a resultant demand for supplements (Steichen et al., 2023). This demand is clearly evident from projected increase in the global SPD supplement market from 194 million US$ in 2023 to 331.1 million US$ by 2030 (Valuate Reports, 2024).

The two modalities of exogenous PA ingestion include synthetic supplements (PA analogues) and natural supplements of PA-rich extracts, respectively. Within the framework of natural plant-derived supplements, SPD-based supplements on the market have mostly been developed from WG, which is one of richest plant-based sources of SPD containing 200–300 μg/g WG with low levels of anti-nutritional PAs (Hirano et al., 2021; Mohajeri et al., 2023a; Muñoz-Esparza et al., 2019). One such clinically tested patented WG SPD supplement, shown to improve cognitive ability and inflammation in the elderly (Madeo et al., 2016; Schwarz et al., 2018; Wirth et al., 2019), was awarded status as a "novel food" by the Austrian Agency for Food Safety (AGES) and the European Food and Safety Agency (EFSA) (López-Rodríguez et al., 2022). The SPD contents in this supplement ranged from 0.8 to 2.4 % of the dried extract (EUR-LEX, 2020). Other WG and soybean supplements are shown to fall within the range of 0.064–0.94 % SPD (Steichen et al., 2023). However, the requisite to produce supplements containing higher concentrations of SPD was highlighted (Hirano et al., 2021; Schwarz et al., 2022; Steichen et al., 2023). Given the interest on the part of the global SPD market (Valuate Reports, 2024), there exists a strong incentive for improvements in SPD extraction protocols.

Acidic-water-based extraction protocols are the classical standard methods in obtaining PAs from plant sources. Traditionally, perchloric acid ($HClO_4$) and trichloroacetic (TCA) extraction protocols were shown to yield PA-enriched extracts (Dai et al., 2014; Ibarra et al., 2015; Magnes et al., 2014; Minocha et al., 1994), but these were not considered suitable for oral formulations. Moreover, extraction of water-soluble PAs in acidic-water extracts was shown to produce highly hygroscopic, viscous molasses-type solutions, due to the recovery in water solution of several additional components of the WG (Yamada et al., 2014). A US patent by Yamada et al. (2014) proposed a high-efficiency laboratory-scale (100 g) PA extraction method from natural products, and was based on the use of weaker edible acids (citric acid), as well as the resort to ethanol with water to remove additional biological components. Currently laboratory-scale investigative research and industrial-scale research for SPD supplement development accept and favor variations based on this method (Madeo et al., 2016; Mohajeri et al., 2023, 2025; Yamada et al., 2014). However, given the existing low concentration of SPD obtained, there is a need for an innovative extraction process, based on a different chemical approach compared to the simple aqueous extraction in acidic conditions. Hence, the aim of the present study was to propose a novel five-phase extraction protocol from WG to yield SPD contents significantly exceeding the range currently found in natural (plant-based) SPD supplements.

The hypothesis underlying this work is that by exploiting the different solubility properties of SPD (free base) in alcohols compared to the protonated form (spermidinium cation, SPDH+), it is possible to obtain a separation and purification of this active ingredient from other components in a natural extract. In fact, while uncharged SPD is highly soluble in ethanol (approximately 40 mg/ml), its salts are not as soluble (< 1 mg/mL). The objective was, therefore, to implement processing conditions to obtain an ethanol extraction of the base form of SPD, followed by the selective precipitation of this active ingredient as a salt. WG was selected as the starting matrix, being a natural source rich in SPD and low in antinutritional and potentially harmful compounds (especially other antinutritional PAs, such as cadaverine and histamine, often abundant in fermented foods) that is readily available in large quantities as a discarded byproduct of the wheat milling industry (Hirano et al., 2021; Mohajeri et al., 2023a; Muñoz-Esparza et al., 2019; Saha Turna et al., 2024). Here, we present the development of the novel five-phase laboratory-scale extraction protocol (100 g WG) for increased SPD, with a comparative efficacy shown for an industrial-scale up (50 kg WG).

## 2. Materials and methods

### 2.1. Materials

Fresh WG, as a byproduct of wheat flour milling, was supplied by the Molino Naldoni milling company (Faenza, Italy). SPD 99 % (N-(3-aminopropyl)butane-1,4-diamine trihydrochloride), SPE 99 % (N,N′-Bis(3-aminopropyl)−1,4-butanediamine tetrahydrochloride) and calcium hydroxide (ACS reagent, purity >95 %) were purchased from Sigma Aldrich (Merck Spa, Milan, Italy). Orthophosphoric acid (purity 85 %), and 95 % ethanol were from Carlo Erba Reagents (Milan, Italy). All other reagents were of analytical grade.

### 2.2. Laboratory-scale extraction of SPD from wheat germ

Ten separate batches (lots) of WG, obtained from the Molino Naldoni milling company, were individually extracted for SPD content. The extraction protocol was subdivided into five sequential phases, namely, milling, alkaline conversion, ethanol extraction, filtration, and acid precipitation, respectively. For phase 1, the WG (100 g) was milled to fine powder (0.2–0.5 mm) using a blade mill (Moulinex AR11, Moulinex Group Italia-SEB, Milano, Italy). The ground powder was then transferred to a 1 L beaker. In phase 2 of the extraction protocol, the milled WG was dry-mixed with 2.0 g calcium hydroxide ($Ca(OH)_2$) (20 mg/g WG) and kneaded following the addition of 50 mL of distilled water (0.5 mL/g WG). The resultant pH of the mix was > 10.5. The homogeneous mixture was kneaded for 30 min at room temperature. Thereafter, in phase 3, 800 mL 95 % ethanol (8 mL/g WG) was added to the mix and stirred vigorously for 1 h to solubilize and extract the PAs. In phase 4, the suspension was then filtrated on Whatman filter paper under vacuum. The solid material was rinsed a second time with an additional 200 ml 95 % ethanol (2 mL/g WG). The liquid phases were then pooled and transferred to a 1 L graduated cylinder. In phase 5, 2.5 mL of concentrated (85 %) phosphoric acid ($H_3PO_4$) (25 μL/g WG) was added to the ethanol containing PAs. There was an immediate formation of opalescence in the solution. The desired pH of 3.5 was checked to ensure the complete precipitation of the PA salts. After approximately 24 h, PA phosphate salt crystals had formed along the edges and base of the cylinder, and could be separated from the remaining solution by filtration. The crystals were then dried for each of the WG batches and the weight recorded. The SPD and SPE contents were determined in each extract.

### 2.3. Industrial scale-up extraction of SPD from wheat germ

The industrial scale-up experiment was performed on a single 50 kg batch of WG in the production line of Abel Nutraceuticals S.R.L (Turin, Italy). The laboratory-scale protocol reported above was followed, requiring minor modifications: $Ca(OH)_2$ at 25 mg/g of WG, $H_2O$ at 0.6 mL/g WG and concentrated (85 %) $H_3PO_4$ at 26 μL/g WG, respectively. All other aspects of the extraction protocol remained constant.

The fresh WG batch and the dried extract from the extraction protocol were tested for 160 known pesticides. The analysis was performed according to the official guidelines (UNI, 2018) by Mérieux NutriSciences laboratory (Milan, Italy).

### 2.4. SPD and SPE quantification in extract

SPD and SPE contents of the extracts were quantified by reverse phase HPLC-ESI-MS. The chromatographic system was composed of a Waters e2695 separation module equipped with an Acquity QDa

detector (Waters corp., Milford, Massachusetts, USA). The stationary phase was a Luna Omega Polar C18 column, 100 Å, 250 × 4,6 mm (Phenomenex, Torrance, CA, USA) maintained at 30 °C. The mobile phase was composed of 15 % solution A, 5 % solution B, 80 % solution C (where solution A was 100 % methanol, solution B was 100 % acetonitrile and solution C was 0.4 % trifluoroacetic acid in water). The isocratic composition of the mobile phase was maintained throughout the elution. Instead, the flowrate was increased from 0.4 mL/min to 0.75 mL/min over the first 5 min, after which it was maintained at a constant rate of 0.75 mL/min until completion of the run in 15 min. An ESI interface provided ionization to the compounds directed into a single quadrupole MS detector. The MS acquisition parameters were as follows: probe temperature = 600 °C, capillary ± 0.8 kV, and cone voltage = 12V. The positive ionization acquisition SIR channels were 146,25 Da and 203,34 Da for SPD (+) and SPE (+), respectively.

The extract samples were prepared following a series of dilution steps. Firstly, 40 mg of the precipitated salt crystal extract was dissolved in 100 mL of distilled water and the solution mixed for 5 min in a sonicating water bath. After filtration at 0.22 µm, an aliquot of 1 mL was then diluted in 10 mL with distilled water. Lastly, an aliquot of 100 µL was transferred into a 1 mL HPLC vial containing 885 µL distilled water and acidified by adding 15 µL solution containing 0.05 M trifluoroacetic acid and 0.05 M perchloric acid, respectively. Thereafter, a sample volume of 10 µL was injected on the HPLC. Standard calibration curves were prepared using synthetic SPD and SPE at 20–50–100–200–500 µg/L.

## 3. Results and discussion

### 3.1. Novel protocol for the extraction of SPD from wheat germ

Within the framework of SPD extraction from plant material for nutritional and medicinal purposes, most protocols involve the use of hydroalcoholic pre-treatment to reduce contaminating cellular components, followed by water extraction in the presence of edible acids (citric acid) (Mohajeri et al., 2025). Despite the pretreatments, the water-soluble components of the WG (sugars, proteins and carbohydrates) are predominant, and consequently the polyamine extraction phase recovers a large amount of other unwanted material. As a consequence, the SPD concentration in extracts obtainable with such methods is low, and the requisite to increase the SPD content for new supplement formulation was then raised (Hirano et al., 2021; Schwarz et al., 2022; Steichen et al., 2023). Within the context of increasing the SPD titer in extracts from plant material, the present work describes the development and underlying rationale for a five-phase laboratory-scale extraction protocol that exploits the chemical properties of SPD using a novel approach that differs from all previous water/acid extraction protocols. The workability of the laboratory-scale protocol was then investigated in an industrial scale-up.

#### 3.1.1. The rationale behind the pilot laboratory-scale protocol

The proposed protocol takes advantage of the high solubility of SPD (free base) in ethanol (approximately 40 mg/ml), compared to that of the protonated form (spermidinium cation, SPDH+) and its salts, which are not as soluble in alcohol (< 1 mg/mL). By implementing conditions to obtain an ethanol extraction of SPD, the recovery of unwanted water-soluble compounds from the plant material is drastically reduced. The subsequent precipitation/crystallization of this active ingredient as a salt from the ethanol solution permits the separation and a further purification of this active ingredient from other components extracted from the natural matrix. In so doing, it is possible to attain a high SPD content extract.

As illustrated in Fig. 1, the five-phase protocol was easily workable, not requiring the use of complex laboratory equipment. Phase 1 involved milling 100 g WG into a fine particle size powder to generate a larger surface area to maximize the extraction efficiency (Fig. 1, Table 1). At physiological pHs, SPD in the cells exists in the form of cations (SPDH+). Phase 2 then involved the alkaline conversion of the cation SPDH+ into the free base form SPD with the underlying objective of rendering it soluble in ethanol in phase 3 of the protocol. Phase 2 was performed by dry-mixing the powdered WG with $Ca(OH)_2$, followed by the addition of a minimal volume of water to act as a medium for the acid/base reaction and to increase the pH to > 10.5. The rise in pH is visually accompanied by the rapid change in color from pale to bright yellow (Fig. 1). The selected pH of > 10.5 was important to ensure the predominance of SPD in base form (pKa value is 10.1) (Table 1). The underlying rationale for the choice of $Ca(OH)_2$ is that it is a strong base with two hydroxyls per formula unit, ensuring the attainment of the desired pH and the deprotonation of SPD with a low mixing ratio to the WG. Moreover, $Ca(OH)_2$ is non-volatile and safer compared to other

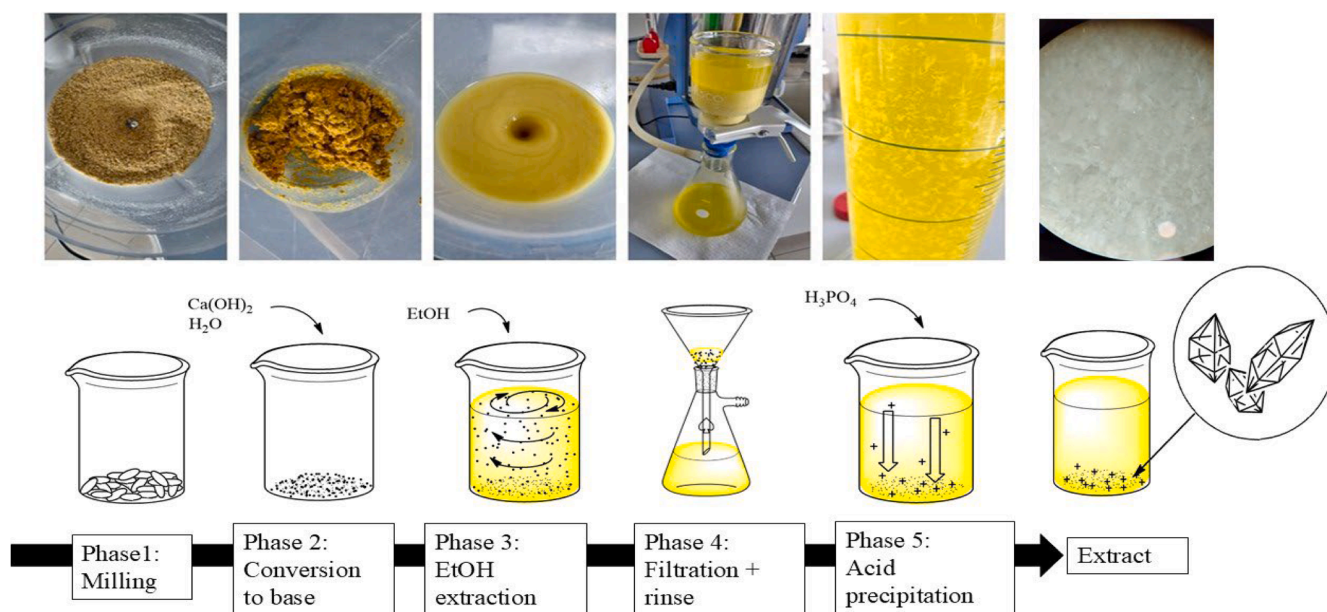



**Fig. 1.** Illustration of each of the five processing phases of the novel SPD extraction protocol from wheat germ (WG) to attain a crystalline end-product extract containing SPD phosphate salts. The accompanying images along the top panel provide a visual representation for each phase of the laboratory-scale protocol.

**Table 1**
The five processing steps to extract SPD from wheat germ (WG) and the rationale behind the novel experimental design.

| Processing step | Method | pH | SPD form | Rationale behind the experimental design |
|---|---|---|---|---|
| Phase 1 Milling | • WG ground to fine powder (0.2–0.5 mm) | 6.5 | cation (SPDH+) | • Increase surface area, thereby improving extractability of the matrix |
| Phase 2 Conversion to base | • $Ca(OH)_2$ added to the WG (20 mg/g WG)<br>• $H_2O$ added (0.5 mL/g WG)<br>• Kneaded for 30 mins | > 10.5 | neutral base form (SPD) | • $H_2O$ allows for proton exchange and acid base reaction occurs<br>• Minimum amount of $H_2O$ is required,<br>• SPD (pKa =10.1) is mostly converted in neutral base form (SPD)<br>• SPD is now soluble in EtOH (40 mg/mL) |
| Phase 3 EtOH extraction | • EtOH (8 mL/g WG) added to mixture and stirred vigorously for 1h | > 10.5 | neutral base form (SPD) | • EtOH purity ≥ 95 % to limit the amount of water in the mixture<br>• SPD is extracted in the EtOH solution |
| Phase 4 Filtration + rinse | • Mix filtered under vacuum. Solid filtrate is rinsed with additional EtOH (2 mL/g WG)<br>• Liquid phase is collected | > 10.5 | neutral base form (SPD) | • EtOH-insoluble fraction of WG (carbohydrates, sugars, proteins, minerals and fibers) separated |
| Phase 5 Acid precipitation | • 85 % $H_3PO_4$ (25 μL/g WG) is rapidly added to the solution | 3.5 | cation (SPDH+) | • SPDH+ is insoluble in EtOH (< 1 mg/mL)<br>• SPD phosphate immediately precipitates, forming crystals within 24 h |

strong base reagents, is admitted for food use, and is also not expensive.

In phase 3, the PAs (SPD) were extracted and solubilized in ethanol under vigorous stirring (Fig. 1, Table 1). The ethanol purity was set at ≥ 95 %. The reason for this mandatory requirement, as well as in administering the lowest possible amount of water in phase 2, was to reduce the total water content of the extraction mixture as much as possible. In fact, during the method development, it was shown increasing the water to ethanol ratio resulted in unwanted phenomena. The latter included: a larger quantity of undesired substances extracted from the WG (phase 3), an increased solubility of SPD salts, thereby limiting the precipitation in response to the decrease in pH (phase 5), and a simultaneous increase in the co-precipitation of lipids (phase 5). Such unwanted phenomena would collectively result in the loss in yield and purity of the final extract. Next, phase 4 was the filtration step, in which the ethanol-soluble PAs were easily separated from the ethanol-insoluble bio-compounds (carbohydrates, sugars, proteins, minerals and fibers). The solid was then rinsed with additional ethanol to recover PAs from the matrix (Table 1, Fig. 1).

Extraction of the PAs from the ethanol solution formed the rationale behind phase 5, which was performed by adding $H_3PO_4$ to lower the pH to *ca* 3.5. This step stimulated the protonation of the PAs and the subsequent precipitation of the PA salts (Table 1, Fig. 1). Opalescence immediately formed within the ethanol solution due to the formation of insoluble PA-phosphate salts (Fig. 1). $H_3PO_4$ was preferred to other acids commonly used in SPD extraction (citric acid, hydrochloric acid or oxalic acid). The advantages of selecting $H_3PO_4$ were that it is soluble in EtOH and is also a strong triprotic acid. Hence, it was possible to obtain the desired pH drop with a smaller volume compared to weaker acids. Moreover, it is non-volatile, non-flammable, non-oxidizing, suitable for the extraction of components for oral consumption, and also economical. These aspects are important when considering both the safety and affordability of a potential industrial scale-up.

After 24 h PA-phosphate crystals had developed and could be separated. After collecting the precipitate or extract, the solid material obtained appeared as white-to-yellowish crystals (Fig. 1).

### 3.1.2. *Extract yield, and SPD and SPE contents*

The extraction protocol was performed on 10 separate batches (lots) of WG, obtained as byproducts of the milling process. From 100 g of WG extracted for each lot, there was an average yield of 0.74 g of solid extract (Table 2). The extract was shown to be comprised PAs in the form of crystalline phosphate salts, mixed with an as of yet uncharacterized lipophilic fraction of WG that co-precipitated with PA salts in phase 5 of the extraction process. Of relevance was the SPD content, that was on average 7.64 ± 1.88 % w/w of the dried extract (Table 2).

Although the emphasis of the present study was on SPD and although no unique role for SPE has been unequivocally identified, SPE has been shown to be involved neurological functions, development, in DNA protection (Muñoz-Esparza et al., 2019; Pegg, 2014). As such the SPE content was also measured in the 10 batches and shown to be 3.1 ± 0.9 % of the dried extract (Table 2). Of interest, a single WG batch yielded a remarkable SPD and SPE contents of 11.12 % and 3.84 % of the dried extract, respectively. The variation in extract yield and SPD and SPE content between the 10 batches (Table 2) was attributable to the variation existing between different batches of WG as a normal compositional variability of natural products. The pilot laboratory-scale protocol of the present study showed that SPD contents of the dried extract significantly exceeded the quantities ranging from 0.064 to 2.08 % reported for patented WG extracts or supplements on the market (EUR-LEX, 2020; Kitazawa et al., 2011; Mohajeri et al., 2023, 2025; Steichen et al., 2023; Yamada et al., 2014). The next objective was to investigate the workability and feasibility of laboratory-scale method in an industrial scale-up.

### 3.1.3. *Industrial-scale up for the extraction of SPD and SPE*

To investigate the potential application of the pilot laboratory protocol in industrial production, it was necessary to scale-up the extraction protocol. The scalability of the extraction process was evaluated with the support of Abel Nutraceuticals S.R.L, specialized in the design of extraction processes and production of plant extract intermediates for the dietary supplements market. A batch of 50 kg of fresh WG was processed in the production line of Abel Nutraceuticals. In comparison to the laboratory extraction protocol, minor changes to the methodology were implemented in the industrial scale-up (Table 3). Of note, a small increase in both $Ca(OH)_2$ and water content were necessary in phase 2. In the industrial mixer, it was reported that the viscosity of the mix made necessary the addition of more water. The slight increment in $Ca(OH)_2$ was made to ensure that the desired pH in the mixture was obtained to

**Table 2**
The extract mass yield with the percentage content of SPD and SPE within each extract of 10 wheat germ batches (WG) of 100g.

| Batch Number | Extract (g) | SPD (% of extract) | SPE (% of extract) |
|---|---|---|---|
| 1 | 0.74 | 10.11 | 4.32 |
| 2 | 0.72 | 5.10 | 1.10 |
| 3 | 0.52 | 8.31 | 3.09 |
| 4 | 1.01 | 7.60 | 3.39 |
| 5 | 0.52 | 6.42 | 2.57 |
| 6 | 0.81 | 6.99 | 3.21 |
| 7 | 0.80 | 11.12 | 3.84 |
| 8 | 0.64 | 8.28 | 3.71 |
| 9 | 0.74 | 6.71 | 3.09 |
| 10 | 0.94 | 5.74 | 2.44 |
| Mean | 0.74 | 7.64 | 3.08 |
| Standard deviation | 0.16 | 1.88 | 0.89 |

**Table 3**
Comparison of the laboratory and industrial scale-up protocols and SPD and SPE contents.

| | Laboratory scale extraction | Industrial scale-up extraction |
|---|---|---|
| Phase 1 | WG: 100 g. | WG: 50 kg |
| Phase 2 | $Ca(OH)_2$: 20 mg/g WG<br>$H_2O$: 0.5 mL/g WG | $Ca(OH)_2$: 25 mg/g WG<br>$H_2O$: 0.6 mL/g WG |
| Phase 3 | 95 % EtOH<br>EtOH: 8 mL/g WG | 96.5 % EtOH<br>EtOH: 8 mL/g WG |
| Phase 4 | EtOH: 2 mL/g WG | EtOH: 2 mL/g WG |
| Phase 5 | 85 % $H_3PO_4$: 25 μL/g WG | 85 % $H_3PO_4$: 26 μL/g WG |
| Extract mass (g) | 0.74 ± 0.16 | 347 |
| % SPD of extract | 7.64 ± 1.88 | 9.57 |
| % SPE of extract | 3.08 ± 0.89 | 3.11 |

account for possible discrepancies in the purity of the reagent (Table 3). In response to the slight excess of $Ca(OH)_2$, an increase in volume of $H_3PO_4$ was administered to obtain a pH of 3.5 (Table 3). The scaled-up method produced a 347 g crystal extract from 50 kg WG, corresponding to an extract yield of 0.69 % which was comparable to the average yield of 0.74 % using the laboratory protocol. SPD and SPE contents were 9.57 % and 3.1 % of the extract, respectively, which were within the range of contents obtained for the laboratory protocol (Table 2).

Of relevance for the production of supplements, the fresh WG (sampled from the same batch used for the extraction), as well as the resulting PA phosphate salt extract, were also subjected to a multi-residue analysis of 160 known pesticides to verify the absence of recovery and concentration phenomena of contaminants. Of relevance to the present study, no pesticides were detected in the PA extract. Nonetheless, the raw WG material was moderately contaminated by six different pesticides as follows: cyproconazole 0.007 ± 0.003 mg/kg, chlorpyrifos 0.008 ± 0.003 mg/kg, diphenylamine (traces), piperonyl butoxide 0.112 ± 0.047 mg/kg, pirimiphos-methyl 0.178 ± 0.075 mg/kg, and tebuconazole 0.035 ± 0.015 mg/kg, respectively.

It is also relevant to mention that, at the conclusion of the extraction procedure, it was possible to recover (via an evaporator-concentrator apparatus) >90 % of the 531 Ls of ethanol utilized in the extraction process. Moreover, as with the laboratory-scale protocol, the procedure was easily performed in an industrial-scale production line with no requirement for complex or specialized equipment.

### *3.1.4. Comparison of the present protocol with previous SPD extraction protocols for oral consumption*

The present research was specifically focused on developing a protocol for the extraction of naturally occurring SPD contents in plant material. Protocols for the extraction of SPD from seedlings cultivated in synthetic SPD solutions and/or fermented organic materials were not taken into consideration when comparing the present protocol with previous protocols aimed at obtaining edible products enriched in SPD. Within the framework of SPD extraction protocols from plant material, only those involving the use of reagents suitable for oral consumption were taken into consideration for comparative purposes (Table 4). The present protocol employed a novel approach that was based on exploiting the acid/base properties and solubility in ethanol of SPD. The present method was not based on refining previous methods based on a similar approach, and, therefore, represents a novelty compared to previous and current SPD extraction protocols for oral consumption. In contrast to the present work, all previous protocols (using WG and in certain instances soybean) illustrated the development of the same extraction method primarily involving the use of a weak organic acid to solubilize SPD.

In the earliest patented protocol (Table 4), citric acid and other organic acids were used to extract SPD from WG at a concentration of 0.10 to 0.20 % of dried extract (Kitazawa et al., 2011), which was significantly higher than the starting contents in WG which range from 0.02 to 0.03 % (Hirano et al., 2021; Mohajeri et al., 2023a; Muñoz-Esparza et al., 2019). A different patented protocol used citric acid to extract SPD, after a pretreatment of the WG to clean-up contaminating bio-compounds with varying ratios of ethanol and water (Yamada et al., 2014). The titer of SPD in the final extract was increased to 0.25–0.48 % (Table 4). The method of Yamada et al. (2014) was considered the cornerstone on which to develop extraction protocols for the industrial extraction of SPD (Mohajeri et al., 2025). Although methodology details were not provided, the citric acid method was considered acceptable for the development of a patented, well-acclaimed, commercial supplement which reported a range of 0.8–2.4 % SPD of the dried extract (EUR-Lex, 2020; Madeo et al., 2017). The citric acid extraction protocol including ethanol/water clean-up procedures with 40 % ethanol was also adopted for the investigation of plant sources for SPD contents (Mohajeri et al., 2023). This method was further modified to include 3 clean-up steps prior to the addition of citric acid which was incubated for extended period of 7 h (Mohajeri et al., 2025) compared to the 1–2 h of previous methods (Mohajeri et al.,

**Table 4**
Extraction protocols to extract SPD from natural sources for oral consumption.

| SPD extraction protocol | SPD content | Advantages | Disadvantages | Refs. |
|---|---|---|---|---|
| Extraction with different organic acids | 0.10–0.20 % of dried extract | • Suitable for oral consumption | • Low SPD content | Kitazawa et al., 2011 |
| EtOH/$H_2O$ pre-treatment → residue extracted with $H_2O$ + citric acid → concentration step | 0.25–0.48 % of dried extract | • Citric acid is a weak acid, suitable for oral consumption<br>• Non-corrosive towards equipment | • Low SPD content<br>• Industrial scale-up not performed<br>• SPD liquid extract would require spray drying/ rotary freeze-drying + clean-up | Yamada et al., 2014 |
| EtOH/$H_2O$ extraction. Citric acid method accepted. Details not provided | 0.8–2.4 % of dried extract | • Highly recognized natural SPD supplement on the market | • Low SPD content | EURO-LEX, 2020; Madeo et al., 2017 |
| EtOH/$H_2O$ pre-treatment → residue extracted with $H_2O$ + citric acid → concentration step. | SPD % of dried extract not provided | • SPD recovery reported to be significantly increased<br>• First production report of SPD-enriched powder for encapsulation purposes | • Scale-up to 500 g required methodology modifications and demonstrated complications.<br>• Increased length of time<br>• Industrial-size scale-up not performed | Mohajerei et al., 2025 |
| Alkaline treatment with $Ca(OH)_2$ + water→ EtOH extraction→filtration → precipitation of SPD with $H_3PO_4$ | 7.6–9.6 % of dried extract | • Highest reported SPD titer in the dry extract<br>• Feasibility of the industrial scale-up with minor method modifications Simple to perform, not requiring complex equipment | • Increased length of time due to precipitation of SPD (24 h)<br>• Crystallization step delicate, susceptible to losses due to environmental factors | Present work |

2023; Yamada et al., 2014). The extract weight was not provided (Mohajeri et al., 2025) as well as the % titer of SPD (Table 4). Instead, the method reported an increased total recovered SPD of 173–185 mg from an original 500 g WG. Using the principle of reporting total extracted SPD used by those authors, the present protocol calculated comparative total recovered SPD that were *ca* 280 mg from 500 g WG.

On an extract basis, the present protocol (both lab-scale and industrial scale-up) reported between 7.6–9.6 % SPD of the dried WG extract, significantly exceeding previous methods (Table 4). This was considered a noteworthy advantage of the present method.

Moreover, unlike previous methods, the current method did not involve significant methodological adjustments for a scale-up (Mohajeri et al., 2025) and was also the only method to report the extraction of industrial quantities (50 kg). For the successful extraction of SPD, no previous methods employed the use of alkaline reagents. To the best of our knowledge, there is only a single method that reported the basic alcoholic extraction of soybean PUT (Wang, 1972). This method involved an initial extraction of PAs in TCA, followed by ether treatment and addition of NaOH to raise the pH. This was followed by an extraction in butanol and finally the precipitation of PAs with HCl. It was shown that the concentration of PUT in the alcohol-extractable fraction was seven times higher than the TCA fraction (Wang, 1972). Interestingly, since SPD and SPE were not present in the alcohol-extractable fraction, it was concluded that the alcohol extraction method was selective and less satisfactory (Wang, 1972). As such, to the best of our knowledge, there were no subsequent attempts to extract PAs in alcohol-extractable fractions. Of significant relevance is that SPD and SPE could be solubilized and extracted from basic pH alcohol in contrast to that reported previously (Wang, 1972), and that SPD and SPE contents in the WG extracts far exceeded those reported previously.

### 3.2. Validation of the analytical method for spermidine quantification in extracts

In the present study, the analysis of SPD and SPE content in the extracts was performed by HPLC-ESI-MS, without a derivatization step. This choice was made to simplify as possible the pre-treatment of the samples in virtue of the expected low level of contaminants in the extracts and to avoid the well-documented complexities associated with this step (Ibarra et al., 2015; Mohajeri et al., 2023; Petrarca et al., 2017). Here, we validate the analytical efficacy for SPD as the PA of interest.

The validation was performed by evaluating peak shape, signal to noise ratio, linearity and range, selectivity, specificity, reproducibility, as well as limit of detection (LOD) and limit of quantification (LoQ). In the previously reported conditions, the chromatographic peak obtained from the injection of a 100 μg/L standard solution of SPD (midpoint of the calibration curve) appeared sharp and defined at a retention time of 5.78 min, with a full width at half maximum (FWHM) of 0.076 min and a signal to noise ratio of 156 (Fig. 2).

The method was validated from a concentration of 20 μg/L to 500 μg/L SPD. Within this range the obtained curve was sufficiently linear with a $R^2 = 0.997$ (Fig. 3A). When adding calibration points at higher concentrations, a deviation from linearity was observed. Due to charge competition, the signal tended to progressively decrease, resulting in the curve demonstrating an exponential trend (Fig. 3B).

The selectivity and specificity of the proposed method was evaluated by building a spike calibration curve. A sample of PA extract, diluted to contain approximately a concentration of 50 μg/L of SPD was spiked with +20μg/L, +50μg/L, +100μg/L, +200μg/L with pure synthetic SPD. The obtained curve results were shown to be well superimposed on the standard curve in terms of slope and linearity, with an intercept value coherent with the analyte concentration in the sample (Fig. 4). The precision and reproducibility of the analytical method was performed on 10 replicate sample analyses. The samples were prepared with a dilution factor of 250.000 as reported previously. The mean value of SPD concentration in the 10 replicates was 303 μg/L with a RSD of 3.5 % (Table 5). LOD and LOQ were 2.1 μg/L and 6.2 μg/L, respectively. These values were calculated from 5 blank samples as $3.3*(S_b/sl)$ and $10*(S_b/sl)$, where $S_b$ is the standard deviation of the blanks, and sl is the slope of the calibration curve.

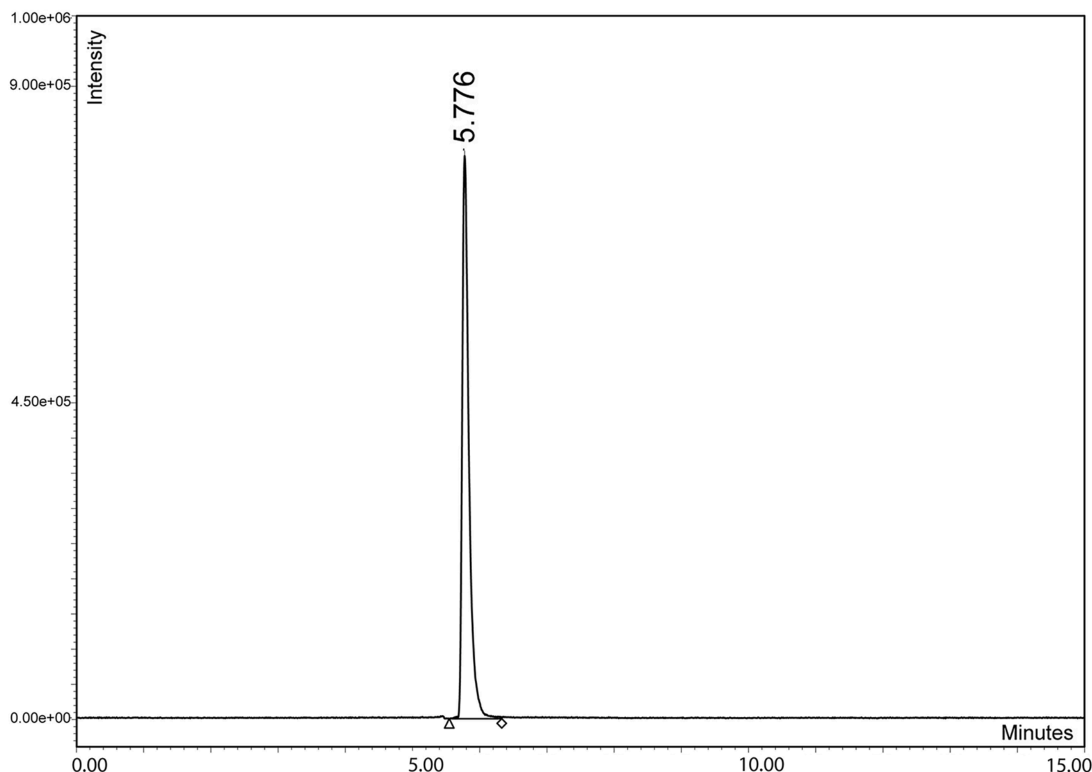



**Fig. 2.** Chromatographic peak of a 100 μg/L standard solution of SPD.

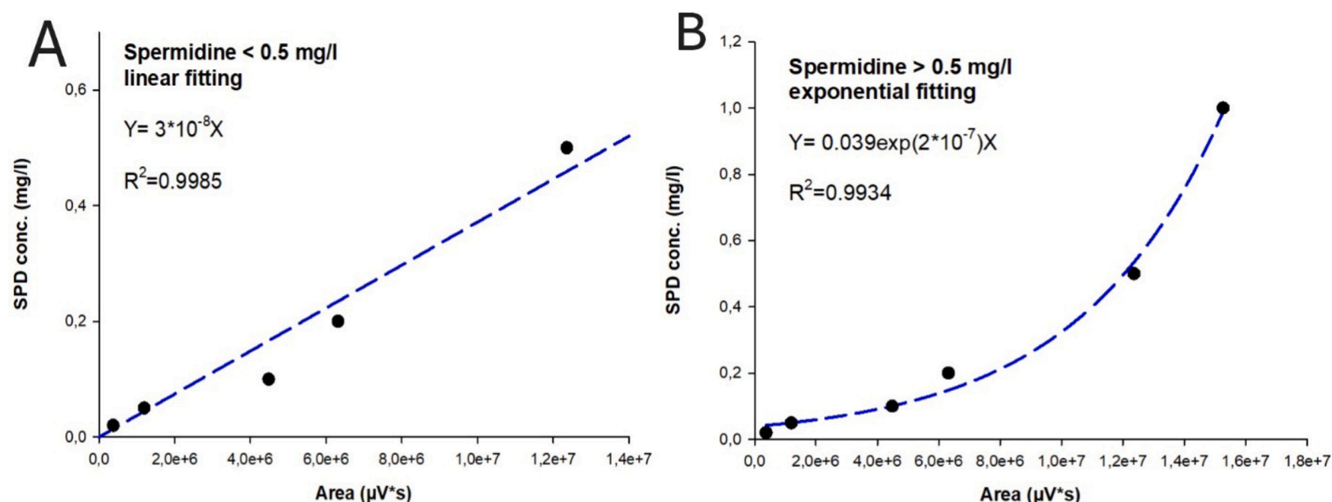



**Fig. 3.** (A) Calibration curve showed linearity in the range 20 μg/L to 500 μg/L spermidine (SPD) and (B) divergence from linearity to exponential behavior for SPD concentrations exceeding 500 μg/L.

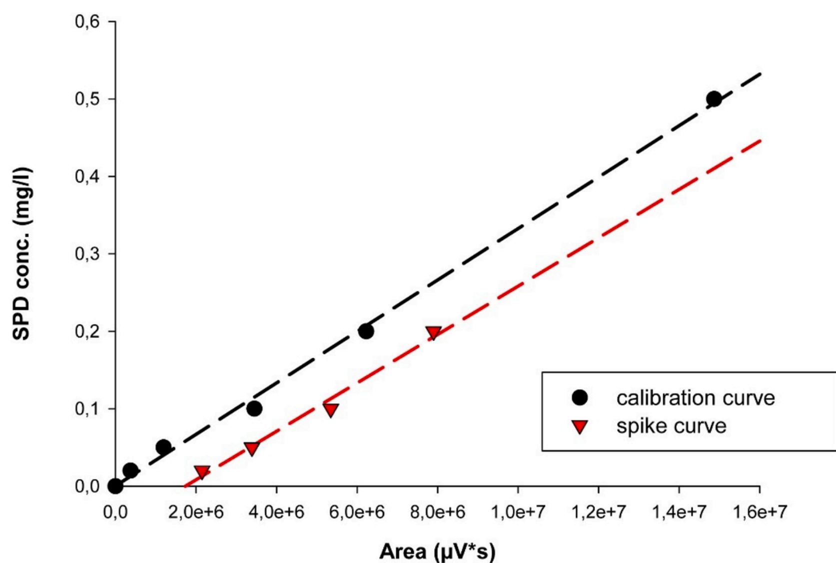



**Fig. 4.** Standard calibration curve (black line) compared with the spike curve (red line); the slope of the lines was comparable, and the intercept value of the spiked curve is in line with the expected value for the SPD extract sample.

**Table 5**
Analytical repetitions of the same extract sample were made to test the reproducibility and the precision of the analysis method. Peak retention time and calculated concentrations of SPD in each sample are reported.

| Repetition | Retention time (min) | SPD content (μg/mL) |
|---|---|---|
| 1 | 5.79 | 302.37 |
| 2 | 5.79 | 303.41 |
| 3 | 5.79 | 325.60 |
| 4 | 5.79 | 298.398 |
| 5 | 5.79 | 287.78 |
| 6 | 5.80 | 312.95 |
| 7 | 5.79 | 299.411 |
| 8 | 5.79 | 301.21 |
| 9 | 5.79 | 291.80 |
| 10 | 5.79 | 307.52 |
| Mean | 5.79 | 303.04 |
| Standard deviation | 0.00276 | 10.67 |
| % RSD | 0.04865 | 3.52 |

## 4. Conclusions

A novel extraction protocol was developed to obtain a higher SPD content, thereby overcoming existing limitations of water-acidic extraction methods. The protocol was shown to be an easily workable procedure based on the ethanol solubility properties of PAs in neutral rather than in cationic form. It can be summarized as follows: alkaline treatment of the WG with $Ca(OH)_2$ in water (conversion of PAs to free bases at pH >10.5), solubilization of neutral-basic PAs in 96 % ethanol, collection and filtration of the extraction solvent, addition of $H_3PO_4$ (to pH 3.5) resulting in the precipitation of SPD phosphate salts. None of the steps in the protocol required the use of complex apparatus. Moreover, the filtration procedure of the alcoholic extraction liquid was uncomplicated as the formation of gel phases of carbohydrates and proteins with water did not occur. The solid extract was obtained in the form of PA phosphate crystals. The SPD and SPE contents of the extract significantly exceeded those of previous water-acid extraction protocols. Future work necessitates a complete analysis of all components within the extract which had not been performed in the present study.

Nonetheless, of great relevance was that the process proved to be easily scalable on an industrial scale, with comparable yields for SPD and SPE obtained from the extract. Additionally, no recovery or concentration phenomena of pesticides from the raw material were recorded, and the industrial procedure also permitted the recycling of over 90 % of the alcohol used as an extraction solvent. SPD content of the extracts was analyzed using HPLC-ESI-MS. We obtained sufficient retention of non-derivatized SPD, characterized by a narrow and well-defined peak with an acceptable signal-to-noise ratio. The linearity of the analytical method was limited to a narrow concentration range, but which allowed it to cover almost two orders of magnitude above the calculated LOD. In WG, the content of SPD has been reported to range from 200 to 300 μg/g WG (or 0.02–0.03 % WG) (Hirano et al., 2021; Mohajeri et al., 2023; Muñoz-Esparza et al., 2019). In the present extraction laboratory and industrial scale experiments, the SPD contents extracted ranged from 0.056 to 0.066 % WG, which exceeds the widely reported values. This difference may be attributable to the following: the use of genotypes with a higher SPD expression, the developmental phase in which the grain was milled, the dry weight of the WG and/or the underestimation of SPD contents using classical acidic-water-based extraction protocols ($HClO_4$, TCA, and citric acid). These aspects are warranting attention.

## Patents

The overall process has been filed as a patent with the application number IT202100007331A1 and identified I0192057 at the Italian patent office.

## CRediT authorship contribution statement

**Eros D'Amen:** Writing – original draft, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **Francesca Truzzi:** Supervision, Funding acquisition, Data curation. **Anne Whittaker:** Writing – review & editing, Supervision. **Giovanni Dinelli:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Giovanni Dinelli reports financial support was provided by EIT Food iVZW. Eros D'Amen, Giovanni Dinelli, Francesca Truzzi has patent Procedimento di estrazione di poliammine da matrici naturali complesse issued to IT202100007331A1. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

## Data availability

Data will be made available on request.

## References

Allison, L. (2024) Spermidine: The science behind longevity supplements. Longevity technology. Retrieved from https://longevity.technology/lifestyle/spermidine-the-science-behind-spermidine-supplements/. Accessed August 17 2024.

Dai, Z., Wu, Z., Wang, J., Wang, X., Jia, S., Bazer, F. W., et al. (2014). Analysis of polyamines in biological samples by HPLC involving pre-column derivatization with o-phthalaldehyde and N-acetyl-L-cysteine. *Amino Acids, 46*(6), 1557–1564. https://doi.org/10.1007/s00726-014-1717-z

Eisenberg, T., Knauer, H., Schauer, A., Büttner, S., Ruckenstuhl, C., Carmona-Gutierrez, D., et al. (2009). Induction of autophagy by spermidine promotes longevity. *Nature Cell Biology, 11*, 1305–1314. https://doi.org/10.1038/ncb1975

EUR-Lex (2020). Commission Implementing Regulation (EU) 2020/443 of 25 March 2020 authorising the change of the specifications of the novel food spermidine-rich wheat germ extract (Triticum aestivum) under Regulation (EU) 2015/2283 of the European Parliament and of the Council and amending Commission Implementing Regulation (EU) 2017/2470. Retrieved from https://eur-lex.europa.eu/legal-content/en/ALL/?uri=CELEX:32020R0443. Accessed August 20 2024,.

Hirano, R., Shirasawa, H., & Kurihara, S. (2021). Health-promoting effects of dietary polyamines. *Medical Sciences, 9*(1), 8. https://doi.org/10.3390/medsci9010008 (Basel).

Ibarra, A. A., Wrobel, K., Escobosa, A. R., Elguera, J. C., Garay-Sevilla, M. E., & Wrobel, K. (2015). Determination of putrescine, cadaverine, spermidine and spermine in different chemical matrices by high performance liquid chromatography-electrospray ionization-ion trap tandem mass spectrometry (HPLC-ESI-ITMS/MS). *Journal of Chromatography B, Analytical Technologies in the Biomedical and Life Sciences, 1002*, 176–184. https://doi.org/10.1016/j.jchromb.2015.08.036

Kitazawa, H., Kasukabe, Y., Kitagawa, M. (2011). Stress-reducing agent including plan derived polyamine-containing extract serving as active component. Patent No. US 2011 0236512A1, United States.

López-Rodríguez, R., Domínguez, L., Fernández-Ruiz, V., & Cámara, M. (2022). Extracts rich in nutrients as novel food ingredients to be used in food supplements: A proposal classification. *Nutrients, 14*(15), 3194. https://doi.org/10.3390/nu14153194

Madeo, F., Eisenberg, T., & Stekovic, S. (2016). Use of spermidine for the enhancement of mitochondrial respiration. Patent No EP3320899A1, European Patent Office.

Madeo, F., Eisenberg, T., Pietrocola, F., & Kroemer, G. (2018). Spermidine in health and disease. *Science, 359*(6374), 2788. https://doi.org/10.1126/science.aan2788 (New York, N.Y.).

Madeo, F., Bauer, M. A., Carmona-Gutierrez, D., & Kroemer, G. (2019). Spermidine: A physiological autophagy inducer acting as an anti-aging vitamin in humans? *Autophagy, 15*(1), 165–168. https://doi.org/10.1080/15548627.2018.1530929

Magnes, C., Fauland, A., Gander, E., Narath, S., Ratzer, M., Eisenberg, T., et al. (2014). Polyamines in biological samples: Rapid and robust quantification by solid-phase extraction online-coupled to liquid chromatography-tandem mass spectrometry. *Journal of Chromatography A, 1331*, 44–51. https://doi.org/10.1016/j.chroma.2013.12.061

Minocha, R., Shortle, W. C., Long, S. L., & Minocha, S. C. (1994). A rapid and reliable procedure for extraction of cellular polyamines and inorganic ions from plant tissues. *Journal of Plant Growth Regulation, 13*, 187–193. https://doi.org/10.1007/BF00226036

Mohajeri, M., Ayatollahi, S. A., Kobarfard, F., Goli, M., Khandan, M., Mokhtari, S., et al. (2023). Wheat germ, a byproduct of the wheat milling industry, as a beneficial source of anti-aging polyamines: A quantitative comparison of various forms. *Food Science & Nutrition, 11*, 7242–7254. https://doi.org/10.1002/fsn3.3650

Mohajeri, M., Mokhtari, S., Khandan, M., Ayatollahi, S. A., Kobarfard, F., & Hudaverdi, A. (2025). Enhancing polyamine enrichment from wheat germs: A study utilizing response surface methodology and liquid chromatography-mass spectrometry. *Food Chemistry, 463*(3), Article 141408. https://doi.org/10.1016/j.foodchem.2024.141408

Muñoz-Esparza, N. C., Latorre-Moratalla, M. L., Comas-Basté, O., Toro-Funes, N., Veciana-Nogués, M. T., & Vidal-Carou, M. C. (2019). Polyamines in food. *Frontiers in Nutrition, 6*, 108. https://doi.org/10.3389/fnut.2019.00108

Park, M. H., & Wolff, E. C. (2018). Hypusine, a polyamine-derived amino acid critical for eukaryotic translation. *Journal of Biological Chemistry, 293*, 18710–18718. https://doi.org/10.1074/jbc.TM118.003341

Pegg, A. E. (2014). The function of spermine. *International Union of Biochemistry and Molecular Biology Life, 66*(1), 8–18. https://doi.org/10.1002/iub.1237

Petrarca, M. H., Fernandes, J. O., Godoy, H. T., & Cunha, S. C. (2017). Determination of polyamines in baby food by gas chromatography-mass spectrometry: Optimization of extraction and microwave-assisted derivatization using response surface methodology. *Food Analytical Methods, 10*, 3548–3557. https://doi.org/10.1007/s12161-017-0918-y

Sagar, N. A., Tarafdar, S., Agarwal, S., Tarafdar, A., & Sharma, S. (2021). Polyamines: Functions, metabolism, and role in human disease management. *Medical Sciences, 9*(2), 44. https://doi.org/10.3390/medsci9020044 (Basel).

Saha Turna, N., Chung, R., & McIntyre, L. (2024). A review of biogenic amines in fermented foods: Occurrence and health effects. *Heliyon, 10*(2), e24501. https://doi.org/10.1016/j.heliyon.2024.e24501

Schwarz, C., Stekovic, S., Wirth, M., Benson, G., Royer, P., Sigrist, S. J., et al. (2018). Safety and tolerability of spermidine supplementation in mice and older adults with subjective cognitive decline. *Aging, 10*, 19–33. https://doi.org/10.18632/aging.101354

Schwarz, C., Benson, G. S., Horn, N., Wurdack, K., Grittner, U., Schilling, R., et al. (2022). Effects of spermidine supplementation on cognition and biomarkers in older adults with subjective cognitive decline: A randomized clinical trial. *JAMA Network Open, 5*(5), Article e2213875. https://doi.org/10.1001/jamanetworkopen.2022.13875

Soda, K., Uemura, T., Sanayama, H., Igarashi, K., & Fukuim, T. (2021). Polyamine-rich diet elevates blood spermine levels and inhibits pro-inflammatory status: An interventional study. *Medical Sciences, 9*(2), 22. https://doi.org/10.3390/medsci9020022 (Basel).

Steichen, P., Gruber, K., Hippe, B., & Haslberger, A. (2023). Spermidine content of selected dietary supplements: Potential for improvement? *Functional Foods in Health and Disease, 13*. https://doi.org/10.31989/ffhd.v13i5.1102

Truzzi, F., Whittaker, A., D'Amen, E., Tibaldi, C., Abate, A., Valerii, M. C., et al. (2022). Wheat germ spermidine and clove eugenol in combination stimulate autophagy *in vitro* showing potential in supporting the immune system against viral infections. *Molecules, 27*, 3425. https://doi.org/10.3390/molecules27113425 (Basel, Switzerland).

Truzzi, F., Whittaker, A., D'Amen, E., Valerii, M. C., Abduazizova, V., Spisni, E., et al. (2023). Spermidine-eugenol supplement preserved inflammation-challenged intestinal cells by stimulating autophagy. *International Journal of Molecular Sciences, 24*, 4131. https://doi.org/10.3390/ijms24044131

UNI (Ente Nazionale Italiano di Unificazione) (2018). UNI EN ISO 15662:2018. Foods of plant origin - multimethod for the determination of pesticide residues using GC- and LC-based analysis following acetonitrile extraction/partitioning and clean-up by dispersive SPE. Retrieved from https://shop.standards.ie/en-ie/standards/uni-en-15662-2018-1096552_saig_uni_uni_2742112/. Accessed on September 10 2024,.

Valuate Reports (2024). Global spermidine supplement market research report. Spermidine Supplement Market size. Retrieved from https://reports.valuates.com/market-reports/QYRE-Auto-34Z12020/global-spermidine-supplement. Accessed August 10 2024,.

Wang, L. C. (1972). Polyamines in soybeans. *Plant Physiology, 50*(1), 152–156.

Wirth, M., Schwarz, C., Benson, G., Horn, N., Buchert, R., Lange, C., et al. (2019). Effects of spermidine supplementation on cognition and biomarkers in older adults with subjective cognitive decline (SmartAge)-study protocol for a randomized controlled trial. *Alzheimer's Research & Therapy, 11*, 36. https://doi.org/10.1186/s13195-019-0484-1

Yamada, C., Hiroaki, K. & Shusaku, Y. (2014). Method for producing polyamine composition from plant. Patent No. USA1201403787.08A1, United States.

Zou, D., Zhao, Z., Li, L., Min, Y., Zhang, D., Ji, A., et al. (2022). A comprehensive review of spermidine: Safety, health effects, absorption and metabolism, food materials evaluation, physical and chemical processing, and bioprocessing. *Comprehensive Reviews in Food Science and Food Safety, 21*(3), 2820–2842. https://doi.org/10.1111/1541-4337.12963