

1 ***In vitro* determination of diamine oxidase activity in food matrices by an enzymatic assay**
2 **coupled to UHPLC-FL**

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22 **Abstract**

23 Intestinal diamine oxidase (DAO) acts as a protective barrier against exogenous histamine. A
24 deficit of DAO activity can lead to the appearance of histamine intolerance, a clinical condition
25 that may be treated by a low-histamine diet and oral DAO supplementation to enhance
26 intestinal histamine degradation. As sources of DAO, porcine kidneys and certain legume
27 seedlings are suitable components for the formulation of a DAO supplement. The aim of this
28 work was to develop a rapid and reliable methodology for the *in vitro* determination of DAO
29 activity in food matrices based on an enzymatic assay coupled to UHPLC-FL. The proposed
30 method showed a satisfactory linearity and sensitivity and provided a relative standard
31 deviation lower than 3%, guaranteeing method precision, and a mean recovery greater than
32 99% both for lyophilized pea sprouts and porcine kidney protein extracts. A high specificity is
33 a key attribute of this method due to the use of histamine as the reaction substrate and the
34 direct quantification of its degradation. Moreover, the lack of interference of catalase and
35 hydrogen peroxide is another advantage in comparison with previously published methods.
36 Lyophilized pea sprouts showed the greatest histamine-degrading activity (0.40 ± 0.01
37 mU/mg), followed by porcine kidney protein extracts (0.23 ± 0.01 mU/mg) and commercial
38 DAO supplements (0.09 ± 0.06 mU/mg). This technique could be used as a tool to validate the
39 DAO activity of food matrices of potential interest for the treatment of histamine intolerance.

40

41 **Keywords**

42 UHPLC-FL; Enzymatic assay; Histamine; Diamine oxidase (DAO) enzyme; Porcine kidney; Pea
43 sprouts.

44 1. Introduction

45 The enzyme with histamine-degrading capacity, discovered in 1929 by Charles H. Best in
46 autolyzing lung tissues, was first known as histaminase [1]. After subsequent studies revealed
47 its ability to deaminate other diamines, such as putrescine and cadaverine, the enzyme was
48 renamed diamine oxidase (DAO) [2,3]. DAO (EC 1.4.3.22), which belongs to the category of
49 copper-containing amine oxidases, is a homodimeric and ubiquitous enzyme found in
50 microorganisms, plants and animals, generally in the range of 140 to 200 kDa [4-8]. In
51 particular, DAO catalyzes the oxidative deamination of the primary amino group of histamine
52 to imidazole acetaldehyde, consuming dioxygen with the concomitant release of
53 stoichiometric amounts of ammonia and hydrogen peroxide (Figure 1) [9, 10].

54 In humans, DAO is mainly located in the intestines, placenta and kidneys [6,11]. Intestinal
55 DAO acts as a protective barrier against exogenous histamine, especially of food origin [12-
56 14]. A deficiency of DAO enzyme may thus lead to excess the normal plasmatic levels of
57 histamine (0.3 - 1.0 ng/mL) and the subsequent appearance of histamine intolerance
58 symptoms [15, 16]. Due to the diverse effects and functions of histamine in multiple organs
59 and systems of the body, histamine intolerance is characterized by a variety of complaints,
60 including gastrointestinal (abdominal pain, diarrhea or vomiting), dermatological (urticaria,
61 dermatitis or pruritus), respiratory (rhinitis, nasal congestion or asthma), cardiovascular
62 (hypotonia or arrhythmias) or neurological (headaches) [14-17]. The most frequently used
63 treatment for histamine intolerance consists of following a low-histamine diet [15, 18]. Only
64 foods with histamine levels below detectable limits can be considered safe for histamine-
65 intolerant patients and unfortunately for this population, histamine is widespread among all
66 food categories in highly variable concentrations [19, 20]. In this context, considering that

67 DAO is the key enzyme in the breakdown of dietary histamine at the intestinal level, orally
68 administered DAO supplements have been proposed as a strategy to enhance histamine
69 degradation and improve the quality of life of intolerant individuals undergoing those dietary
70 restrictions [21, 22]. As sources of DAO, porcine kidneys and certain legume seedlings are
71 suitable components of such an enzymatic supplement [21, 23].

72 A wide range of methods to detect *in vitro* DAO activity are described in the literature. With
73 the aim of measuring the rate of substrate degradation or the generation of by-products of
74 this enzymatic reaction, most methods are based on the detection of hydrogen peroxide,
75 aldehyde or dioxygen by spectrophotometric [13, 21, 24, 25], fluorometric [26], polarographic
76 [27, 28] or amperometric [29, 30] techniques. Radioimmunoassay techniques have also been
77 extensively described, consisting of the radioactive labeling of the substrate and the
78 scintillation counting of its consumption [3, 11, 31]. Although chromatographic analytical
79 procedures are widely used, this approach has only been applied to measure histamine or
80 other biogenic amine degradation capacity in microbial starter cultures involved in food
81 fermenting processes [10, 32, 33]. Despite some of these methods may be advantageous in
82 terms of rapidity or automation, they generally have a limited sensitivity, require a laborious
83 experimental set-up or entail a high cost in the correct storage and handling of radioactive
84 waste. Moreover, in those methods in which the DAO activity is estimated through the
85 determination of hydrogen peroxide or dioxygen, the action of other enzymes, such as
86 catalase, may interfere by H₂O₂ consuming or O₂ releasing [34, 35]. Additionally, the most
87 extensively used reaction substrates in the methods reported so far are putrescine and
88 cadaverine, which have different affinity or kinetic parameters to histamine [36, 37].

89 Therefore, the aim of this work was to develop a reliable, rapid and highly sensitive
90 methodology for the determination of *in vitro* DAO activity of several matrices using
91 histamine as the substrate and based on the direct quantification of its degradation during
92 the reaction process. Specifically, an enzymatic assay coupled to an ultra-high performance
93 liquid chromatography and fluorimetric (UHPLC-FL) detection method was proposed,
94 validated and tested for applicability in porcine kidney protein extracts, legume sprouts and
95 commercialized DAO supplements.

96

97 **2. Material and methods**

98 *2.1. Reagents and chemicals*

99 Histamine dihydrochloride, purified DAO from porcine kidney and catalase from bovine liver
100 were purchased from Sigma-Aldrich (St. Louis, MO, USA). UHPLC-grade methanol and
101 acetonitrile, hydrochloric acid 0.1M, perchloric acid 70%, sodium di-hydrogen phosphate
102 anhydrous and di-sodium hydrogen phosphate anhydrous were obtained from PanReac
103 Química (Castellar del Vallès, Spain). Acetic acid, boric acid, 1-octanesulfonic acid sodium salt,
104 ammonium formate, phthaldialdehyde (OPA) and brij® L23 solution were acquired from
105 Sigma-Aldrich (St. Louis, MO, USA); and formic acid, sodium acetate anhydrous, potassium
106 hydroxide and 2-mercaptoethanol from Merck (Darmstadt, Germany). A LaboStar System
107 from Evoqua Water Technologies (Warrendale, PA, USA) was used to produce ultrapure water
108 (18.2 MΩcm).

109

110 *2.2. Samples*

111 For the analytical method development and validation, porcine kidney protein extracts and
112 lyophilized pea sprouts (*Pisum sativum*) were used. Porcine kidney extracts were provided by
113 a biotechnology company specialized in the extraction of biomolecules from animal tissues.
114 These extracts consisted of a homogenate powder obtained by an acetonic extraction
115 followed by a drying process. Porcine kidney extracts consisted of 84% of protein, estimated
116 by applying the nitrogen-to-protein conversion factor (6.25) to the total nitrogen determined
117 following the Kjeldahl method (2200 Kjeltex[®] Auto Distillation Unit, Foss Iberia S.A.U.,
118 Barcelona, Spain). Etiolated pea sprouts were obtained in our laboratory through the
119 germination of peas at 27°C and 70% HR. After the sprouts were freeze-dried (Cryodos-50,
120 Telstar, Terrassa, Spain), a lyophilized extract consisting of 39% of protein was obtained.
121 Samples were kept under refrigeration (4-8 °C) until analysis.

122 The applicability of the method was assayed with 13 different production batches of porcine
123 kidney protein extract, 7 batches of lyophilized pea sprouts and 6 commercialized DAO
124 supplements available in the market. These dietary supplements were in the form of gastro-
125 resistant coated capsules or tablets, all of them containing 4.2 mg of porcine kidney protein
126 extract.

127

128 *2.3. In vitro determination of DAO activity*

129 The capacity of the DAO enzyme to degrade histamine in a working solution with a defined
130 initial concentration of histamine was tested under controlled optimal conditions (37°C, pH
131 7.2). The subsequent analysis of degraded histamine during the reaction time was performed
132 by UHPLC-FL. Specific DAO activity is expressed in mU/mg, referring to the amount of

133 histamine that is degraded by a mg of sample per minute (nmol of degraded histamine per
134 minute/mg of sample).

135

136 2.3.1. Enzymatic assay

137 Figure 2 illustrates in a schematic manner the experimental procedure of the enzymatic assay
138 for the *in vitro* determination of DAO activity. In detail, 1 to 20 mg of porcine kidney protein
139 extract, lyophilized pea sprouts or the content of one tablet or capsule of dietary supplement
140 were thoroughly homogenised in 20 mL of 0.05M phosphate buffer solution (pH 7.2) and
141 placed in a shaker incubator (Ivymen® 100-D, JP SELECTA S.A., Abrera, Spain) for at least 30
142 min (37°C, 200 rpm). The addition of a histamine standard solution to reach an initial
143 concentration of 45 µM in the homogenized sample marked the start of the enzymatic
144 reaction. The enzyme in contact with its substrate was kept in constant incubation and 500
145 µL aliquots were progressively extracted at different sampling times (t=0, 1, 2, 4 and 6 h). To
146 stop the enzymatic reaction, 15 µL of 2N perchloric acid solution was added to the extracted
147 aliquot, vigorously mixed with a vortex mixer and centrifuged (4°C, 5 min, 15000 rpm). The
148 supernatant was filtered through a 0.22 µm GHP filter and stored at 4°C until UHPLC analysis.
149 Each sample was analyzed in duplicate and a positive control was performed with 1 mg of
150 purified DAO.

151 To assay the potential interference effect of catalase on the DAO activity determination,
152 porcine kidney protein extract was assayed with the addition of catalase enzyme at two
153 different concentrations (100 and 500 U/mL) using the same experimental procedure.

154

155 2.3.2. UHPLC-FL analysis

156 Chromatographic separation was performed using a UHPLC-FL system consisting of a Waters
157 Acquity™ Ultra Performance Liquid Chromatography apparatus, which comprised a
158 quaternary pump, an auto-sampler and a fluorescence detector, and a post-column reagent
159 manager connected to a zero dead volume union between the column outlet and the
160 detector. Data acquisition was performed using the Empower™ 3 software (Waters Corp.,
161 Milford, MA, USA).

162 The chromatographic determination of histamine was performed by ion-pair reverse-phase
163 UHPLC coupled with post-column online derivatization with OPA and fluorescence detection.
164 Elution time was 7 min. Chromatographic conditions were as previously described by Latorre-
165 Moratalla et al. [38], briefly summarized in Table 1.

166

167 *2.4. Statistical analysis*

168 The software package IBM SPSS Statistics (IBM Corporation, Armonk, NY, USA) for Windows
169 (version 22) was used for the statistical analysis of data. The reliability of the method was
170 tested by means of analysis of variance for linear regression and the data sets were compared
171 using the Student's t-test. Cochran's C test was used to assess the homogeneity of variances.

172

173 **3. Results and discussion**

174 The method developed in this work is based on the direct addition of a defined amount of
175 histamine to a food matrix homogenized in an aqueous solution. During the incubation period
176 of the mixture, the DAO enzyme potentially present in the sample progressively degrades the
177 substrate. DAO activity was determined by comparing the absolute amount of histamine

178 degraded during the reaction time with the initial substrate concentration. The absence of
179 histamine degradation when assaying the same amount of substrate but lacking the DAO
180 enzyme or samples as a negative control proved that the degradation of histamine in the
181 proposed method is exclusively mediated by the enzyme.

182 The UHPLC-FL method allowed us to unequivocally determine the remaining histamine in the
183 samples with a chromatographic elution time of 7 minutes and without the need for tedious
184 pre-column derivatization procedures. The selected substrate concentration was 45 μ M of
185 histamine, in accordance with published kinetic data for DAO activity on this specific amine,
186 to ensure optimal performance of the enzymatic reaction [9, 39, 40]. The degradation of
187 histamine was monitored for 48 hours to study the enzymatic reaction. A linear histamine
188 degradation rate was observed in the first 6 hours of the assay ($r > 0.9990$) for both porcine
189 and legume matrices.

190

191 **3.1. Method reliability**

192 The linearity of the method was assessed by performing in triplicate seven determinations of
193 different enzymatic activities using purified DAO and verified by analysis of the variance of
194 the regression. A correlation coefficient of $r=0.9998$ and a coefficient of determination (r^2)
195 higher than 99% were obtained ($p < 0.001$), demonstrating the satisfactory performance of the
196 method within the DAO activity range of 0.7 to 4.5 mU. Regarding method sensitivity, the
197 limit of detection (LOD) and the limit of quantification (LOQ) were estimated using a
198 regression curve with low DAO activity values and considering the mean response of a blank
199 plus three or ten times the standard deviation of the blank, respectively [41]. Specifically, the
200 value obtained for LOD was 0.025 mU and for the LOQ it was 0.038 mU.

201 The precision and recovery of the proposed method for routine analysis of DAO activity were
202 assessed with different batches of porcine kidney protein extract and lyophilized pea sprouts.
203 The precision was evaluated by performing 7 independent determinations of DAO activity for
204 each food matrix (Table 2). The relative standard deviation was in both cases lower than 3 %,
205 showing a satisfactory level of precision. The Horwitz equation for intra-laboratory studies
206 confirmed the acceptability of this precision data [42]. Recovery was evaluated by performing
207 7 independent determinations of porcine kidney extract and lyophilized pea sprouts,
208 considering 3 addition levels with purified DAO (Table 2). The recovery values obtained for
209 the three levels of addition were satisfactory and no statistical differences from the
210 theoretical value 100% were found ($p > 0.05$) [42]. The variance of the recovery values was not
211 dependent on the content of the analyte according to Cochran's C test ($p > 0.05$).

212 Among the range of methodologies described in the literature that challenge the
213 determination of DAO activity, the majority are based on the measurement of the liberation
214 of hydrogen peroxide or the consumption of oxygen occurring along the oxidative
215 deamination reaction [13, 21, 24-30]. Those largely used approaches face an important
216 drawback, as the presence of hydrogen peroxide and dioxygen may be markedly influenced
217 by the concomitant presence of other enzymatic capacities in certain complex biological
218 matrices [34, 35]. This is the case of catalase, an enzyme commonly found in plant and animal
219 tissues, which can lead to the underestimation of DAO activity by consuming H_2O_2 and
220 releasing O_2 [34, 35]. Therefore, the frequent occurrence of catalase in DAO-positive matrices
221 makes those techniques unadvisable due to major interference effect. In this sense,
222 Ahmadifar et al. [35] have recently proposed a zymographic approach consisting in an
223 electrophoretic separation of DAO enzyme followed by its densitometric image analysis
224 capable to evaluate the DAO activity of a sample in the presence of interfering catalase.

225 Concurrently, all those methods consisting in the monitoring of hydrogen peroxide release
226 through a coupled reaction with peroxidase entail further complexities, such as a potential
227 partial substrate inhibition produced by excess of hydrogen peroxide [43]. In general, coupled
228 peroxidase assays may be targeted as unreliable when working with purified DAO enzyme
229 and totally inadvisable when studying non-purified complex samples due to the presence of
230 peroxidase inhibitors or other enzymatic activities [34, 44]. In fact, Calinescu et al. [34]
231 evaluated the DAO capacity of formulation containing a vegetal extract with the presence of
232 catalase, using both a peroxidase coupled assay and an alternative assay non-related to
233 peroxidase enzyme. In this context, the authors described the unsuitability of the peroxidase
234 coupled assay due to the diminution of released H₂O₂ by catalase enzyme, emphasising the
235 need to seek for enzymatic tests not affected by the presence of catalase [34]. In this sense,
236 methods based in the direct measurement of the degradation of the amine substrate,
237 hitherto scarcely described in the literature, may overcome this limitation. In the proposed
238 method, DAO activity of the porcine kidney extract did not significantly differ ($p>0.05$) when
239 was determined with or without the addition of catalase, and independently of the
240 concentration of this enzyme added to the sample. Therefore, the proposed method herein
241 is not influenced by the presence of catalase present in the analyzed food matrices, since it is
242 based in the direct determination of histamine.

243 Although the largely used spectrophotometric techniques seem to be sensitive enough for
244 the analysis of samples with an elevated degree of purification, there is a lack of a reliable
245 and sensitive methods that allow to determine DAO activity in complex biological or food
246 matrices, which will not only contain several potential interferences but will also show
247 relatively low enzymatic rate. In this case, radiochemical detection techniques based on the
248 use of C¹⁴-labelled putrescine becomes the preferred approach [44]. However, while a high

249 sensitivity may be attributed to the latter, serious concerns related to the hazardous potential
250 in the handling of radioactive material and the high cost and unsuitability of its storage need
251 to be considered. The proposed method shows the advantages of sensitivity, reproducibility
252 and automatization of an UHPLC approach while avoiding user-related hazardous potential
253 and becomes a suitable approach to analyze DAO activity in complex non-purified matrices.

254

255 **3.2. Suitability of the method for the determination of DAO activity in porcine kidney** 256 **protein extracts, lyophilized pea sprouts and DAO supplements**

257 The applicability of the developed method was tested by analyzing several production
258 batches of porcine kidney protein extract and lyophilized pea sprouts. Additionally, the
259 enzymatic capacity of porcine kidney extract in DAO supplements available in the market was
260 studied. All analyzed products showed *in vitro* histamine-degrading capacity (Figure 3).
261 Lyophilized pea sprouts were the most effective, with a mean enzymatic activity of 0.40
262 (± 0.01) mU/mg, compared to 0.23 (± 0.01) mU/mg for porcine kidney protein extracts. It is
263 worth highlighting that the DAO activity of both products showed minimal variation among
264 different production batches. These results are in good agreement with previously published
265 data indicating a higher catalytic turnover rate for plant- than animal-derived DAO [5, 23, 37].
266 The amine-degrading capacity described in the literature for these food matrices is highly
267 variable, with values ranging from 0.1 to 500 mU/mg. Different behavior toward the same
268 amino substrates has been reported for DAO enzymes depending on whether they are of
269 animal or plant origin [37]. This heterogeneity could also be explained by differences in
270 methodology between studies, as a range of detection techniques and substrates have been
271 used. Thus, Kivirand et al. [7] suggested that the substrate specificity data available for DAO

272 varied according to the experimental method and recognized an important difficulty to find
273 comparable data due to the evidenced dispersion of methodological procedures. Concretely,
274 the wide range of used substrates (i.e. putrescine, cadaverine, agmatine, histamine,
275 spermidine and spermine) may easily lead to differences in the reported enzymatic activities,
276 as the affinity of DAO for each substrate varies [36, 37]. Due to the evidenced differences in
277 kinetic parameters depending on the amino substrates, histamine is the optimal substrate in
278 order to have an available methodology to determine the enzymatic activity of potential new
279 sources of DAO, considering the degradation of this target substrate and no other amines.

280 Porcine kidneys and pea seedlings are the main sources of DAO according to the literature
281 [21, 23], but it can also be found in other food products, such as certain legumes (*Cicer*
282 *ariantum*, *Lathyrus sativus*, *Lens esculenta*), barley (*Hordeum vulgare*), maize (*Zea mays*) and
283 tea (*Thea sinensis*) [21, 23, 36]. The method proposed here could be applied to validate the
284 *in vitro* enzymatic capacity of these food matrices and to screen for new potential sources of
285 DAO.

286 The DAO activity of the six commercial DAO supplements ranged widely from 0.04 to 0.20
287 mU/mg, despite all being formulated with the same amount of porcine kidney extract (4.2
288 mg) (Figure 3). In comparison with the raw porcine extract (0.23 ± 0.01 mU/mg), a markedly
289 lower DAO activity was generally observed in these supplements. The application of different
290 galenic formulation processes may influence the enzymatic capacity of the kidney extract,
291 which would explain both the variability and loss of activity of the DAO supplements. Further
292 studies are required to understand how different technological parameters linked to the
293 manufacturing process of these supplements influence the enzymatic activity. The variable
294 activity of commercial DAO supplements could help explain the different efficacy rates

295 reported by clinical studies evaluating the use of exogenous DAO to treat symptoms
296 associated with histamine intolerance [22, 45-47].

297 Few studies have estimated the intestinal DAO activity in a healthy population. An enzymatic
298 activity of 0.001 - 0.03 mU/mg has been reported in the intestinal mucosa, with higher values
299 given for intestinal protein (0.2 - 0.33 mU/mg) [31, 48-50]. As indicated by the manufacturers,
300 the usual posology of DAO supplements is 1 capsule before each meal, which provides an
301 enzymatic activity in the range of 0.17 to 0.84 mU, depending on the product. In view of these
302 results, more accurate studies are needed in order to establish the effective dosage of DAO
303 that can provide a complementary intestinal protective barrier for histamine-intolerant
304 individuals.

305

306 **4. Conclusion**

307 The proposed method, consisting of an enzymatic assay coupled to a UHPLC-FL technique,
308 allowed the *in vitro* determination of DAO activity in food matrices using histamine as the
309 reaction substrate. This method provided satisfactory experimental performance in terms of
310 linearity, sensitivity, precision and recovery, and its suitability was tested on different food
311 matrices reported as sources of DAO. The DAO activity of lyophilized pea seedlings was nearly
312 two-fold higher than that of porcine kidney protein extracts. The histamine-degrading
313 capacity of the six DAO supplements available in the market was variable and lower compared
314 to the other analyzed matrices. Due to the growing awareness of histamine intolerance, it is
315 important to have effective methods for validating the DAO activity of supplements and foods
316 of potential interest for the treatment of this disorder.

317

318 **Compliance with Ethical Standards**

319 The authors declare that they have no conflict of interest.

ACCEPTED MANUSCRIPT

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472 **Figure captions**

473 **Fig. 1** Oxidative deamination of histamine by DAO

474 **Fig. 2** Schematic experimental procedure of the enzymatic assay for the *in vitro*

475 determination of DAO activity

476 **Fig. 3** *In vitro* DAO activity of several production batches of porcine kidney protein extract,

477 lyophilized pea sprouts and different commercial DAO supplements

478

479 **Table 1.** Chromatographic conditions for the UHPLC-FL determination of histamine

Stationary phase	
Column	Acquity UPLC™ BEH C18 column (1.7 μm, 2.1 mm x 50 mm)
Column temperature	42 °C
Mobile phase	
Eluent A	H ₂ O solution with 0.1 M sodium acetate and 10 mM sodium octanesulphonate (adjusted to pH 4.8 with acetic acid)
Eluent B	H ₂ O solution with 0.2 M sodium acetate and 10 mM sodium octanesulphonate (adjusted to pH 4.5 with acetic acid) :
	Acetonitrile (6.6:3.4)
Linear gradient	0 min, 80% A; 2 min, 80% A; 3 min, 60% A; 4 min 50% A; 5 min, 40% A; 6 min, 20% A; 6.40 min, 80% A; 7 min, 80% A.
Flow rate	0.8 mL/min
Injection volume	1 μL
Fluorescence detection	
Derivatization reagent	OPA (0.2 mg/mL), brij®, 2-mercaptoethanol, methanol, potassium hydroxide and boric acid
Excitation and emission wavelengths	340 nm and 445 nm
Flow rate	0.4 mL/min

480

481 **Table 2.** Precision and recovery results for porcine kidney extracts and lyophilized pea sprouts

482

	Precision		Recovery ^c			
	RSD (%) ^a	RSDH (%) ^b	Addition level I	Addition level II	Addition level III	Cochran's test C_{exp} ^d
Porcine kidney extract	2.76	3.45-4.60	100.54 (4.98)	102.69 (5.44)	99.14 (2.52)	0.41
Lyophilized pea sprouts	2.80	3.27-4.36	101.28 (0.90)	100.00 (0.76)	100.51 (2.61)	0.05

483

484 ^a Relative standard deviation (RSD) for seven determinations.

485 ^b Acceptable range for relative standard deviations according to the Horwitz equation for intra-laboratory studies (1/2 - 2/3 of the
486 interlaboratory study calculate by the formula).

487 ^c Mean recovery percentages and standard deviation in parentheses for three addition levels corresponding to enzymatic activities of 0.5, 1.0
488 and 2.0 mU for porcine kidney extract and 1.0, 2.0 and 4.0 mU for lyophilized pea sprouts.

489 ^d Cochran's C variance outlier test, $C_{tab}(6,2,0.05) = 0.8534$.

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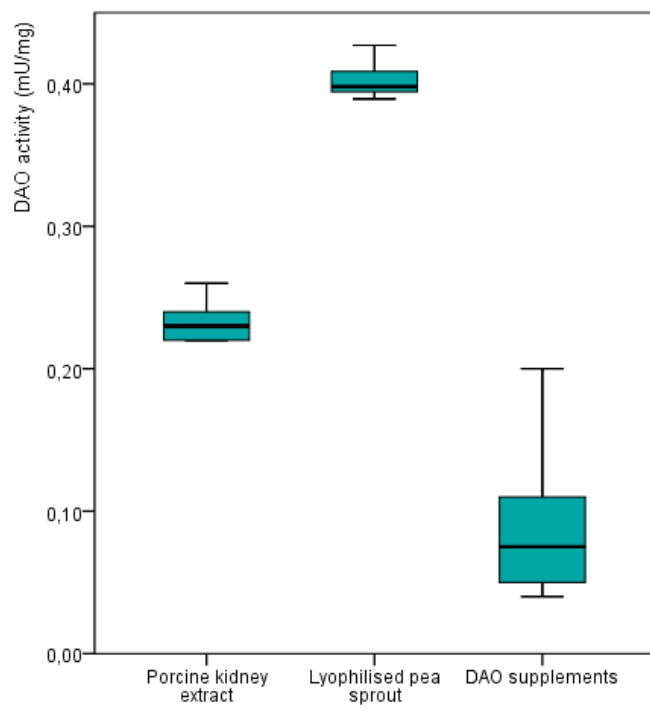
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