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A reverse phase high performance liquid chromatography method for analyzing of neurotoxin β-*N*-oxalyl-L-α,β-diaminopropanoic acid in legume seeds

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Abstract

A RP-HPLC method for detecting and quantifying β-*N*-oxalyl-L-α,β-diaminopropanoic acid (β-ODAP) the neurotoxin in grasspea (*Lathyrus sativus*) seeds by pre-column derivatization was developed. Seed extracts of grasspea were derivatized with *o*-phthalaldehyde in the presence of β-mercaptoethanol and subjected to RP-HPLC on a C-18 column (250 l × 4.6 i.d. mm). The derivatized β-ODAP could be detected either by UV at 340 nm or by fluorescence. The relatively instantaneous derivatization procedure and high sensitivity make this method useful for detection of the less expensive and drought tolerant grasspea seeds a common adulterant in edible legume seeds such as chickpea (*Cicer arietinum*) and redgram (*Cajanus cajan*). The sensitivity of this method for the detection of β-ODAP in legume seed flour was 3.5 ± 0.1 ppm. The accuracy, precision, linearity and limit of detection were consistent with that previously reported in literature. This method can be potentially applied for the detection of the neurotoxin in low-toxin varieties of the grasspea seeds being developed and those reported in other plants.

Keywords: Neurotoxin; β-ODAP; Grasspea; Pre-column derivatization; *o*-phthalaldehyde; HPLC

1. Introduction

Grasspea (*Lathyrus sativus*) is an annual legume crop belonging to the family Fabaceae and the tribe Vicieae. Grasspea, known by a wide range of common names, include chickling vetch, Indian vetch and khesari or batura dhal (in India and Bangladesh), thrives under adverse environmental conditions rendering it a popular drought tolerant crop in drought areas of Asia and Africa (Spencer, Roy, Palmer, & Dwivedi, 1986; Tekle-Haimanot & Lambein, 1997). Grasspea is primarily grown as a winter pulse crop for stock-feed and human consumption (McCutchan, 2003), and thus considered as the ''poor man's meat'' in the central region of India (Singh & Mishra, 1985). The seeds of grasspea are primarily valued as a nutritious staple food due to its high protein content, 18–34% of the dry seed weight (Almeida, 1980), 17% in mature leaves and high lysine content (Rosa, Ferreira, & Teixeira, 2000). The use of the seeds has been hampered by the fact that the seeds contain a major antinutritional constituent β-*N*-oxalyl-L-α,β-diaminopropanoic acid (β-ODAP) or its synonym L-2-amino-3-oxalyl aminopropanoic acid (IUPAC rules). β-ODAP a non-protein acidic amino acid causes neurological lesions by neuronal over-excitation and eventual cytotoxicity of neurons manifesting as spastic paralysis of the leg muscles together with muscular rigidity and weakness (Grela, Studzinski, & Winiarska, 2000; Hanbury, White, Mullan, & Siddique, 2000). Prolonged or excessive ingestion of the seeds can eventually lead to an upper motor neuron or degenerative disease known as neurolathyrism. This disease was recorded in India (Shah, 1939). For this reason its cultivation is prohibited in some places. β-ODAP

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first isolated and characterized independently by Rao, Adiga, and Sarma (1964) and Murthi, Seshadri, and Venkitasubramanian (1964), is derived from 2,3-diaminopropanoic acid and oxalyl-coenzyme A (Malathi, Padmanaban, & Sarma, 1970).

According to the Prevention of Food Adulteration Rules, 1955 of India (Chadha, 2004), the use of khesari dhal and its products or a mixture with any other gram is prohibited in items of food for human consumption. Grasspea is the cheapest legume seed available in countries like Bangladesh, India and Ethiopia. In addition the crop is produced with minimum amount of care and can be successfully grown in a variety of adverse climates. A valid concern in food safety is the intentional or unintentional presence of grasspea in the more expensive edible legumes such as chickpea (*Cicer arietenum*) and pigeonpea (*Cajanas cajan*). This advocates the need for a fast and accurate method of detection and estimation of β-ODAP in mixtures of legume seeds. Although a number of analytical methods have been developed to assess various effects of detoxification of the crop in the field and post harvest, grasspea adulteration has not been addressed previously. The most commonly used spectrophotometric method involves the alkaline hydrolysis of β-ODAP to yield 2,3-diaminopropanoic acid, which in turn is complexed with *o*-phthalaldehyde in the presence of ethanethiol to form a colored adduct that is quantitated at 420 nm (Rao, 1978). Post and pre-column derivatization HPLC methods have been developed for screening plant samples for ODAP (Shah, Younie, Adlard, & Evans, 1992). Pre-column derivatization with phenylisothiocyanate (PITC) although sensitive is time intensive, requiring 5–6 h for derivatization and complete removal of excess coupling reagent and by products (Khan, Kebede, Kuo, Lambein, & De Bruyn, 1993). In addition, PITC is easily degraded and sensitive to light. 9-Fluorenyl methyl chloroformate (FMOC) derivatization detects β-ODAP at picomolar levels, however incomplete removal of excess reagent interferes with resolution and is detrimental to the column performance (Geda, Briggs, & Venkataram, 1993). Pre-column derivatization with 1-fluoro-2,4-dinitrobenzene (FDNB) prior to HPLC has become an important analytical technique for the assay of amino acids (Morton & Gerber, 1988; Vander Horst, Teeuwsen, Holthuis, & Brinkman, 1990). However, a major drawback is the toxicity of FDNB requiring careful handling and protective apparel. A capillary zone electrophoresis method for detecting β-ODAP however requires no derivatization (Arentoft & Greirson, 1995), 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) reacts quantitatively with primary and secondary amino acids to form stable derivatives (Chen, Wang, Chen, Qin, & Li, 2000), the detection limits being ~1.8 ng for β-ODAP. However, this method requires a complicated ternary gradient for resolution in addition to 10 min at 55 °C for the derivatization reaction. We report here a relatively instantaneous pre-column derivatization using *o*-phthalaldehyde followed by RP-HPLC to detect β-ODAP. This method is a simple, rapid and accurate method that can be effectively used to detect the adulteration of legume seeds with grasspea. The detection level was as low as one grasspea seed in a 1000 legume seeds (equivalent to 3.5 ± 0.1 ppm β-ODAP).

2. Experimental

2.1. Materials

o-Phthalaldehyde, β-mercaptoethanol and triethylamine (TEA) were obtained from Sigma–Aldrich Co., St. Louis, MO, USA. Aspartic acid and glutamic acid were from Pierce Chemical Co., Rockford, IL, USA. Acetonitrile and methanol were HPLC grade from Ranbaxy Fine Chemicals limited, New Delhi, India. β-ODAP was a gift from Dr. Rajagopal Rao, Mysore, India synthesized according to Rao (1975). Boric acid was from Amresco, OH, USA and sodium acetate from SD Fine Chemicals, Mumbai, India. Grasspea seeds were a gift from Ms. Usharani, Mysore, India. All solutions were prepared with water purified with a Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

Sulfosalicylic acid (2.5 mL, 3% w/v) was added to 500 mg of finely powdered grasspea seeds (60 mesh) and extracted by agitation for 1 h at 25 ± 2 °C. The solution was centrifuged at 15,000*g* for 15 min at 4 °C. The clear supernatant was subsequently filtered through a 0.45 μm membrane.

2.3. Pre-column derivatization

Derivatization reagent was prepared fresh everyday. Five milligram of OPA dissolved in 0.05 ml methanol was added to 0.45 ml of 0.4 M sodium borate buffer pH 10.5 followed by 0.025 ml of β-mercaptoethanol. A 10-μL aliquot of the sample extract was mixed with an equal volume of OPA reagent and incubated at 25 ± 2 °C for exactly 2 min and then subjected to HPLC analysis as described in the following section. Standard solutions containing 2.5 mM each of β-ODAP, aspartic acid and glutamic acid were derivatized as described above.

A standard solution of β-ODAP (2.5 mM) in water was prepared and incubated in a boiling water bath for 30 min (Khan et al., 1993) and derivatized as described above. β-ODAP was converted to DAP by alkaline hydrolysis according to the method of Rao (1978). The resulting solution was subjected to derivatization with OPA as described and detected at 420 nm.

2.4. RP-HPLC analysis

The analysis was performed on a Shimadzu, Model LC-10ATVP HPLC system (Shimadzu, Japan) equipped with a

Rheodyne injector with a 20 μL loop, LC 10 separation module and SPD-M10AVP PDA detector set to monitor the derivatized amino acids at 340 nm. The derivatized amino acids were also detected by fluorescence using the RF-10AXL fluorescence detector (Ex: 340 nm and Em: 450 nm). A reverse phase Jupiter 5 μ C18 300 Å (250 L × 4.6 i.d. mm) column was used.

The concentration of the optimized mobile phase A was 0.14 M CH_3COONa containing 0.05% (v/v) TEM adjusted to pH 6.8 with glacial acetic acid and methanol (90:10). Mobile phase B was 60:40 acetonitrile in water. The mobile phase was filtered through a 0.22 μm membrane filter and degassed prior to use. The optimized binary gradient elution program listed in Table 1 was operated at a flow-rate of 1.0 mL/min.

2.5. HPLC method validation

Validation tests were performed for accuracy, precision, linearity range and limit of detection. The accuracy of the method was evaluated by recovery where chickpea flour, which is devoid of β-ODAP, was mixed with grasspea flour at 0.2%, 0.5% and 1.0% (w/w) and compared with chickpea flour alone. The % recovery was calculated from the mean concentration of three replicates each. The precision of the method was evaluated within day ($n = 10$) and between days ($n = 10$ for 7 days) for both the retention time and peak area. The precision of the method is expressed by standard deviation (SD), relative standard deviation (RSD) or coefficient of variation (CV). The linearity of the method was evaluated by injecting 20 μL of derivatized standard β-ODAP at 0.048–25 nmol level. Each standard was analyzed in triplicate.

Calibration curves for both the UV and fluorescent detection method were constructed individually by plotting peak areas versus concentration. Using the software Origin version 4.1, regression analysis was performed to relate standard concentration to peak area and obtain the regression correlation coefficient. A correlation of 0.999 is acceptable for most methods.

The limit of detection (LOD) for the standard β-ODAP was determined by analyzing β-ODAP at levels ranging from 0 to 25 nmol range. The detection limit of β-ODAP in legume flours were evaluated by making admixtures containing 0.01%, 0.1%, 0.5% and 1% (w/w) of grasspea flour. Recoveries were calculated from peak area ratio of the analyte in extracts of the spiked chickpea flours. In addition, admixtures of a single grasspea seed with either 99 or 999 chickpea seeds were also prepared and β-ODAP was detected in the extracts.

3. Results and discussion

The factors that influence the retention and efficiency of the chromatographic separation of β-ODAP from aspartate and glutamate i.e. pH, organic modifier and ion-pairing agent, TEM content were optimized. The maximum percentage of reaction measured as the A_{340} response between β-ODAP and OPA as attained within 2 min at 25 °C. A slow but measurable decrease in yield occurred as the reaction was allowed to proceed for a longer time (Table 2). Therefore, the reaction time used in all the derivatizations was limited to 2 min. The amount of OPA selected was based on previous reports for standard amino acids. The OPA derivatives of β-ODAP and standard aspartic and glutamic acid were prepared as described for pre-column derivatization. Fig. 1 reports the LC-UV chromatogram for the derivatized standard β-ODAP and a mixture of β-ODAP, Asp and Glu at concentrations of 10 nmol each using the optimized mobile phase composition and gradient as described in Section 2.4. The three amino acids were well resolved with retention times of 12.8, 13.6 and 14.3 min for Asp, β-ODAP and Glu, respectively. Injecting individual standard amino acids identified each component. Analysis of dilutions of β-ODAP from 0.048 nmol to 25 nmol showed a linear response in this range with a $R^2 = 0.999$ (Fig. 2a), when detected at 340 nm. A linear response in the range 0.003–12.5 nmol was obtained when detected by fluorescence (Ex: 340 nm and Em: 450 nm, Fig. 2b). The limit of detection by fluorescence was 0.0025 nmol. The limit of detection using a chromatographic-biosensor system was 2 μM with respect to ODAP (Yigzaw, Larsson, Gorton, Ruzgas, & Solomon, 2001). Regression equations for both the detection systems with R^2 of 0.999 and 0.998 could be used to predict concentration from peak area. Peak area repeatability obtained from $n = 10$ injections within the day was 2.3% CV and between days ($n = 10$ over seven days) was 2.3–4.4% CV. The same standards analyzed by fluorescence detection indicated a fivefold higher sensitivity.

The isomerization of β-ODAP to α-ODAP was achieved by incubating β-ODAP at 98 ± 2 °C for 30 min. The OPA derivatized mixture resolved into two peaks with retention times of 13.6 and 16.5 min for β- and α-ODAP, respectively

Table 1
Gradient programme used for the separation of β-ODAP

Time (min)	*A* (%)	*B* (%)
0	100	0
15.00	25	75
16.00	0	100
20.00	0	100
21.00	100	0
25.00	100	0

Table 2
Peak area yield for OPA derivatization of β-ODAP

Reaction time (min)	Area
1	1844586
2	2147940
5	1705721
10	1673026
20	1420899
60	500893

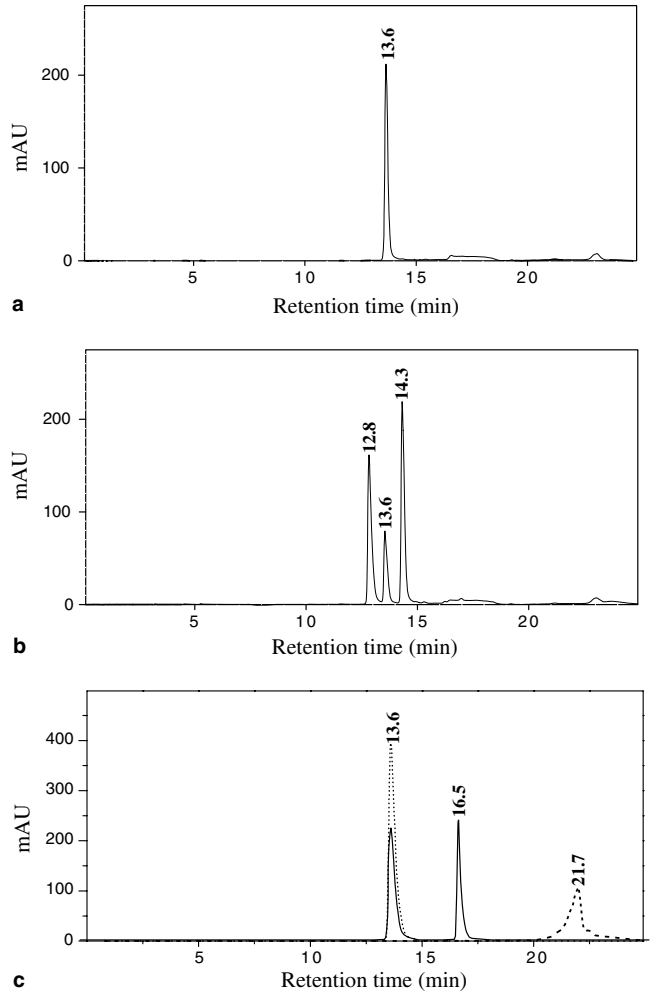

Fig. 1. A typical chromatogram of the OPA derivatized amino acid standard mixture. Detection was by UV at 340 nm: (a) β-ODAP, (b) a standard mixture of aspartic acid, β-ODAP and glutamic acid and (c) separation of α-, β-ODAP and DAP after derivatization with OPA. (···) β-ODAP, (—) isomerization of β-ODAP to α-ODAP and (----) alkaline hydrolysis of β-ODAP to DAP. DAP was detected at 420 nm.

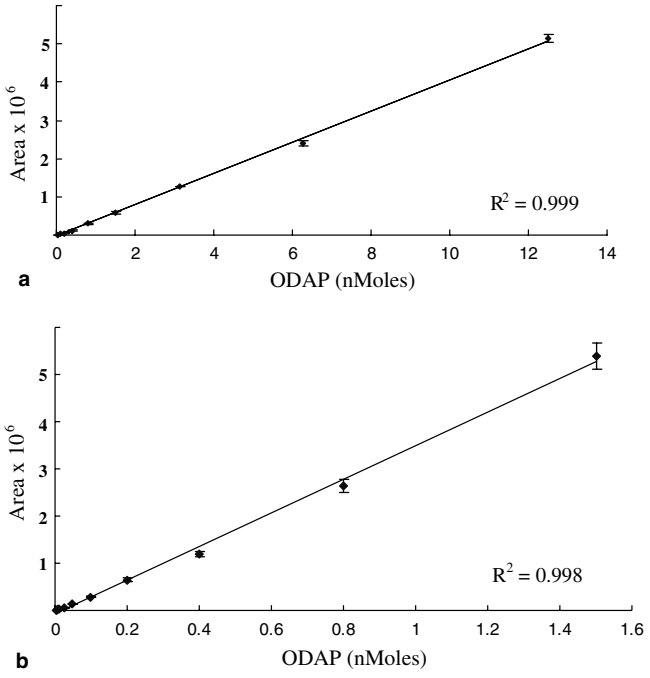

Fig. 2. Calibration curve for β-ODAP determination: (a) Detection by UV at 340 nm and (b) detection by fluorescence (Ex: 340 nm and Em: 450 nm).

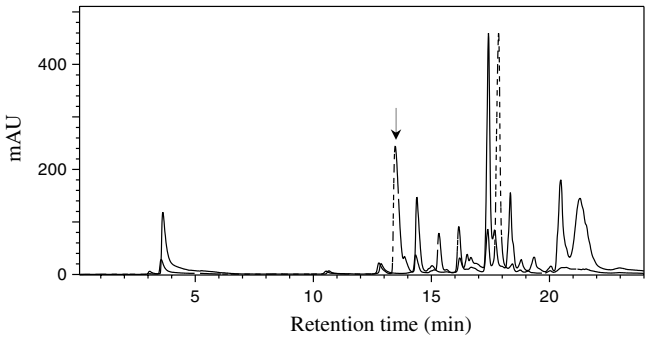

Fig. 3. A typical chromatogram of the OPA derivatized grasspea seed and chickpea seed extract. Sample preparation and chromatography are as described in the text. Detection was by UV at 340 nm. β-ODAP is indicated by an arrow.

(Fig. 1c). These results indicate the separation of α, β isomers by this method. The increase in the α-ODAP was ~33%. Khan et al. (1993) have demonstrated that the isomerization of β-ODAP to α-ODAP reached an equilibrium ratio of 35:65 (α:β). The retention time of OPA derivatized DAP, a product of alkaline hydrolysis of ODAP detected at 420 nm, was 21.7 min (Fig. 1c).

The precision for the retention time of β-ODAP over 10 runs showed a SD of ±0.078% and 0.68% CV within and between days. These results suggest that under the given storage conditions β-ODAP standard was stable with no isomerization as evidenced by the absence of α-ODAP ($R_t = 16.5$ min), reproducibility of the peak areas, precision of retention times and absence of DAP ($R_t = 21.7$ min).

The applicability of the methods was evaluated using extracts of grasspea seed flour. Fresh extracts were derivatized as described and subjected to HPLC. The chromatogram (Fig. 3) indicates that β-ODAP is a major amino acid with a retention time of 13.6 min. The sulfosalicylic acid extraction procedure was rapid and the yield of 7.6 ± 0.05 mg β-ODAP/gm was higher compared to ethanol extraction (6.1 ± 0.05 mg β-ODAP/gm). The optimized mobile phase and gradient elution programme permitted to resolve β-ODAP, the target compound with no significant interference from endogenous amino acids in either of the extracts. This was further confirmed by spiking the extract with standard β-ODAP. Notable in the extracts of chickpea flour is the absence of β-ODAP (Fig. 3). The β-ODAP content of the seeds calculated from the calibration curve was 7.6 ± 0.05 mg/gm (equivalent to 7.6 ppm dry seed weight). This value is in close agreement to the values 0.52–0.76 g% determined for various grasspea varieties using

post column refractive index detection in combination with biochemical detection (Yigzaw et al., 2001). The traditional spectrophotometric method of Rao (1978) was used to validate these results. The toxin level calculated on dry mass basis by this method was 0.70 g%. The occurrence of β-ODAP in grasspea seed extracts has been reported to be 95% of the total ODAP content (Khan et al., 1993; Belay, Moges, Solomon, & Johansson, 1997). This level is dictated by the extraction method and speed of analysis. The β-ODAP content of five independent extractions was 0.03% RSD. The absence of α-ODAP peak ($R_t = 16.5$ min) observed in all the extractions suggests that no conversion to the α-isomer has occurred (Fig. 3). In addition, the 2.3% CV for peak area repeatability and reproducibility of the areas also suggests that no such isomerization to α-ODAP occurs during the extraction procedure or storage.

The sensitivity of the method was evaluated by preparing admixtures of one grasspea seed in a total of 100 and 1000 seeds. The admixture was powdered and used for extraction of β-ODAP, derivatized and subjected to HPLC. β-ODAP could be detected in both the admixtures (Fig. 4b and c), but was distinctly absent in the virgin chickpea flour (Fig. 4a). The concentration of β-ODAP was found to 35.31 and 3.55 μg/g of flour for the 1 in 100 and 1 in 1000 seed admixture, respectively. These results advocate that adulterations at the levels of 1 in 1000 seeds (equivalent to 3.6 ppm β-ODAP) can be reliably detected by this method.

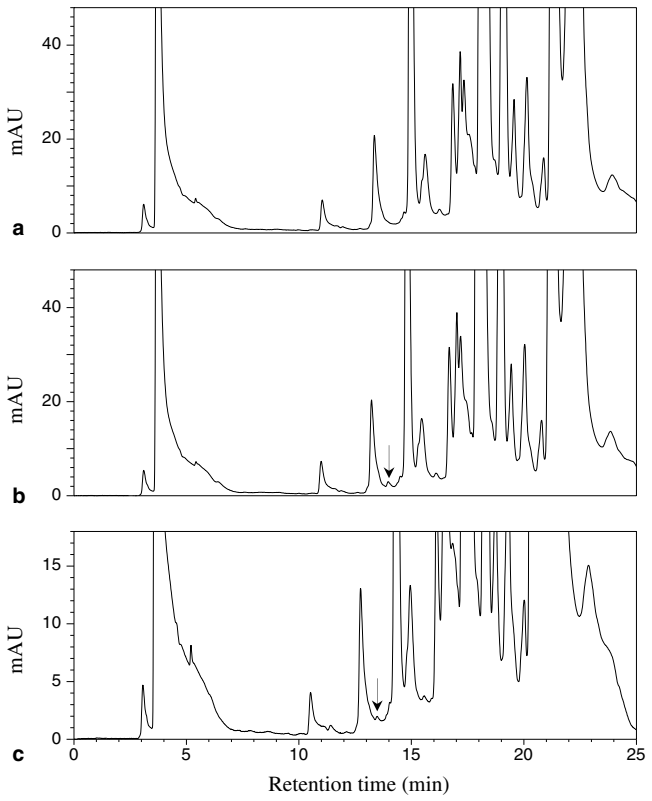

Fig. 4. Chromatogram for the detection of grasspea in admixtures with chickpea: (a) chickpea flour, (b) 0.1% grasspea flour and (c) 1:999, grasspea:chickpea seeds.

The accuracy of the method is also supported by the recovery of β-ODAP from spiked chickpea seed flour. Pure grasspea flour was added to freshly ground chickpea flour to obtain an admixture of 0.1%, 1% and 5% (w/w) grasspea. The β-ODAP content after extraction and derivatization was 0.75 mg/100 g. The obtained recovery ($n = 5$) of the β-ODAP was 98.7 ± 0.8% and remained unchanged passing from 0.1% to 5% (w/w) level. Screening of 10 different grasspea seed samples carried out by measuring the β-ODAP content using a biosensor based on crosslinking horse radish peroxidase and an Os-containing mediating polymer with poly (ethylene glycol-400) diglycidyl ether and then immobilizing L-glutamate oxidase resulted in 98% recovery (Yigzaw et al., 2001). When an accurate amount of the toxin β-ODAP was added to seeds, about 99% was recovered after extraction and derivatization with FDNB (Wang, Chen, Chen, Qin, & Li, 2000). Therefore, the method described is consistent with previously reported procedures.

Freshly prepared OPA reagent in the presence of a thiol is a speedy alternative pre-column derivatizing reagent for the determination of β-ODAP. The potential of the described method has been demonstrated by the application to chickpea samples adulterated with grasspea. The relatively instantaneous derivatization procedure and high sensitivity detection of 1 grass pea seed in 1000 seeds (3.6 ppm) makes this method a useful tool for detection of the less expensive grasspea as an adulterant in items of food for human consumption and is most applicable to finely milled pulses where the adulteration is generally obscure. This method can also be effectively used for screening the low-toxin varieties of the *L. sativus* being developed. In addition, the method may be potentially applied to the detection of β-ODAP reported in the seed plant of *Panax ginseng* (Kuo, Ikegami, & Lambein, 2003), which has now become popular in herbal pharmaceuticals.

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