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Amino acids interfere with the ninhydrin assay for asparagine

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A colorimetric ninhydrin assay, reportedly specific for asparagine, has been published (Sheng *et al.* (1993). *Anal. Biochem.*, **211**, 242–9). The suitability of this assay for determining asparagine in food samples was tested by using it to measure the asparagine levels in tips of harvested asparagus spears. Compared with our standard chromatographic procedure (x), the colorimetric assay (y) severely overestimated asparagine in asparagus ($y = 129{\cdot}6 + 1{\cdot}12x$, $S_{y/x} = 32{\cdot}2$) even though the results from the two methods were highly correlated ($r = 0{\cdot}997$, $P = 0{\cdot}003$). By testing other amino acids in the assay, we showed that they also react with ninhydrin under the conditions of Sheng *et al.* In particular, proline gave a response two-fold greater than asparagine and the response with phenylalanine was half that of asparagine. It is believed that the overestimation of asparagine by the ninhydrin method is due to the additive response of the other amino acids present in asparagus. It is concluded that the assay is unsuitable for the routine determination of asparagine in asparagus, or in complex mixtures of amino acids in other foods.

INTRODUCTION

A colorimetric assay, reportedly specific for L-asparagine in the presence of glutamine, glutamate, aspartate and ammonium, has been described (Sheng *et al.*, 1993). In this procedure, asparagine at pH 6·0 reacts with dilute ethanolic ninhydrin at 37°C to yield a pale yellow solution with an absorption maximum at 340–350 nm. The authors used the assay to measure the activities of purified asparagine synthetase and asparaginase in buffered solutions. However, the authors suggested other amino acids such as proline and cysteine may also react with ninhydrin under their assay conditions and although they cautioned using the assay for determining asparagine concentrations in biological samples, they did not test them in their assay. We were attracted to the assay because with our studies on the postharvest metabolism of asparagus we frequently measure asparagine. Hitherto, we have used time-consuming chromatographic methods (Hurst & Clark, 1993; Hurst *et al.*, 1993*a*,*b*), therefore, a rapid colorimetric assay for asparagine would be of considerable benefit to us. The present study demonstrates that other amino acids as well as proline and cysteine interfere with the assay. The assay failed to quantify accurately asparagine in asparagus extracts, thus indicating that the assay is unsuitable for food or biological samples.

MATERIALS AND METHODS

L-Asparagine, L-cysteine, L-glutamine and L-glutamic acid were from Sigma Chemical Co. (St Louis, MO, USA). L-Alanine was from Boehringer Mannheim GmbH (Mannheim, Germany). L-Phenylalanine, L-lysine and L-proline and AnalaR® ninhydrin were from BDH Laboratory Supplies (Poole, Dorset, UK). Free amino acids were extracted with 1 ml of 62·5% (v/v) methanol from 10 mg samples of freeze-dried tips (0–30 mm portion of spear measured from the apex) of 18 cm long asparagus spears that had been held at 20°C for up to 3 days (Hurst & Clark, 1993; Hurst *et al.*, 1993*a*). Asparagine in these extracts was quantified by HPLC as the *o*-phthaldialdehyde derivative (Martin *et al.*, 1982) as previously described (Hurst *et al.*, 1993*a*) except the isocratic methanol–phosphate buffer elution regime of Martin *et al.* was replaced by a methanol–phosphate buffer gradient elution regime designed to improve the amino acid separation. For the colorimetric method, extracts were diluted 1 : 4 with methanol : 0.133 M Tris–HCl pH 6·0 solution 62·5 : 37·5 (v/v) in order to conform closely with the assay conditions of Sheng *et al.* (1993). Asparagine in these diluted asparagus extracts and response of other amino acids were measured according to Sheng *et al.* In brief, 100 μl of extract or

amino acid standard solutions (0·5, 1·0, 1·5, 2·0 and 2·5 mM in methanol-Tris) was mixed with 900 μl of 0·05% (w/v) ethanolic ninhydrin solution in 1·5 ml microfuge tubes and incubated at 37°C for 3 h. Solutions were then microfuged for 1–2 min and the absorbance spectra (300–800 nm) and absorbance at 340 nm (A_{340}) of the supernatants were measured using a Shimadzu UV-160A double-beam spectrophotometer. Blanks contained 100 μl of methanol-Tris and 900 μl of ethanolic ninhydrin.

RESULTS AND DISCUSSION

Asparagine levels of spear tips from the colorimetric assay were highly correlated with those obtained by HPLC (r = 0·997, P = 0·003), but the colorimetric assay (Table 1) greatly overestimated the asparagine content of asparagus spear tips (colorimetric (y) = 129·6 + 1·12 × HPLC (x), $S_{y.x}$ = 32·2). Moreover, the precision of the colorimetric method was less than that of the HPLC method. Asparagus spear tip asparagine levels determined by HPLC in this study (Table 1) were in line with our previous work that used three different chromatographic procedures. Clearly, this validates our modified HPLC method. Since other amino acids also accumulate in harvested asparagus (Hurst & Clark, 1993; Hurst *et al.*, 1993*b*), we tested a range of amino acids (Table 2), representative of aliphatic, sulfur-containing, acidic, basic, aromatic and imino amino acids, to see if they reacted in the ninhydrin method and could, therefore, account for the overestimation of asparagine. All amino acids tested produced some A_{340} and linear standard curves (Table 2). On a molar basis, proline gave a response two-fold greater than asparagine, whereas the response with phenylalanine was half that of asparagine; the other amino acids tested gave lesser responses.

Scanning the amino acid–ninhydrin mixtures confirmed the observation of Sheng *et al.* that with asparagine there was a single absorption maximum at 340–350 nm. However, proline-ninhydrin also showed a single but somewhat sharper peak at 340 nm. All of the other amino acid-ninhydrin mixtures showed two absorption maxima at 405–410 and 570–580 nm similar to those reported by Sheng *et al.*, except the peak at 405–410 nm was accompanied by a shoulder at shorter wavelengths. It was this shoulder, that varied in magnitude depending on the amino acid, that gave the A_{340} shown in Table 2. Asparagus extract–ninhydrin mixtures gave a complex absorption spectrum with maxima at 340–350, 405–410 and 570–580 nm reflecting the complex amino acid composition of asparagus (Hurst & Clark 1993; Hurst *et al.*, 1993*b*).

In order to confirm that the A_{340} observed with these amino acid solutions and asparagus extracts was due to reaction with ninhydrin and not due to their native A_{340} *per se*, we diluted amino acid standard solutions and asparagus extracts 1:9 with ethanol and measured their A_{340}. Neither amino acid standards nor asparagus extracts had any A_{340}. Since monosaccharides can react with ninhydrin under certain conditions (Magné & Larher, 1992), and since our asparagus extracts would have contained glucose and fructose (Irving & Hurst, 1993), we tested glucose and fructose (up to 2 mM in methanol-Tris) in the method. No A_{340} was detected, thus eliminating monosaccharide interference.

Our results with a representative selection of amino acids indicates that, irrespective of structure, all amino acids will react to some degree with ninhydrin under the conditions of Sheng *et al.* Furthermore, the data in Table 2 indicates that imino and aromatic are likely to interfere most in the assay. Although proline reacts strongly in the assay, it cannot by itself account for the disparity between the HPLC and ninhydrin values for asparagine because proline levels in asparagus spear tips drop after harvest (Table 3; Hurst & Clark, 1993; Hurst *et al.*, 1993*b*). Rather, we believe the overestimation of asparagine by the ninhydrin method is due to

Table 1. Asparagine content (μmol g^{-1} dry weight) of tips of harvested asparagus spears determined by *o*-phthaldialdehyde derivative HPLC and colorimetric ninhydrin methods (mean ± SE, n = 2)

Days after harvest	HPLC method	Colorimetric method
0	85 ± 3	196 ± 20
1	181 ± 10	367 ± 23
2	472 ± 19	654 ± 61
3	714 ± 31	924 ± 33

Table 2. Relative absorbance values at 340 nm after ninhydrin reaction with selected amino acids (1 mM), based on asparagine = 100%[a] (mean ± SE, n = 4)

Amino acid	Relative absorbance
Alanine	4
Asparagine	100
Cysteine	8
Glutamic acid	7
Glutamine	6
Lysine	10
Phenylalanine	47
Proline	207

[a]Reaction of 100 μl of 1 mM L-asparagine with 900 μl of 0·05% (w/v) ninhydrin in ethanol gave an absorbance of 0·587 ± 0·018 at 340 nm after 3 h incubation at 37°C.

Table 3. Changes in the concentration (μmol g^{-1} dry weight) of amino acids used in interference studies in asparagus spear tips during storage at 20°C[a] (means, n = 3)

Amino acid	Days after harvest			
	0	1	2	3
Alanine	36	27	36	29
Asparagine	53	188	376	594
Cysteine	2·6	3·2	8·0	8·7
Glutamic acid	58	52	46	36
Glutamine	144	67	54	74
Lysine	4	17	31	43
Phenylalanine	3·7	12	32	49
Proline	14	2·0	4·9	4·2

[a]Data are taken from Hurst and Clark (1993).

the additive response of all other amino acids, some of which (notably his, ile, leu, lys, phe, ser, trp, val) increase several fold after harvest (Table 3; Hurst & Clark, 1993; Hurst *et al.*, 1993*b*). With increasing time after harvest the values for asparagine by the two methods converge (Table 1). This is merely a reflection of the fact that, with increasing time after harvest, asparagine accounts for an increasing proportion of the total free amino acid pool in spear tips (10% at harvest, 40% after 3 days; Hurst & Clark, 1993), and hence the specificity of the assay for asparagine appears to increase.

In conclusion, we have shown that the ninhydrin method, reportedly specific for asparagine (Sheng *et al.*, 1993), is severely interfered with by several other amino acids. We have found it unsuitable for measuring the asparagine content of asparagus. We advise food scientists and other potential users of the assay to evaluate it critically under their own conditions.

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