Analytical Methods

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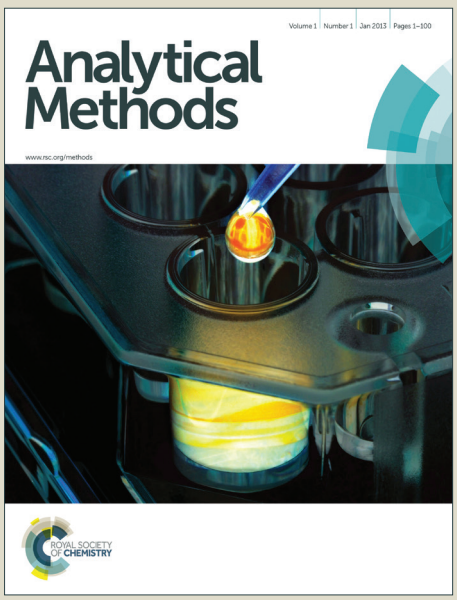

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ARTICLE

A rapid method for the determination of free alpha-amino acids in pea (*Pisum sativum*) using Ultra High Performance Liquid Chromatography – High Resolution Accurate Mass - Mass Spectrometry

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M. Dickinson [a], M. Parker [a], F. Schoutsen [b] and A.J. Charlton [a]

The analysis of alpha-amino acids in biological matrices usually requires time-consuming ion exchange liquid chromatography with post column derivatisation. Using Ultra High Performance Liquid Chromatography coupled to High Resolution Accurate Mass - Mass Spectrometry (UHPLC-HRAM-MS) we describe a rapid, accurate and sensitive method for the analysis of the 21 common free amino acids in pea seeds (*Pisum sativum*). Total analysis time including extraction, separation by LC and detection was less than an hour per sample. Extraction efficiency and repeatability were assessed using surrogate standards and were found to be acceptable with recoveries of the standards between 84 % and 109 % and relative standard deviations of less than 8% between replicates. Mass accuracy of the ions detected (between 2-4 ppm), linearity of standards and sensitivity were excellent with limits of detection (LOD) less than 30 ng g^{-1} and r^2 values greater than 0.96 for all amino acids tested.

1. Introduction

Alpha-amino acids (α-AAs) contain an amine group (NH_2), a carboxylic acid group (COOH), and a side-chain (R) that is specific to each amino acid and have the generic formula H2NCHRCOOH[1] as shown in Figure 1. Their critical role in ensuring growth performance in humans and animals necessitates an interest in the development of efficient measurement techniques, particularly for the formulation of foods, food supplements, infant formulae and animal feed.

The development of suitable analytical methodologies for α-AAs determination is addressing a worldwide protein deficit[2] by allowing novel feed stocks to be rapidly assessed for key amino acids, such as lysine and methionine, that are critical to human and animal nutrition[3].

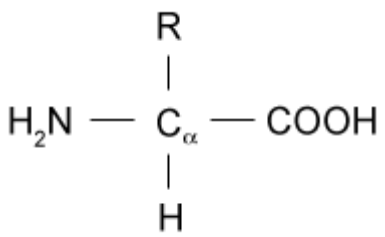

Figure 1. General structure of an alpha-amino acid (α-AA)[4]

The analysis of amino acids historically requires the use of ion exchange chromatography[5] followed by post column derivatisation with ninhydrin[6]. Detection is achieved using spectrophotometry at 570 and 440 nm. One major drawback of this method is that it requires a lengthy chromatographic gradient run of up to 2.5 hours duration in order to obtain acceptable chromatographic resolution. The efficiency of the ninhydrin derivatisation may also vary depending on the amino acid / matrix combination. The ninhydrin reagent is also known to be relatively unstable, hence when automated this method is reported to demonstrate poor between-run precision[7]. Gas Chromatography (GC, often with Mass Spectrometric detection) has also been used for amino acid detection. However, due to the relative low volatility of the amino acids' this necessitates sample derivatisation (trimethylsilylation)[8] of the acids. The efficiency of this procedure has been problematic in our laboratory in certain complex matrices. There are also documented problems of the derivatisation reagent potentially creating artefacts[9].

In this communication we describe a rapid and relatively facile analysis for the detection and accurate quantification of 21 free amino acids in peas using UHPLC-HRAM-MS following simple water extraction. MS is achieved using electrospray ionisation (ESI) in positive mode, without the need for derivatisation. While this method has been developed and

validated for peas, it may be applicable across a wide variety of matrices.

In comparison to Triple Quadrupole LC-MS/MS, LC-HRAM-MS has the advantage of spectral high resolution in combination with high mass accuracy where isobaric matrix effects are minimised. Analyte peaks can therefore be readily distinguished from background signals. Analyte confirmation is achieved using a combination of accurate mass measurement, retention time (against a known analytical standard), isotope pattern and/or library matching. In common with single or triple quadrupole MS detection, quantification of the α-AAs can be achieved by standard addition using both solvent and / or matrix calibration standards.

2. Experimental

2.1 Chemicals and Reagents

All 21 amino acid analytical standards (≥98% purity) and the surrogate standards 5-methyl-DL tryptophan (≥95% purity) and DL-norleucine (≥98% purity) were purchased from Sigma-Aldrich (Gillingham, UK).

Methanol, acetonitrile, water and formic acid for the LC mobile phase were all purchased from Fisher Scientific (Loughborough, UK).

2.2 Extraction conditions

Each pea sample (minimum 5g) was lyophilised following freezing at -20ºC then ground into a fine powder using a pestle and mortar. 10 mL of deionised water was then added to 1 g of pea powder and the sample was shaken for 10 minutes followed by centrifugation at 22,000 x *g* for 10 minutes. The supernatant was then diluted 1:9 (v/v) with deionised water followed by UPLC-HRAM-MS analysis. Total extraction time per sample was approximately 45 minutes.

A 5-point standard addition approach was used for accurate quantification of the acids; 5 replicate sample extracts were fortified with a 21 standard α-AA mixture covering the concentration range 0.09 – 1.2 μg g^{-1} (depending upon the α-AA). Solvent calibration was achieved using 5 concentrations between 0 and 1.6 μg ml^{-1}.

2.3 Ultra High Performance Liquid Chromatography – High Resolution Accurate Mass – Mass Spectrometry Analysis

LC analysis was performed on a Accela High Speed LC system from Thermo Fisher Scientific (Waltham, Massachusetts, USA). The analytical column used was an ACE Excel AQ (Advanced Chromatography Technologies, UK) 150 mm x 3 mm, 100 Å. Mobile phase A (MPA) was 0.1% formic acid in HPLC water, mobile phase B (MPB) was 0.1% formic acid in acetonitrile. Linear gradient elution was used over 10 minutes from 100% MPA to 100% MPB. The gradient was then held for 2 minutes at 100% B before re-equilibration with 100% A for a further 2 minutes.

The LC flow rate was 0.4 ml min^{-1} and the column temperature was 30ºC. Sample injection volume was 5 μl.

The MS used was an Exactive™ Orbitrap™ high resolution mass spectrometer from Thermo Fisher Scientific (Waltham, Massachusetts, USA) with mass resolution of 25,000 at *m/z* 200 and the advanced gain control set to "Balanced." Maximum injection time was 50 ms. Ionisation was by ESI and responses were assessed in both positive and negative mode with sheath gas set to 60 and aux gas at 10 (arbitrary units). The capillary temperature was 350°C. Chromatographic run times were 12 minutes per injection.

ExactFinder and Xcalibur™ software both from Thermo Fisher Scientific (Waltham, Massachusetts, USA) were each assessed for both detection and quantification of the amino acids.

2.4 Validation Parameters

For validation purposes, linearity of response with respect to amino acid concentration (R^2 values) in both solvent and matrix calibrants were recorded. Limits of detection (LOD) in matrix were estimated for each acid based on the signal to noise ratio of the peak of the lowest concentration standard. The difference between estimated LOD and concentration of lowest standard was not greater than 50 fold. Limits of quantification (LOQ) in matrix have also been estimated using a 10:1 signal to noise factor of the peak response in the lowest standard.

To assess extraction efficiency, two surrogate standards (5-methyl-DL-tryptophan and DL-norleucine) were fortified into 5 samples of ground lyphophilised pea at 1 μg g^{-1}.

To assess the mass accuracy of the MS, the detected *m/z* was compared to the theoretical *m/z* based on the chemical formula of each α-AA. For each acid the difference was recorded in parts per million (Δppm).

3. Results and Discussion

Chromatography for all 21 amino acids in both solvent and matrix extracts was achieved. Figures 2, 3 and 4 show peaks for all 21 α-AAs in the same pea extract (data smoothed using 5 point "Boxcar" smoothing), displaying relative retention times. Although chromatographic separation has not been achieved for all compounds the increased selectivity of the accurate mass MS provides sufficient resolution for detection of each individual compound. Figure 4 (top panel) shows chromatographic separation of the critical analyte pair isomers leucine and isoleucine that cannot be differentiated by accurate mass alone.

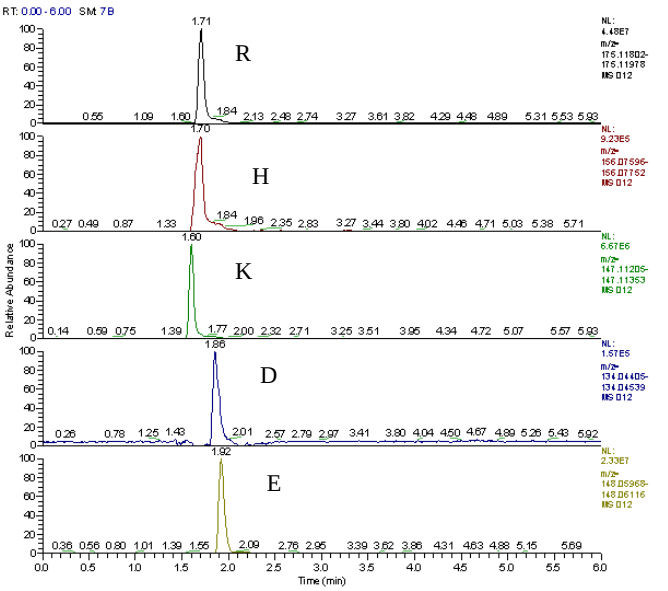

Figure 2. Extracted ion chromatograms for amino acids with charged side chains in pea extract. From top to bottom; Arginine (R), Histidine (H), Lysine (K), Aspartic acid (D) and Glutamic acid (E).

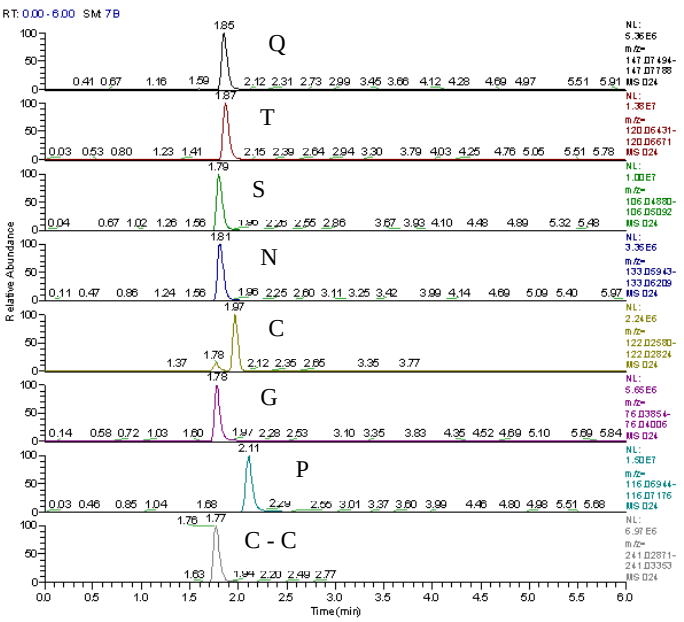

Figure 3. Extracted ion chromatograms for amino acids with polar uncharged side chains and other miscellaneous side chains in pea extract. From top to bottom; Glutamine (Q), Threonine (T), Serine (S), Asparagine (N) , Cysteine (C), Glycine (G), Proline (P) and Cystine (C-C).

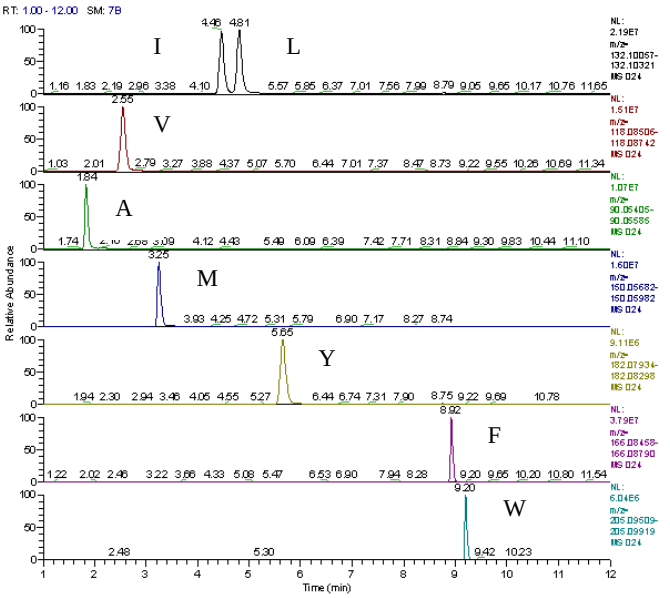

Figure 4. Example extracted ion chromatograms for amino acids with hydrophobic side chains in pea extract. From top to bottom; Isoleucine (I), Leucine (L), Valine (V), Alanine (A), Methionine (M), Tyrosine (Y), Phenylalanine (F) and Tryptophan (W).

Figure 5 shows an example extracted ion chromatogram for tryptophan in pea extract from ExactFinder software (data not smoothed). As can be seen from this figure the added selectivity of accurate mass and high sensitivity of the MS gives excellent signal to noise ratio parameters for all analytes.

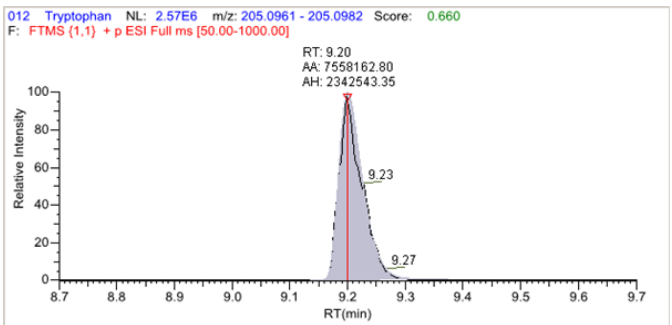

Figure 5. Extracted ion chromatogram of tryptophan (*m/z* 205.09714) in pea extract using ExactFinder software.

Acceptable linearity of standards for quantification of each amino acid was achieved using the method described with R^2 in the range 0.961-0.999. Figure 6 shows an example standard

addition calibration curve in pea for glutamine. Standards were spiked into 5 separate samples covering the range 0 – 0.33 µg g^{-1}.

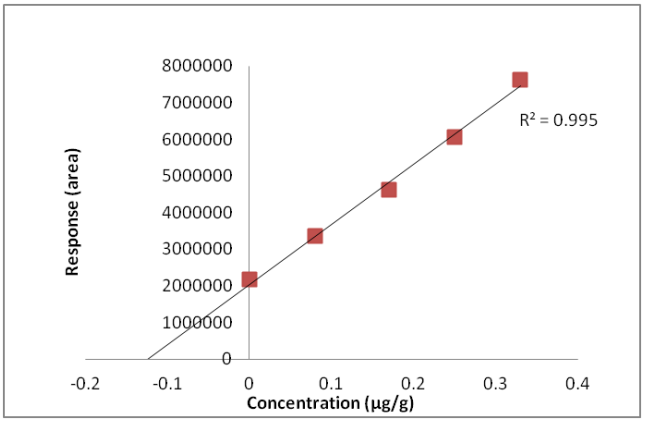

Figure 6. Standard addition calibration curve for accurate quantification of glutamine in pea (0.12 µg g^{-1}). Peak responses collated in Exactfinder software and plotted in Microsoft-Excel (Microsoft Corporation, Washington, USA).

Details of theoretical accurate mass ion $(M+H)^+$, linearity in solvent standards, retention time, Δppm and estimated limit of detection (LOD) for each amino acid are summarised in Table 1.

The extraction efficiency of the method was considered satisfactory with good extraction repeatability between the five replicates. The mean recovery of norleuicne was 109% with 6% RSD between fortified replicates. The mean recovery of 5-methyl tryptophan was 84% with 7% RSD between fortified replicates. Both of these amino acids are non-polar and they served as surrogate standards for the third group of target α-AAs (see Figure 4) i.e. those with a hydrophobic chain. Since the extraction using water was efficient for these two non-polar surrogates then it was assumed that the extraction efficiency was also good for the charged (Figure 2) and the polar (Figure 3) α-AAs of interest. In any case, using the approach of a 5-point standard addition, a good recovery was desirable for sensitivity reasons but the recovery figure is not used in the quantitation calculations.

The sensitivity (LOD) of the method was considered fit for purpose since all α-AAs were detectable in matrix extract at a concentration of less than 30 ng g^{-1}.

The peak response for the majority of analytes was greater in positive ionisation mode than in the negative ionisation mode, as expected with 0.1% formic acid in the mobile phase. The mass accuracy of the M+H ion detected for each α-AA was to within ± 4 ppm. From the 21 α-AAs, only two (glycine and alanine) were outside a ± 2 ppm accuracy window.

Table 1. List of α-AAs with their theoretical accurate mass M+H ion, retention time, R^2 value, difference in measured accurate mass from theoretical and the estimated limit of detection in pea seeds.

Amino acid	$(M+H)^+$	RT (mins)	R^2	Δppm	Estimated. LOD (µg g^{-1})	Estimated. LOQ (µg g^{-1})
Arginine	175.11894	1.71	0.993	-1.14	0.010	0.033
Histidine	156.07674	1.70	0.999	-0.64	0.003	0.010
Lysine	147.11279	1.60	0.999	-0.68	0.004	0.013
Aspartic acid	134.04477	1.86	0.998	-0.74	0.002	0.007
Glutamic acid	148.06042	1.92	0.994	-1.35	0.006	0.020
Glutamine	147.07641	1.85	0.995	-0.68	0.020	0.066
Threonine	120.06551	1.87	0.990	0.84	0.002	0.007
Serine	106.04986	1.79	0.987	0.94	0.002	0.007
Asparagine	133.06076	1.81	0.992	-0.75	0.002	0.007
Cysteine	122.0270	1.97	0.991	0.82	0.001	0.003
Glycine	76.03930	1.78	0.988	3.94	0.015	0.050
Cystine	241.03112	1.77	0.994	-1.24	0.001	0.003
Proline	116.07060	2.11	0.999	0.86	0.001	0.003
Leucine	132.10189	4.46	0.994	-0.76	0.025	0.083
Isoleucine	132.10189	4.81	0.994	-0.76	0.015	0.050
Valine	118.08624	2.55	0.992	0.85	0.007	0.023
Alanine	90.06496	1.84	0.961	2.22	0.004	0.013
Methionine	150.05832	3.25	0.995	-0.67	0.001	0.003
Tyrosine	182.08116	5.65	0.995	-0.55	0.001	0.003
Phenylalanine	166.08624	8.92	0.999	-0.60	0.001	0.003
Tryptophan	205.09714	9.20	0.997	-0.98	0.001	0.003

Conclusions

The use of high resolution accurate mass - mass spectrometry with ultra high performance liquid chromatography has been demonstrated to be an accurate, rapid, selective and sensitive determination method for the analysis of the main 21 amino acids in pea seeds without the need for derivatisation. Following simple, rapid extraction with water all analytes can be detected by high resolution mass spectrometry using a 12 minute liquid chromatography separation, with total analysis time less than an hour per sample. This method has been used routinely in our labs over the past 12 months to analyse and quantify dozens of samples without problems or lengthy maintenance procedures.

Quantitation is achieved using a standard addition approach and limits of detection are less than 30 ng g^{-1}. The extraction efficiency has been evaluated using surrogate standards and is

fit for purpose with good repeatability between extraction replicates.

The mass accuracy of the MS was excellent, with all analytes accurate within ± 4 ppm. Both data processing platforms ExactFinder and Xcalibur™ were suitable for the detection and quantification of the amino acids, although at the time of this work ExactFinder could not be used for calculating standard addition quantification (Thermo Fisher Scientific have a new software TraceFinder™ that can now calculate this).

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Notes and references

[a] *The Food and Environment Research Agency, Sand Hutton, York, YO41 1LZ, UK. E-mail: michael.dickinson@fera.gsi.gov.uk*

[b] *Thermo Fisher Scientific, Takkebijsters 1, 4817 BL Breda, The Netherlands.*

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