Linnæus University
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Bachelor's Thesis

Amino Acid Analysis

Establishment of a GC-FID method for Amino Acid Analysis in Legumes

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Term: VT22
Subject: Chemistry
Level: First Cycle

Abstract

Demands for plant-based diets sufficient in all required amino acids are increasing due to health and environmental advantages. As legumes offer a complementing source of quality protein to the already heavily consumed cereals, methods for determining amino acid compositions in legumes are needed. The aim of this thesis was to establish a GC-FID method for analyzing amino acids in legumes and to apply the method to analyze six locally grown legumes: Black beans, brown beans, red kidney beans, white beans, yellow and grey peas. White bean flour was used as an in-house control sample. Legumes were hydrolyzed with acid and alkaline treatments; hydrolysates were purified and derivatized using an amino acid analysis kit (EZ:faast, Phenomenex) and quantified with GC-FID. All legumes were rich in Asp + Asn, Glu + Gln, Lys and Leu while limited in Met, Cys and Trp. Quality control trials showed a generally good precision of the method (between day CVs were ≤ 10% for 12 of 16 amino acids) and good linearity (r^2 were 0,99 or above for 16 of 19 calibration curves). Limits of detection and quantification ranged from 2-46 nmol/mL and 7-154 nmol/mL, respectively, depending on the amino acid. No accuracy trial was performed. In conclusion, a rapid GC-FID method for amino acid analysis was established and amino acid compositions of locally grown legumes was tentatively determined. However, further quality control trials are required to validate the method.

Sammanfattning

Efterfrågan av växt-baserade dieter med komplett innehåll av aminosyror ökar på grund av både hälso- och miljömässiga fördelar. Då baljväxter erbjuder en kompletterande källa till protein av hög kvalitet till cerealier, som redan konsumeras i hög grad, krävs metoder för att analysera aminosyra innehåll i baljväxter. Målet med detta arbete var att etablera en GC-FID metod för att analysera aminosyror i baljväxter och att applicera metoden för att analysera aminosyror för att analysera sex lokala baljväxter: Svarta bönor, bruna bönor, röda kidney bönor, vita bönor, gula och gråa ärtor. Mjöl på vita bönor användes som in-house kontroll prov. Baljväxterna hydrolyserades med både syra och bas behandlingar, hydrolysaterna renades och derivatiserades med ett kit för aminosyraanalys (EZ:faast, Phenomenex) och kvantifierades med GC-FID. Alla baljväxter hade höga halter av Asp + Asn, Glu + Gln, Lys och Leu men låga halter av Met, Cys och Trp. Kvalitetskontroller visade på en generellt hög precision (CV mellan dagar ≤ 10% för 12 av 16 aminosyror) och god linjäritet (r^2 var 0,99 eller högre för 16 av 19 kalibreringskurvor). Detektions- och kvantifierings-gränser varierade mellan 2-46 nmol / mL respektive 7 – 154 nmol / mL beroende på aminosyran. Inga tester gjordes för att bestämma metodens riktighet. Slutsatsen drogs att en snabb GC-FID metod för aminosyra analys har framtagits och indikativa värden av lokala baljväxters aminosyra innehåll fastställts. Däremot krävs flera kvalitetskontroller för att validera metoden.

Key words

Amino Acids, GC-FID, Hydrolysis, Legumes

Acknowledgments

I would like to thank my supervisors Prof. Dr. Cornelia Witthöft and Assoc. Prof. Dr. Mohammed Hefni for welcoming me into the food-science team and for their great advice. My especial thanks to Assoc. Prof. Dr. Mohammed Hefni for his generosity with his time and energy. Your support and guidance have been tremendous.

Thanks to Dr. Maria Bergström and the Nutrilivs program. You have not only taught me a great deal over these past three years but also helped me develop a deep appreciation for chemistry and food science.

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List of Abbreviations

Amino Acids

Table 1. Amino Acids and their abbreviations

Amino Acid	Abbreviations
Alanine	Ala
Arginine	Arg
Asparagine	Asn
Aspartic Acid	Asp
Cysteine	Cys
Glutamic Acid	Glu
Glutamine	Gln
Glycine	Gly
Histidine	His[1]
Hydroxylysine	Hly
4-Hydroxyproline	Hyp
Isoleucine	Ile[1]
Leucine	Leu[1]
Lysine	Lys[1]
Methionine	Met[1]
Phenylalanine	Phe[1]
Proline	Pro
Serine	Ser
Threonine	Thr[1]
Tryptophan	Trp[1]
Tyrosine	Tyr
Valine	Val[1]

General

Table 2. General Abbreviations

CV	Coefficient of variation
FAO	Food and Agriculture Organization of the United Nations
FID	Flame Ionization Detector
GC	Gas Chromatography
IAA	Indispensable amino acid
IS	Internal Standard
LOD	Limit of detection
LOQ	Limit of quantification
NaOH	Sodium Hydroxide
r^2	Coefficient of determination
SD	Standard Deviation
SPE	Solid Phase Extraction

1 Introduction

To evaluate a food as a source of protein one must not only consider the quantity of protein in the food but also the quality. Methods for determining a protein's quality, such as the commonly used, and by FAO-recommended, PDCAAS (protein digestibility-corrected amino acid score) and DIAAS (digestible indispensable amino acid score) take two factors into consideration: the amounts and relative composition of the indispensable amino acids (IAAs) as well as the digestibility. IAAs are amino acids which the human body requires for normal physiological function yet is unable to synthesize either at all or in sufficient amounts and must consequently be supplied through the diet. The quality of a protein source is therefore a measure of its ability to provide the human body with the necessary amino acids, and a lower quality protein source will have limited amounts of some or all IAA(s) (1–4). The amino acids that are indispensable are: His, Ile, Leu, Lys, Met, Phe, Thr, Trp and Val (1).

Animal-based products represent a major source of protein. While this is especially true in the developed countries of the West, meat consumption in developing countries is also on the rise (5–7). As both health and environmental advantages have been linked to a more plant-based diet, and as plant-proteins typically are of a lower quality than meat, especial care is needed to ensure that intake of all IAA(s) remains adequate during a transition to plant-based foods (1,5,6). Knowledge of the amino acid composition of plant-proteins is needed to determine their quality as protein sources. Thus, in order to construct plant-based diets sufficient in all IAA(s), robust methods for analyzing amino acids are required.

To analyze proteins, amino acids first need to be isolated, which is done by hydrolyzing the proteins. Once the amino acids have been freed a subsequent separation and quantification is required. Both hydrolysis and the following separation are complex procedures as various hydrolyzing agents and conditions as well as separation techniques can be used, all with respective advantages and disadvantages. Additionally, depending on the method additional sample preparation such as purification and derivatization may also be required (2,3,8). Methods for amino acid analysis is discussed in further detail in section 1.2.

1.1 Amino Acids in Legumes

Plant foods, especially cereals, account for the highest portion of global protein intake (5,7). However, cereals are as most plant foods limited as quality protein sources due to inadequate amounts of some amino acids: typically lysine, tryptophan, and threonine for cereals (1,6). While animal-based protein, such as meat and dairy are of high quality and consumption is high in developed countries and increasing in developing countries, animal-protein sources have limitations as a sustainable sole source of protein to satisfy the need of the current, and future, world population (5–7,9). Legumes offer an alternative or complementary protein source to meat and pairs well with cereals. Although legumes, like cereals, have a limited amino acid composition the two food sources' respective amino acid composition are complementary. Legumes are rich in lysine but contain limited amounts of the sulfuric amino acids methionine and cysteine, which cereals in turn are rich in (1,6,7). Legumes are additionally a good protein source due to high quantities of protein as

typical contents range from 20 to 30% in dried legumes. Comparatively, usual protein content in cereals range from 6 to 15% (6,7).

As amino acid composition differs between legume varieties, amino acid analysis of the legume of interest is needed for accurate data (1,10). Additionally, amino acid composition of a legume further varies due to variations of different factors such as the cultivar used, soil composition and precipitation levels, germination, and use of fertilizer as well as other geographical, climatic, and agricultural differences (1,10). For instance, a study that analyzed amino acid compositions of soybeans grown in different regions of Brazil found an average variation from the mean of 2,1 percentage points for IAAs and 3.3 percentage points for non-essential amino acids with variations as high as 8 percentage points for individual amino acids (11). Since amino acid composition varies depending on growing conditions of the legume, geographical, climatic and agricultural differences as well as the cultivar used, each country should therefore, if possible, assess amino acid composition of local legumes instead of relying on previously determined values for another region.

1.2 Analysis of Amino Acids

The analysis of amino acids can be divided into two main parts: Hydrolysis of the peptide/protein to free the amino acids and a subsequent separation and quantification of the released amino acids (12,13). The importance of a successful hydrolysis cannot be overemphasized as it is crucial for accurate quantification, and variations of an amino acid composition are often caused by unsuccessful hydrolysis (12). Despite its importance no method exists that allows full recovery of all amino acids (12,14) and the hydrolysis method thus have to be chosen according to the targeted amino acid(s) (15).

Methods for hydrolyzing proteins can either be chemical, by acid or alkaline treatment, or enzymatic. All methods offer their own advantages and disadvantages, yet no method can recover all amino acids. The amino acids which can be recovered differ between methods. A method can either be applied on its own, thereby limiting the analysis to the method's limitations, or together with complementing methods increasing the number of amino acids that can be analyzed (2,12,16).

Acid hydrolysis is the most commonly applied hydrolysis method as it allows for full recovery of many amino acids in foods (2,8,14,16). Different conditions of acid hydrolysis applied to food samples are summarized in table 3, where vapor-or liquid-phase 6M hydrochloric acid (HCl) hydrolysis at 110°C for 24 hours with vacuum or nitrogen atmosphere with or without added phenol are common. Incubating samples with hydrochloric acid (HCl) at 110°C for 24 hours after removing oxygen has been the conventional acid hydrolysis method since its development during the 1950s. Although developments such as micro-wave assisted hydrolysis resulting in a considerable reduced hydrolysis time have been made, the conventional method still remains, and is likely to remain, as the standard method for amino acid analysis (2,8,16,17). HCl is a versatile hydrolysis agent as it is volatile, allowing evaporation of the acid post-hydrolysis and for HCl to be used both for liquid- and gas-phase hydrolysis (12,15).

While the conventional acid hydrolysis allows for full recovery of many amino acids it results in either a partial or full conversion or destruction of some amino acids (8,16,18). Asparagine (Asn) is fully converted to aspartic acid (Asp) and glutamine (Gln) to glutamate (Glu) and can thus not be analyzed by acid hydrolysis, and alternative methods using enzymatic hydrolysis are needed for quantification (12,16,18). The sulfuric amino acids cysteine and methionine are also affected by acid hydrolysis, as cysteine is converted to cystine and methionine oxidized to methionine sulfoxide and methionine sulfone (2,16,18). Quantification of the sulfuric amino acids are thus often oxidized with performic acid prior to acid hydrolysis with hydrochloric acid and analyzed as methionine sulfone and cysteic acid respectively (16,18–21). However, pre-treatment with performic acid adds additional labor-extensive and expensive steps to the process (18,22). Furthermore, the amino acid analysis kit EZ:faast, supplied by Phenomenex, used in this study for sample clean-up and derivatization of the amino acids is unable to quantify cysteic acid (23). Consequently, another method than pre-treatment with performic acid is needed for quantification of cysteine and methionine. Removing oxygen from the hydrolysis tube, by creating vacuum or flushing with nitrogen, prior to a conventional hydrolysis with hydrochloric acid has been shown to successfully recover and analyze both methionine and cysteine (16,19,22,24,25). Removing oxygen and performing a conventional hydrolysis might therefore offer a faster, easier, and cheaper alternative for quantification of the sulfuric acids than pre-treatment with performic acid.

Furthermore, tryptophan (Trp) is completely destroyed by the standard acid hydrolysis. While some protective substances such as thiols and phenol can be added to increase acid hydrolysis' recovery of Trp (16,26) Trp is often analyzed by alkaline hydrolysis as a complement to the acid hydrolysis (2,12,16). Alkaline hydrolysis is specifically common for tryptophan analysis of carbohydrate rich samples and is thus often applied for analysis of foods (12,19,27–30). Different conditions of alkaline hydrolysis applied for food samples are summarized in table 3. In all presented literature NaOH (4,2-5 M) is used as hydrolyzation agent incubated at 100-120°C for 4-26 hours at vacuum or nitrogen atmosphere. Due to partial destruction of other amino acids including Arg, Asn, Cys, Gln, Met, Ser and Thr an alkaline hydrolysis is however not applied for quantification of amino acids other than Trp (12,18).

Table 3. Methods for acid and alkaline hydrolysis.

Hydrolysis Conditions	Food Matric	Analyzed amino acids	Limitations	Ref.	Comments
Acid Hydrolysis					
24h gas-phase hydrolysis with 6M HCl at nitrogen atmosphere with 1% phenol with 0.1% phenol	Soybean , egg-white for validation Wheat flours	Ala, Arg, Asp + Asn, Cys + half cysteine, Glu + Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val	Measures Asp + Asn, Glu+ Gln and Cys + half-cysteine as units. Cannot measure Trp.	(22) (24)	Overall good results from validation (22). Doesn't require pre-treatment with performic acid to measure Cys and Met. Allows for Cys and Met to be measured together with the bulk of the amino acids.
24h gas-phase hydrolysis with 6M HCl	Lotus seeds	Ala, Arg, Asp + Asn, Cys, Glu + Gln, Gly, His, Ile, Leu, Lys, Met, Phe, Pro Ser, Thr, Tyr, Val,	Measures Asp + Asn, Glu+ Gln. Cannot measure Trp	(31)	Analyzes Trp with NaOH hydrolysis, but hydrolysis conditions unspecified. Successfully analyzes Cys and Met without performic acid treatment
24h hydrolysis with 6M HCl at nitrogen atmosphere	Roselle Seeds	His, Ile, leu, Lys, Met, Cys, Phe, Tyr, Thr, Val	Only analyzes indispensable amino acids.	(25)	Successfully analyzes Cys and Met without performic acid treatment
~2h (Including warm up and cool down, 10 min hydrolysis at 160°C) microwave-assisted hydrolysis with 6M HCl and 0.5% Phenol at 160°C, vacuum.	Quinoa, Amaranth, Buckwheat and Rice	Ala, Arg, Asp, Cys, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Tyr, Val	Cannot measure Asn, Gln or Trp. Requires special microwave oven	(17)	Offers an alternative, faster, hydrolysis method allowing for a substantially faster hydrolysis and thus analysis.
24h hydrolysis with 6M HCl at 110°C under vacuum with 0.1% phenol added.	Goat Milk Formulations	Ala, Arg, Asp, Cys[1], Glu, Gly, His, Ile, Leu, Lys, Met, Met[1], Phe, Pro, Ser, Thr, Tyr, Val	Cannot measure Asn, Gln or Trp. Cys must be treated with performic acid prior to hydrolysis	(19)	Analyzed methionine with and without pre-treatment with performic acid. Methionine measured without pretreatment gave similar or better values than methionine treated with performic acid, as average difference between the methods were 0,5% for methionine but 18,7% for methionine sulfone (19). Indicates that methionine can be measured without pre-treatment and with, possibly, better results.

24h hydrolysis with 6M HCl at 110°C, nitrogen atmosphere	Chickpea Duck	Ala, Arg, Asn, Cys[1], Glu, Gly, His, Ile, Leu, Lys, Met[1], Phe, Pro, Ser, Thr, Tyr, Val	Cannot measure Asn, Gln or Trp. Cys must be treated with performic acid prior to hydrolysis	(20) (21)	
Alkaline Hydrolysis					
26h hydrolysis with 4,2M NaOH at 110°C , vacuum	Sufu (Chinese fermented Soybean)	Trp	Only used to measure Trp.	(30)	
12h hydrolysis with 5M NaOH at 120°C at nitrogen atmohsphere	Cereals and Legumes	Trp	Only used to measure Trp.	(27)	The authors tested variations of the hydrolysis time (4h, 12h, 16h and 24h). Recovery was lowest for 4h but the other durations gave similar results (27)
4h hydrolysis with 4M NaOH at 100°C at nitrogen atmosphere	Feedstuff (Seed flour)	Trp	Only used to measure Trp.	(29)	An later article comparing variations of hydrolysis times found less recovery of Trp from 4h hydrolysis compared to longer (27)
20h hydrolysis with 4,2M NaOH at 110°C, nitrogen atmosphere	Kinema	Trp	Only used to measure Trp.	(28)	

[1]Oxidized with performic acid prior to hydrolysis

There are multiple methods that may be used for separation and quantification of free amino acids including ion exchange chromatography, capillary electrophoresis, high pressure liquid chromatography and gas chromatography (GC). Reverse-phase high pressure liquid chromatography (RP-HPLC) and automated cation-exchange chromatography, often referred to as amino acid analyzers, are commonly used. Methods for amino acid analysis using GC equipped with various detectors are however also well established and offers precise, accurate and sensitive analysis, typically more accurate than liquid chromatography and ion-exchange chromatography (2,3,16,32). Due to the nature of gas chromatography extensive preparations are however required prior to injection such as sample preparation, isolation and, as is the case with amino acids, derivatization (3,33). Since samples are injected onto the GC at high temperatures substances must be thermally stable and volatile to enable analysis and to further avoid degradation of non-volatile substances and the resulting false peaks of degradation products. Additionally, to achieve sufficient separation substances must also have a low polarity. Amino acids are both highly polar and non-volatile and must be derivatized to counteract this (33). Silylation is the most frequently used derivatization agent, but derivatives are susceptible to humidity and further causes instability of Arg and Glu derivatives. Alternative methods for derivatization exist, such as chloroformate mediated indirect alkylation of amino acids in solutions containing pyridine and alcohol, allowing rapid derivatization as well as fast subsequent GC-FD-analysis of as low as 6 minutes. As Arg is retained on the column Arg can however not be quantified using this method (3,8,34).

Purification of amino acids is essential to maximize performance. Methods for purification include solid-phase extraction (SPE) and liquid-liquid extraction. SPE is typically performed by drawing a liquid sample, containing the compound of interest, through a chromatographic packed column. Various materials can be used for the chromatographic packing and the material chosen should have an affinity for the analyte. When passing liquid through the column compounds having affinity to the column will be retained in the packing while other substances pass through the column unhindered. After all liquid and compounds lacking affinity has been removed from the column the analyte, amino acids in this case, can be extracted with an eluting medium. A liquid – liquid extraction on the other hand consists of separating compounds into two liquid phases: of which one phase is polar and the other non-polar. During sample preparation for GC this is typically done to isolate volatile substances (33). While amino acids, due to high polarity, typically are found in the polar phase they will, after chloroformate derivatization, be found in the solvent phase from where they can be extracted and injected into the GC (33,34).

GC-analysis can be run equipped with either a packed or capillary column. While packed columns were initially popular, improvements to capillary columns have since then made capillary columns the most used column due to quick run times and high resolutions. Almost all capillary columns are constructed of fused silica coated with a liquid stationary phase (33,35) and has been successfully applied to quantify amino acids in food (3). Different stationary phases may be used based on the analyte and

its polarity. Various column lengths and diameters can additionally be used and offers different advantages. While special lengths and diameters can be used to achieve desired condition, such as especially fast, extra high resolutions or high capacity analyses, columns are typically 15-60 meters long with internal diameters of 0.25 – 0.32 mm as it offers the best combination of all factors (33,35). Gas chromatography utilizes gases (nitrogen, helium, or hydrogen) as the mobile phase, or carrier gas. All three gases offer different advantages and disadvantages. Nitrogen allows for the high resolution, yet slow, analysis. Both helium and hydrogen offer significantly quicker run times while only slightly less efficient. While hydrogen has some advantages over helium it is also flammable and may interact with analytes (33). GC equipped with a mass spectrometry (MS) is the most utilized gas chromatography method (8,32) however a flame ionization detector (FID) may also be used for rapid amino acid analysis with high accuracy (2,3,36). Detection with FID is done by measuring organic ions released when organic compounds are burned in the detectors' flame, making it a highly suitable detector for food analysis as most food analytes are organic (33).

This study utilizes the EZ:faast amino acid analysis kit supplied by Phenomenex for sample clean-up and derivatization. In the kit a column for amino acid analysis and recommended settings for the GC is also included. The kit, compromised of a solid-phase extraction, derivatization and a liquid-liquid extraction offers a quick sample preparation enabling a high-throughput when coupled with the 8 minute run time of the GC (23).

1.3 Aim of the Study

The aim of this study is to establish a GC-FID method for amino acid analysis of legumes using the Phenomenex EZ:Faast amino acid analysis kit for sample clean-up and derivatization, to assess parameters for acid and alkaline hydrolysis and to apply this method to analyze amino acid compositions of some common Swedish legumes.

2 Materials and Method

2.1 Chemicals and Reagents

Hydrochloric acid (37%) and Sodium Hydroxide were of p.a grade and were purchased from Sigma-Aldrich (St. Louis, USA). Water was purified using a MilliQ Water Purification system (Merck Millipore, USA)

Reagent 1 (Internal Standard solution containing 0,2 mM Norvaline, 10% n-propanol and 20 mM HCl), Reagent 2 (Sodium carbonate), Reagent 3a (Eluting Medium Component I containing 0,33M NaOH), Reagent 3b (Eluting Medium Component II containing 80% n-propanol and 20% 3-picoline), Reagent 4 (Organic Solution I containing 60% chloroform, 20% propyl-chloroformate and 20% octane), Reagent 5 (Organic Solution II containing Iso-octane), Reagent 6 (Acid solution containing hydrochloric acid 1M), Amino Acid Standard Mixtures (for composition see 2.3.3.5) were all included in the EZ:Faast kit supplied by Phenomenex.

2.2 Food Samples

Black beans (*Phaseolus vulgaris,* Zorro), red kidney beans (*Phaseolus vulgaris,* Montclam), brown beans (*Phaseolus vulgaris,* Katja), yellow peas (*Lathyrus aphaca,* Clara), grey peas (*Cajanus cajan,* Latvian variety) and white beans (*Phaseolus vulgaris,* T9905) were all donated from Kalmar-Ölands Trädgårdsprodukter and produced locally (Kalmar/Öland). All samples were from the 2016 harvest year and stored at room temperature in their original packages. Before analysis all food samples were milled using the Retsch Ultra Centrifugal Mill ZM 200 with a 0.5 mm ring sieve. Milled food samples were stored in the freezer, except for the white beans which were stored in the fridge.

The protein content (table 4) of all legumes samples has been determined previously (37) in Sandra Ohlströms Bachelors Thesis in Chemistry ‘’Betain, kolin och protein i baljväxter från Öland med olika skördesår’’ in the spring of 2017.

Table 4. Protein content of all food samples (g protein / 100 g sample), harvest year 2016).

Legume	Gram protein / 100 gram sample
Black Beans	22
Red Kidney Beans	28
Brown Beans	19
Yellow Pea	21
Grey Pea	22
White Bean	26

2.3 Quantification of Amino Acids in Legume Samples

2.3.1 Acid Hydrolysis

6 mL of 6M HCl was added to 225 mg of food sample (n=2), flushed with nitrogen for a minute before capping the bottle The bottle was placed in an oven at 110°C for 24 hours. During the 24-hour period the bottle was shaken twice: Once two hours after insertion and then again 19 hours after insertion. After 24 hours the tube was taken out of the oven and allowed to cool down to room temperature. The weight was adjusted to 10 grams with milliQ-water to compensate for evaporation during the hydrolysis to standardize results. 1 mL was transferred to a 10 mL volumetric flask and the volume adjusted to 10 mL with milliQ-water. 1 mL of the solution was filtered using a 0.45 µmfilter (0.45 µm pore size, polypropylene membrane, 13 mm diameter, Captiva Econofilter, Agilent Technologies, Santa Clara, USA). 200 µL of the filtered extract was transferred into another tube and the pH adjusted to 1.5 - 5 by adding 25 µL of Reagent 2 and controlled with pH-paper. 50 µL of the solution was pipetted into a sample preparation vial and then purified and derivatized according to steps 2.3.3.

2.3.2 Alkaline Hydrolysis

2 mL of 4.2M NaOH was added to 90 mg of food sample (n=2), flushed with nitrogen gas for a minute before capping the bottle. The bottle was placed in an oven at 105°C for 20 hours. During the 20-hour period the bottle was shaken twice: Once two hours

after insertion and then again 15 hours after insertion. After 24 the tube was taken out of the oven and allowed to cool down to room temperature. The weight was adjusted to 5 grams with milliQ-water to compensate for evaporation during the hydrolysis to standardize results. To neutralize the solution 2 mL of 4M HCl was then added to the tube. Thereafter, 1 mL of the solution was added to an Eppendorf vial which was centrifuged for 5 minutes at 10 000 rpm. 1 mL of the supernatant was filtered using a 0.45 µM filter (0.45 µM pore size, polypropylene membrane, 13 mm diameter, Captiva Econofilter, Agilent Technologies, Santa Clara, USA). 50 µL of the solution was pipetted into a sample preparation vial and then purified and derivatized according to step 2.3.3.

2.3.3 Purification and derivatization

Purification and derivatization were done in accordance with the EZ:faast manual, with the exception that reaction times during 2.3.3.2 were extended from 60 to 90 seconds.

2.3.3.1 Solid Phase Extraction

A fresh elution medium was prepared prior to use by mixing an appropriate volume (200 µL/sample) of Reagent 3a & Reagent 3b in a 3:2 ratio.

100 µL of Reagent 1, containing the Internal Standard (IS) Norvaline, was pipetted into each sample vial. Sample was slowly drawn through a sorbent tip, followed by 200 µL of milliQ water. Air was thereafter drawn into the tip until dryness. Sorbent contained amino acids were then removed from the tip using 200 µL of elution medium.

2.3.3.2 Derivatization and liquid-liquid extraction

50 µL of Reagent 4 was first added to each sample vial. The liquid was emulsified by vortexing the vial for a few seconds until the liquid turned white and allowed to react for 90 seconds. The liquid was then re-emulsified by vortexing for a few seconds and then allowed to react for another 90 seconds. 100 µL of Reagent 5 was then added to the vial and the liquid vortexed. After an additional reaction time of 90 seconds 100 µL of Reagent 6 was pipetted into the vial and the sample vortexed. 30 µL of the liquid in the emulsions' upper layer was then transferred to an autosampler vial which was in turn applied to the GC and run according to 2.3.4

2.3.4 Quantification using GC-FID

Amino acids were quantified using an Agilent 7980B GC-system equipped with a splitless/split injector used in split mode, a Zebron PLUS Liner for Agilent & Thermo (4mm ID Single-Taper Z-Liner) and a Flame-Ionization Detector (GC-FID). Separation was done using a 10m x 0.25 mm ID Zebron ZB-AAA GC column for amino acid analysis supplied by Phenomenex with helium as the carrier gas.. 2 µL of sample was injected at a 1:10 split and injection temperature of 250°C. The initial oven temperature was set at 110°C and was gradually increased to 320°C at a rate of 32°C/min during the 7,6-minute run. The FID operated at 320°C and at detector flow rates of 400, 40 and 25 mL/ min for air, hydrogen gas and helium respectively.

Injections with different split injections and injection temperatures were tried while establishing the method: 1:5 and 1:10 splits at 200°C as well as 1:10 and 1:15 at 250°C. To find the most effective split conditions a solution of the amino acid standard mixture (see 2.3.5) with a concentration of 100 nmol / mL was prepared and ran with all four settings. The 1:10 split injected at 250°C was found to be most effective and was used for preparing the calibration curve and for quantification of amino acids in food samples.

2.3.5 Calibration Curve

The amino acid standard mixtures supplied by Phenomenex in the EZ:faast kit were used to create calibration curves for the supplied amino acids. 19 amino acids were included in the mixture: Ala, Asp, Cys, Glu, Gly, His, Hly, Hyp, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val.

Data for the calibration curve was acquired as instructed by the EZ:faast manual. Three levels were prepared by adding 25, 50 and 100 µL (corresponding to a final concentration of 50, 100 and 200 nmol of each amino acid / mL) to three sample preparation vials and then purified and derivatized samples according to the procedure in 2.3.3. Blank samples consisted of iso-propanol. After manually integrating peaks from the GC the peak areas for all amino acids were used for the calibration curve. Before using the data, all peak areas were divided by the area of the internal standard to improve the procedures' reproducibility.

The calibration curves were constructed using averages from two sets of calibration samples prepared and run on two separate days (n=2, duplicate analyses and duplicate injections).

2.4 Quality Control of Analytical Method

Milled white beans (*Phaseolus vulgaris,* T9905) were used as the in-house control sample to determine within-day and between-day variations for the acid hydrolyzed samples. The milled white beans were stored in the fridge. Fresh samples were hydrolyzed and prepared in duplicate for each occasion (n=3). Within-day variations were determined by the average CV of samples prepared the same day and between-day variations by the CV of the average concentrations of each day.

The signal limit of detection (yLOD) and quantification (yLOQ) was determined from the calibration curve for each individual amino acid using their respective calibration curves by multiplying the standard error of regression ($S_{y/x}$) by 3 and 10, respectively, and then adding the value of the Y-intercept. Concentration limit of detection (LOD) and quantification (LOQ) were interpolated to nmol / mL using each amino acid's respective calibration curve.

2.5 Statistical Analysis

Data were presented as mean±SD. Standard deviation (SD), coefficient of variation (CV) and coefficient of determination (r^2) were determined using Microsoft Excel. Calibration curves were also constructed using Excel. By using the data from the calibration curves the food samples respective amino acid concentrations were

determined using the interpolation feature of GraphPad Prism, and to determine each standard curves' $S_{y/x}$ and Y-intercept.

Standard deviations calculated from two values are limited as the determined value will be a measure of the spread of the two values which may not reflect the actual standard deviation. SD values in this study are thus only used as an indicator of the deviation between samples.

3 Results

3.1 Calibration Curves

All 19 amino acids included in the standard mixture and norvaline (IS) were successfully separated and identified. A chromatogram prepared at level 200 nmol / mL showing all amino acids is displayed in figure 1.

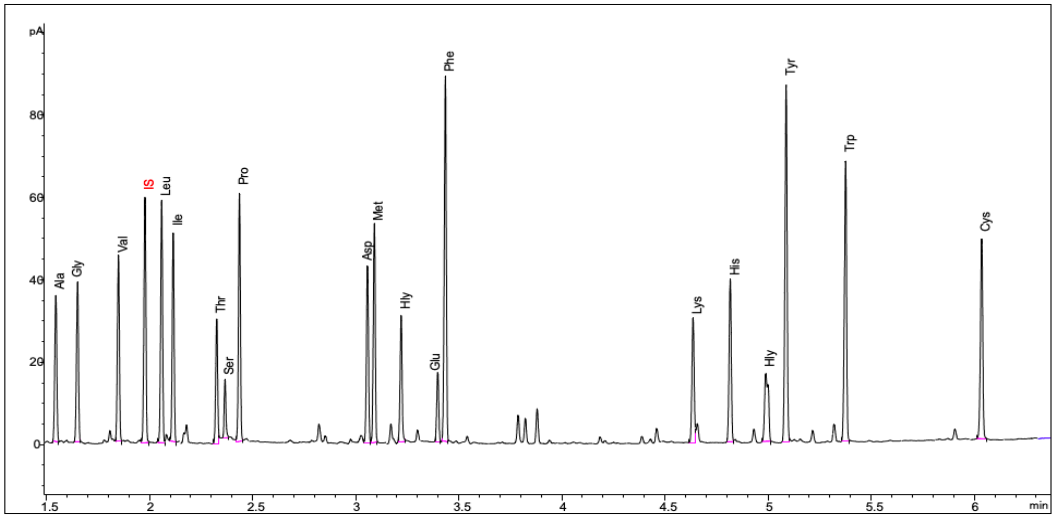

Figure 1. Chromatogram of amino acid standard (level 200 nmol / mL) showing all 19 amino acids included in the EZ:faast kit,. as well as IS Norvaline, marked red in the figure.

Coefficient of determination (r^2) values for all calibration curves and coefficient of variation (CV)-values for all three calibration levels of all amino acids are presented in table 5. All r^2-values for the calibration curves prepared from averages (n=2) were 0,99 or above for all amino acids except three: His (0.988), Hyp (0,979) and Hly (0.979). CV between the two sets of data for the respective concentrations were $\leq$ 10% for most amino acids and concentrations, with ten exceptions of which His 100 nmol / mL was the highest.

Table 5. r^2-value for the calibration curves and CV% values for the average peak area / area of internal standard for all measured amino acids at concentrations 50, 100 and 200 nmol / mL (n=2).

Amino Acid	r^2	Equation	50 nmol / mL CV(%)	100 nmol/ mL	200 nmol/ mL
Ala	1,000	Y = 0,0031*X + 0,0018	3	1	4
Asp	0,997	Y = 0,0037*X + 0,0009	3	18	9
Cys	0,998	Y = 0,0046*X - 0,0157	2	2	11
Glu	0,999	Y = 0,0015*X - 0,0022	0	23	5
Gly	1,000	Y = 0,0032*X + 0,0029	4	3	8
His	0,987	Y = 0,0032*X - 0,0229	11	34	27
Hly	0,979	Y = 0,0026*X - 0,0211	5	1	14
Hyp	0,979	Y = 0,0024*X - 0,03000	4	4	2
Ile	0,999	Y = 0,0038*X + 0,0088	5	1	16
Leu	1,000	Y = 0,0048*X + 0,0067	0	4	7
Lys	0,999	Y = 0,0029*X - 0,0031	9	9	5
Met	1,000	Y = 0,0044*X - 0,0002	0	1	9
Phe	0,999	Y = 0,0074*X + 0,0074	3	4	8
Pro	0,999	Y = 0,0047*X - 0,0052	1	6	14
Ser	0,991	Y = 0,0009*X - 0,0019	4	3	9
Thr	0,993	Y = 0,0024*X - 0,0110	10	6	10
Trp	0,997	Y = 0,0060*X - 0,0259	10	13	17
Tyr	1,000	Y = 0,0080*X + 0,0111	8	0	10
Val	1,000	Y = 0,0035*X + 0,0004	1	5	11

3.2 Quality Control of Analytical Method

Limit of Detection (LOD) was determined to values between 2 -16 nmol / mL for 13 of the 19 amino acids, and for the remaining six to a range of 18-46 nmol / mL of which Hly and Hyp had the highest values (49 nmol / mL). Limit of Quantification (LOQ) for the 13 amino acids was determined to a range of 7 – 53 nmol / mL and 60 – 154 nmol / mL for the other six of which Hly and Hyp had the highest values (165 nmol / mL). All LOD and LOQ values are presented in table 6. When expressed as g / 100 g methionine had a LOD of 0.05-0.07 g / 100 g protein depending on the legume.

Within day variations (3*(n=2)) were ≤ 10% for 11 amino acids and 11 and 12% for His and Met respectively. Higher variations were found in Glu + Gln (21%), Lys (23%) and Ser (46%). Between day variations (n=6) were ≤ 10% for 12 amino acids. Thr and Lys varied with 13% and 20% respectively and highest variations were found in Glu-Gln (32%) and Ser (39%). Within- and between-day variations for all amino acids are summarized in table 7.

Table 6. LOD and LOQ-of analyzed amino acids, nmol / mL (n=2)

Amino Acid	LOD nmol / mL	LOQ
Ala	3	8
Asp	16	53
Cys	13	45
Glu	9	29
Gly	3	11
His	37	122
Hly	46	154
Hyp	46	154
Ile	8	28
Leu	6	21
Lys	10	33
Met	2	7
Phe	9	30
Pro	10	32
Ser	31	102
Thr	27	89
Trp	18	60
Tyr	5	18
Val	6	19

Table 7. With-in Day Variation and Between Day variation (n=6) of the in-house control sample.

Amino Acid	With-in Day Variation CV (%)	Between Day Variation
Ala	7	8
Asp + Asn[1]	10	7
Cys	4	2
Glu + Gln[1]	21	32
Gly	7	5
His	11	6
Ile	9	9
Leu	6	8
Lys	23	20
Met	12	5
Phe	7	10
Pro	6	6
Ser	46	39
Thr	6	13
Tyr	9	10
Val	9	5

[1]Asn and Gln are completely converted to Asp and Glu, respectively, and are therefore presented as a pair.
[2]Hly and Hyp were not identified by the GC and could not be quantified.
[3] Due to late changes to parameters of the alkaline hydrolysis with-in day and between day variations could not be determined due to lack of data.

3.3 Amino Acids in Legumes

16 of the 19 amino acids were detected in samples prepared by acid hydrolysis and used for quantification. Hly, Hyp and Trp peaks were not detected in any of the acid hydrolyzed samples. Additionally, Cys was not detected in one duplicate sample of brown bean sample and one duplicate sample of grey pea, and Ser in one of the red kidney bean duplicate samples.

Trp was detected in all of samples prepared by alkaline hydrolysis and used for quantification.

Chromatograms for white bean prepared by acid (top) and alkaline treatment (bottom) are shown in figure 2. All chromatograms for all legumes prepared by acid treatment can be found in supplementary figures 1a and 1b of the appendix, and legumes prepared by alkaline treatment in supplementary figures 2a and 2b.

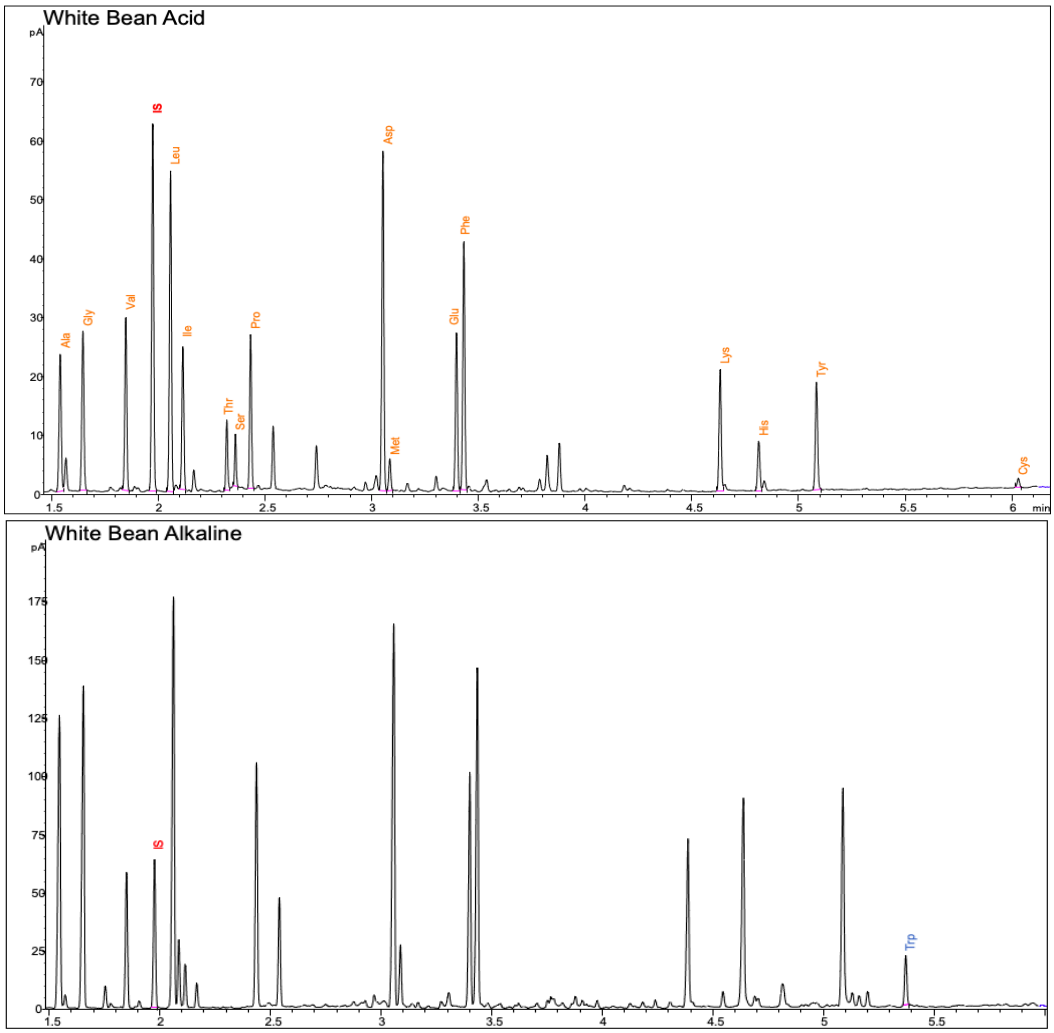

Figure 2. Chromatograms of white bean prepared by acid hydrolysis (upper) and alkaline hydrolysis (lower). All peaks used for quantification in the acid sample are marked with the associated amino acid in orange, peaks in alkaline samples in blue. IS marked is red.

The amino acid composition of analyzed legumes are presented in table 8. Glutamic acid and glutamine (Glu+Gln), measured together, were most in abundance in all legumes ranging from 5.4 g/ 100 g protein in black beans to 9.3 g / 100 g protein in white beans. High values of aspartic acid + asparagine (Asp + Asn), leucine (Leu) and lysine (Lys) of 4.5 – 6.0, 4.0 – 5.2 and 3.1 – 4 g / 100 g protein, respectively, were also found. Lowest amounts were of tryptophan (Trp), methionine (Met) and cysteine (Cys) at 0.4 – 0.6, 0.5 – 0.6 and 0.3 – 0.4 g / 100 g protein respectively. Concentrations of hydroxylysine (Hly) and hydroxyproline (Hyp) could not be determined as they were not identified by the GC. Furthermore, Cys, His, and Thr-concentrations for all legumes were below their respective LOQ-values as well as all Ser-concentrations except for white and red kidney beans.

Table 8. Amino acid content (mean (SD), g/100g protein) in legumes (n=2).

Amino Acid[3]	Black Bean	Brown Bean	Red Kidney Bean	White Bean	Grey Pea	Yellow Pea
	Mean (SD) g / 100 g protein					
Ala	2.1 (0.2)	2.7 (0.2)	2.2 (0)	2.3 (0.1)	2.3 (0.4)	2.4 (0.2)
Asp+Asn[1]	4.9 (0.2)	6.0 (0.8)	4.5 (0.5)	6.0 (0.5)	5.0 (0.8)	5.6 (0.7)
Cys	0.3 (0.0) [5]	0.4 (-)[2, 5]	0.3 (0.0)[5]	0.3 (0.0)[5]	0.4 (-)[2, 5]	0.4 (0.0)[5]
Glu+Gln[1]	5.4 (0.7)	6.5 (0.3)	5.7 (0.7)	9.3 (1.0)	7.4 (0.3)	6.6 (0.5)
Gly	2.0 (0.1)	2.2 (0.6)	2.0 (0.1)	2.1 (0.0)	2.2 (0.5)	2.6 (0.3)
His	2.0 (0.1)[5]	2.3 (0.4)[5]	2.0 (0.3) [5]	2.0 (0.1) [5]	1.8 (0.3)[5]	2.0 (0.2)[5]
Ile	2.7 (0.1)	2.9 (0.7)	2.7 (0.3)	2.5 (0.1)	2.3 (0.6)	2.9 (0.6)
Leu	4.4 (0.3)	5.2 (1.0)	4.9 (0.6)	4.6 (0.1)	4.0 (1.1)	4.6 (0.7)
Lys	3.1 (0.2)	3.8 (0.1)	3.3 (0.1)	3.9 (0.4)	4.0 (0.5)	3.4 (0.1)
Met	0.5 (0.0)	0.6 (0.1)	0.6 (0.0)	0.6 (0.0)	0.5 (0.1)	0.5 (0.0)
Phe	2.8 (0.2)	3.2 (0.9)	3.3 (0.6)	2.9 (0.1)	2.4 (0.7)	2.9 (0.5)
Pro	2.0 (0.1)	2.3 (0.3)	1.9 (0.0)	2.0 (0.0)	2.2 (0.6)	2.6 (0.4)
Ser	3.0 (0.2)[5]	2.1 (0.9) [5]	3.13 (-)[2]	3.4 (1.2)	1.2 (0.2)[5]	1.9 (0.5)[5]
Thr	2.1 (0.1) [5]	2.0 (0.4) [5]	1.8 (0.2)[5]	2.0 (0.2)[5]	1.5 (0.3)[5]	1.7 (0.3)[5]
Trp[4]	0.5 (0.1)	0.4 (0.0)	0.6 (0.2)	0.4 (0.1)	0.4 (0.0)	0.4 (0.0)
Tyr	1.8 (0.7)	1.3 (0.3)	1.5 (0.4)	1.5 (0.2)	1.4 (0.6)	1.6 (0.1)
Val	3.1 (0.2)	3.5 (0.6)	3.1 (0.2)	3.1 (0.1)	2.7 (0.6)	3.2 (0.1)

[1]Asn and Gln are completely converted to Asp and Glu, respectively, and are therefore presented as a pair.
[2]Peak not found in one of the duplicate samples, SD not determined.
[3]Hly and Hyp were not identified by the GC and could not be quantified.
[4]Values determined by alkaline hydrolysis.
[5] Concentrations lower than LOQ, exact values uncertain.

Variation of amino acid composition between duplicates for all legumes, expressed as CV, are summarized in table 9. Low variations were found between duplicates of black beans, red kidney beans and white beans. CV-values were $\leq$ 15% except for analysis of Tyr in black beans and red kidney beans (41% and 26% respectively), of His (16%) and Phe (18%) in red kidney beans and of Ser in white beans (35%). Analysis of 13 amino acids had CVs under 10% for both black and white beans, and of 6 amino acids for red kidney beans.

Higher variations were found in the yellow pea as CV-values ≤ 15% for most amino acids of which 6 were ≤ 10%. Analysis of 6 amino acids were exceptions as 5 had a CV of 16-19% and Ser of 28%. The highest variation between duplicate samples were found in brown beans and grey peas as analysis of 9 and 6 amino acids had CVs of 16-26% respectively. Furthermore, analysis of Phe varied by 28% (Brown bean) and 31% (Grey Pea), of Ser by 42% in brown beans and of Tyr by 40% in grey peas.

Table 9. CV (%) of the analysis of analyzed legumes (n=2).

Amino Acid[3]	Black Bean	Brown Bean	Red Kidney Bean	White Bean	Grey Pea	Yellow Pea
	CV (%)					
Ala	9	6	0	3	18	10
Asp+Asn[1]	3	13	11	8	17	13
Cys	0	-[2]	8	4	-[2]	2
Glu+Gln[1]	12	4	13	13	3	7
Gly	7	26	6	0	25	13
His	7	16	16	4	14	12
Ile	5	23	11	2	26	19
Leu	6	19	12	2	26	15
Lys	7	3	2	9	13	3
Met	4	18	3	3	27	3
Phe	8	28	18	4	31	17
Pro	3	13	0	2	25	14
Ser	6	42	-[2]	35	13	28
Thr	7	21	14	11	18	18
Trp[4]	12	26	1	7	4	16
Tyr	41	24	26	12	40	8
Val	6	18	7	3	22	16

[1]Asn and Gln are completely converted to Asp and Glu, respectively, and are therefore presented as a pair.
[2]Peak not found in one of the duplicate samples, CV not determined.
[3]Hly and Hyp were not identified by the GC and could not be quantified.
[4]Values determined by alkaline hydrolysis.

4 Discussion

4.1 Calibration Curve

All 19 amino acids included in the standard mixture were detected and identified by the GC, showing that all 19 amino acids can be detected after sample preparation with EZ:faast using the established GC-parameters.

As 16 of the 19 calibration curves had r^2-values determined as equal to or above 0,99, linearity is shown, and indicates that data calculated using the calibration curves are reliable. Since all calibration curves were prepared from a standard mixture supposed to contain the same concentration of all amino acids, it is unclear why linearity is lower for His, Hly and Hyp. This could be due to stability differences between amino acids, leading to degradation of some amino acids. That some amino acids are

unstable in the standard mixture is stated by Phenomenex in the EZ:faast-manual (23), making this a probable explanation.

The variations between duplicate analysis (n=2) used to calculate the standard curve were additionally low (≤10%) for most amino acids with few exceptions, except for His where variation between the values was higher (11-34%). This is a further indication that most calibration curves were constructed with accurate and linear data.

4.2 Quality Control of Analytical Method

When a method has been established, validation trials are important to ensure that data determined by the method are reliable and true. There are multiple trials, and aspects, of interest when validating an analytical method. These include the method's accuracy, precision and limit of detection and quantification (38,39).

The limit of detection determines the lowest concentration where an analyte can be reliably distinguished from background noise while the limit of quantification measures the lowest concentration where the analyte can be quantitatively determined with accuracy and precision (38,40). Thus, if an analyte is found in a concentration higher than the LOD yet lower than the LOQ, the analyte can be reliably detected yet not quantified. There are multiple methods for determining LOD/LOQ including utilizing the calibration curve(s), signal:noise ratios, visual evaluations and variations of the blank sample (38,40,41). The method used for determining LOD/LOQ should therefore always be presented in the study (41).

The amino acids' limits of detection, presented in the EZ:faast kit and determined as 3 times the signal:noise ratio, are between 0,2 – 10 nmol / mL (23). Comparatively the LOD values determined in this study are quite high (2-46 nmol / mL). The higher values could be due to variations between methods. Furthermore, construction of the calibration curve was limited to the three levels proposed by Phenomenex. When determining LOD/LOQ-values with the standard curve multiple determinations should be made at five concentrations in the range of the LOD and LOQ-values (42). As the concentrations used for constructing the calibration curve were higher (50 – 200 nmol / mL) than the proposed LOD values (0,2 – 10 nmol / mL), and only four levels were used, the calibration curves may in this case not be ideal for LOD/LOQ calculations which could cause the relatively high values.

Unsurprisingly the three highest LOD and LOQ-values determined were the three amino acids with the lowest r^2-value. Since LOD and LOQ were calculated partly using the standard deviation of the (linear) standard curve, and r^2 is a measure of how linear a curves' values are, decreased linearity will usually correlate with higher standard deviation. As LOQ values were higher than measured concentrations for four amino acids (Cys, His, Thr, Ser) in all legumes, except for Ser in white and red kidney bean, those values cannot be reliably quantified. Concentrations of all other amino acids were higher than their respective LOQ for all legumes and these values can therefore be reliably quantified.

A methods precision refers to its ability to repeatably produce similar results with no or small variations (38,39,41). Precision can be divided into repeatability, the precision of values determined simultaneously (with-in day variation), and

reproducibility, precision of values determined at different times (between-day variation) (41).With-in day and between-day variations were low (≤10%) for analysis of most amino acids. As quantification of samples prepared at the same day and at different days have low variations this indicates a high repeatability and reproducibility of the method for quantifying most amino acids (41). Higher with-in and between-day variations was however found quantifying Glu + Gln (21% and 32%), Lys (23% and 20%) and Ser (46%) and the method's repeatability and reproducibility is less certain for these amino acids.

The accuracy of a method is instead its ability to produce values close to the true value. A method might be precise, determining values with a low variation, yet inaccurate if values are far from the actual value (38,39). Accuracy can be determined by analyzing a reference material of known compositions and comparing results to the true value (38). If accuracy cannot be determined a related recovery trial may instead be performed by analyzing a sample spiked with a known concentration. The method's recovery can then be calculated by determining how much of the spiked amount was found in the analyzed sample (39). However, neither the accuracy nor the recovery could be determined in this study due to lack of reference samples and not enough standard mixture supplied with the kit to spike samples (when accounting for dilutions). Consequently, it is unknown how close quantified amino acid compositions are to the true values and further quality control trials are needed.

4.3 Amino Acids in Legumes

Since the accuracy of the quantification, as mentioned in the above paragraph, is unknown, amino acid compositions determined in the study can only be used as indicative data on the legumes' amino acid composition, not as definite values. This is especially the case with regard to Cys, His, Ser, and Thr, as measured concentrations were below their respective LOQ-values in all legumes, except Ser in white and red kidney bean. As Tyr-values had a large variance (CV 24-41%) in black, brown, red kidney beans and grey peas, and overall large variations were found between duplicate samples of brown beans and grey peas, indicative data regarding these amino acids are also uncertain.

All studied legumes had low amounts of tryptophan and the sulfuric acids cysteine and methionine, yet were rich in lysine, in accordance with previous studies (1,6,7). The amino acid compositions of brown bean and red kidney bean, determined in this study and in previous studies, are presented in table 10 and of white bean and yellow pea in table 11. Similarly to values determined in this study the amino acid profiles of legumes previously determined also had limited amounts of methionine and cysteine. All legumes were additionally rich in Asp or Asp+Asn, Glu or Glu+Gln, Leu and Lys (43–46) with the exception of Asp in brown bean in literature (43). While Trp was not determined in 3 of the 4 presented literature studies (44–46) only limited amounts were found in the brown bean (43)

Hydroxylysine (Hly) and Hyp (Hydroxyproline) were not detected in this study. Measurement of the two amino acids are typically found in collagen-relate foods (47,48) but rarely analyzed in other foods. For instance, no studies presented in the introduction nor discussion of this study analyzed Hly and Hyp contents, and little is

therefore known of their content in non-collagen related foods. One study analyzed the amino acid composition, including Hly and Hyp, of beans of unspecified variety. Hly and Hyp were not found in most samples, and even when found only at values of 0.1 – 0.2 g/16 g nitrogen (49). Thus, Hly and Hyp might either not have been present in the analyzed food samples, or in too low concentrations to be detected.

Table 10. Determined and literature amino acid compositions of brown bean and red kidney bean (g / 100 g protein)

Amino Acid	Brown Bean	Brown Bean, Literature	Red Kidney Bean	Red Kidney Bean, Literature
	Mean g / 100 g protein			
Ala	2.7	3.9	2.2	3.8
Asp	6.0[1]	1.2	4.5[1]	10.9
Cys	0.4[2]	1.4	0.3[2]	0.9
Glu	6.5[1]	12.9	5.7[1]	15.3
Gly	2.2	3.7	2.0	3.6
His	2.3[5]	2.5	2.0[2]	3.4
Ile	2.9	4.5	2.7	5.2
Leu	5.2	7.6	4.9	8.5
Lys	3.8	6.5	3.3	4.9
Met	0.6	0.8	0.6	1.6
Phe	3.2	5.0	3.3	5.9
Pro	2.3	3.6	1.9	3.0
Ser	2.1[2]	6.1	3.13	4.6
Thr	2.0[2]	4.1	1.8[2]	3.2
Trp	0.4	1.3	0.6	n.d[3]
Tyr	1.3	2.2	1.5	3.2
Val	3.5	10.0	3.1	5.3
Ref.		(43)		(44)

[1]Asn and Gln are completely converted to Asp and Glu, respectively, and are therefore presented as a pair.

[2] Concentrations lower than LOQ, exact values uncertain.

[3]Not determined

Table 11. Determined and literature amino acid compositions of white bean and yellow pea (g / 100 g protein)

Amino Acid[3]	White Bean	White Bean, Literature	Yellow Pea	Yellow Pea, Literature
	Mean g / 100 g protein			
Ala	2.3	4.7	2.4	4.3
Asp+Asn	6.0[1]	13.6[1]	5.6[1]	11.4
Cys	0.32	1.1	0.4[2]	3.0
Glu+Gln	9.3[1]	10.6[1]	6.6[1]	16.6
Gly	2.1	4.9	2.6	4.2
His	2.0 [2]	3.2	2.0[2]	2.7
Ile	2.5	5.0	2.9	3.8
Leu	4.6	9.1	4.6	7.7
Lys	3.9	7.2	3.4	7.0
Met	0.6	1.0	0.5	1.2
Phe	2.9	6.4	2.9	4.9
Pro	2.0	n.d[3]	2.6	n.d[3]
Ser	3.4	6.9	1.9[2]	4.7
Thr	2.0[2]	5.13	1.7[2]	4.0
Trp[4]	0.4	n.d[3]	0.4	n.d[3]
Tyr	1.5	3.9	1.6	3.0
Val	3.1	6.1	3.2	4.9

Amino Acid[3]	White Bean	White Bean, Literature	Yellow Pea	Yellow Pea, Literature
	Mean g / 100 g protein			
Ref.		(45)		(46)

[1]Asn and Gln are completely converted to Asp and Glu, respectively, and are therefore presented as a pair.
[2] Concentrations lower than LOQ, exact values uncertain.
[3]Not determined

Including studies presented in this study (43–46), only a limited number of earlier studies have studied the amino acid compositions of unprocessed black, brown, red kidney and white beans or yellow and grey pea. Furthermore, nutritional databases such as the U.S Department of Agriculturals FoodData Central and the Swedish Food Agency's Food Database do not display amino acid composition. This highlights the need for additional studies for analyzing the amino acid profiles of these legumes.

4.4 Future Method Optimization

During development of hydrolysis conditions, problems were encountered with significant evaporation during the 20-/ 24- hour incubation were encountered. To counteract this, an extra step was added by adjusting all samples' weights to a specific total weight (10 grams for acid treatment, 5 grams for alkaline treatment) was added. It is however unsure if and how this affected treatments. If a lot of acid/alkaline evaporated quickly the remaining acid/alkaline might not have been enough to fully hydrolyze all proteins in the food sample. Furthermore, methionine and cysteine recovery are dependent on removing oxygen prior to incubation (15,19,22,24,25). The evaporation of liquid out of the bottles during incubation is an indicator that the bottles may not have been sealed enough, and that oxygen possibly could have entered bottles during the incubation. Since no recovery tests were done it is unknown if, and how, this affected the quantification of Cys and Met. To optimize the procedure using bottles which may be better sealed could be beneficial. If evaporation could be stopped it would also remove the need of the additional dilution step, lessening sample dilution. This would be beneficial when performing recovery trials as trials using spiked in-house control samples were hindered due to insufficient standard mixture since a large quantity was needed due to a high dilution factor (112,5 times). Although analytical variations were low for tyrosine in the in-house control samples, it was high in most legume samples. Using phenol during the conventional acid hydrolysis has been shown to increase recovery, possibly lessening variations (16).

Additional quality control trials are further required to optimize the method. If LOD and LOQ are calculated using the calibration curves the curves should be constructed using multiple levels to, hopefully, reach a lower limit of detection and quantification. Accuracy tests are additionally required to determine the methods' accuracy and the authenticity of its results.

4.5 Ethical and Societal Aspects

No experiments conducted in this study included animal or human subjects nor was any personal data collected or presented. Consequently, no ethical permits or special considerations were needed.

Vegetarian and vegan foods and diets are becoming increasingly popular, due to health-related and environmental reasons (1,5) . It is therefore essential to ensure that these plant-based diets are nutritionally sound and can supply all nutrients required by the human body. As most plant-based foods have limitations with regard to of some indispensable amino acids (1,6), establishing , and applying, robust methods for amino acid analysis are important in order to evaluate plant-based foods as protein sources, both as individual foods and as part of a larger diet.

5 Conclusion

A method for analyzing amino acids in legumes using GC-FID has been established. The method, allowing measurement of 19 amino acids, has good repeatability and reproducibility, although limits of detection and quantification are high and trials determining the accuracy or recovery are lacking. Analyzed legumes were rich in amino acids such as aspartic acid and asparagine, glutamatic acid and glutamine, leucine and lysine while limited in methionine, cysteine, and tryptophan.

6 References

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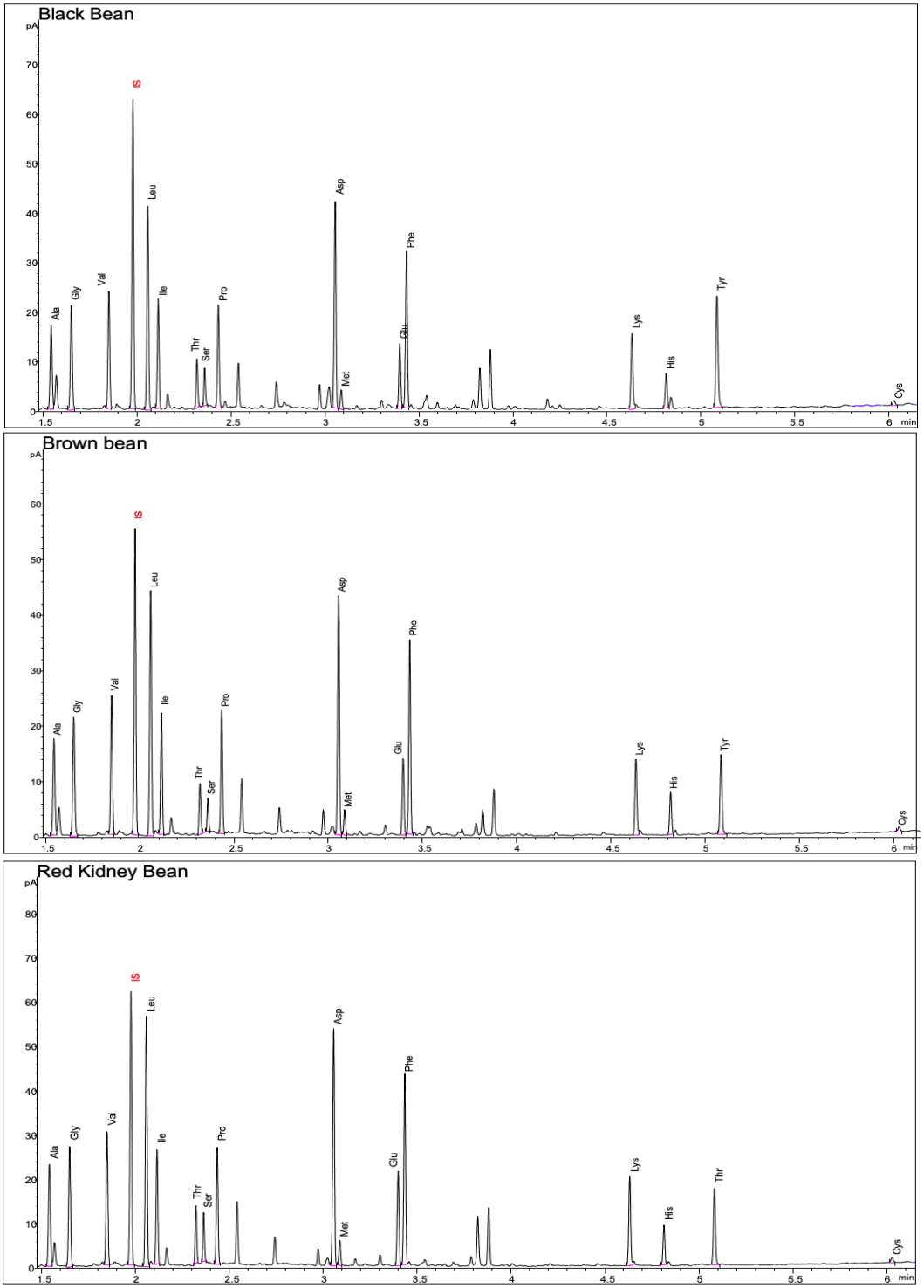
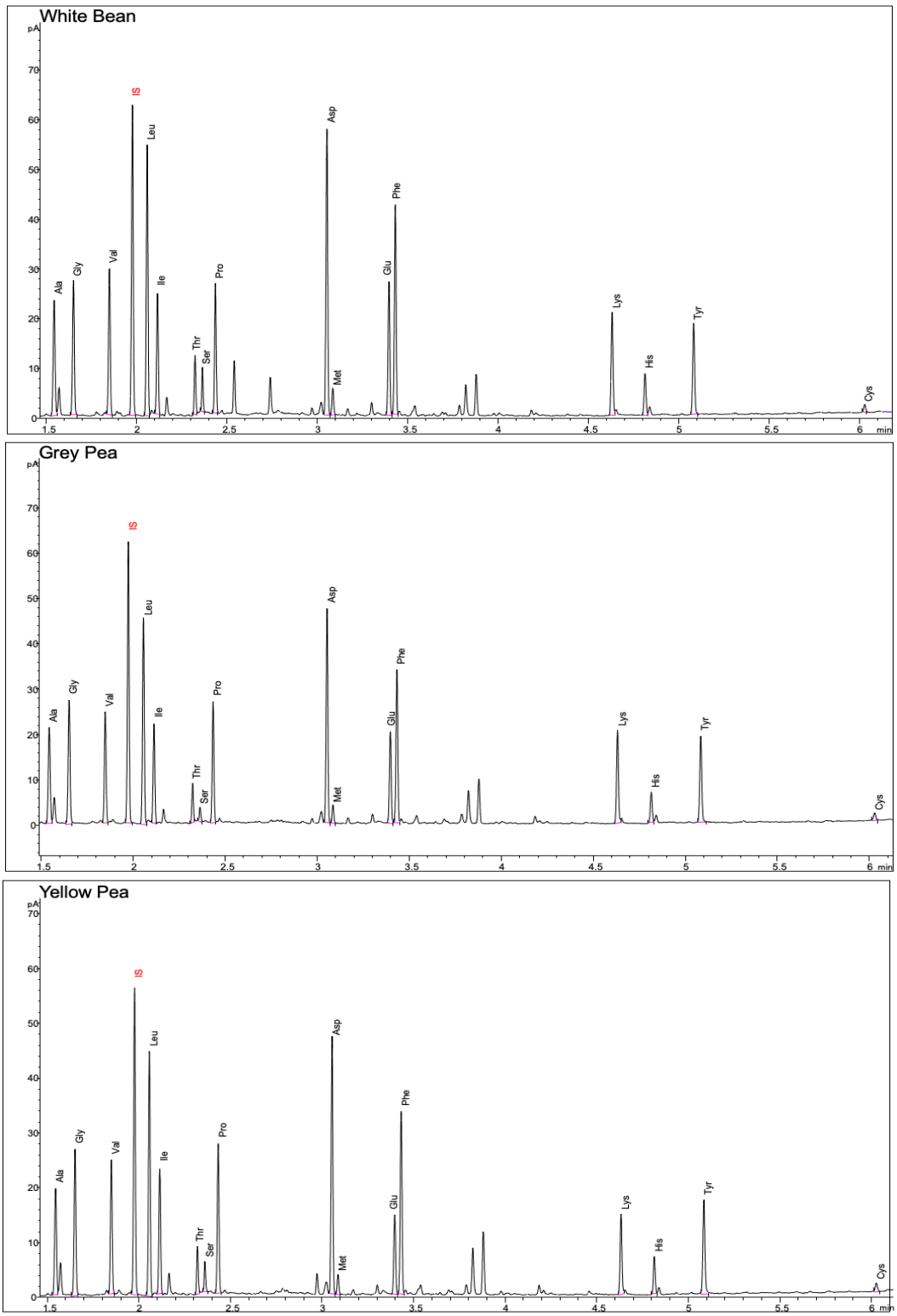
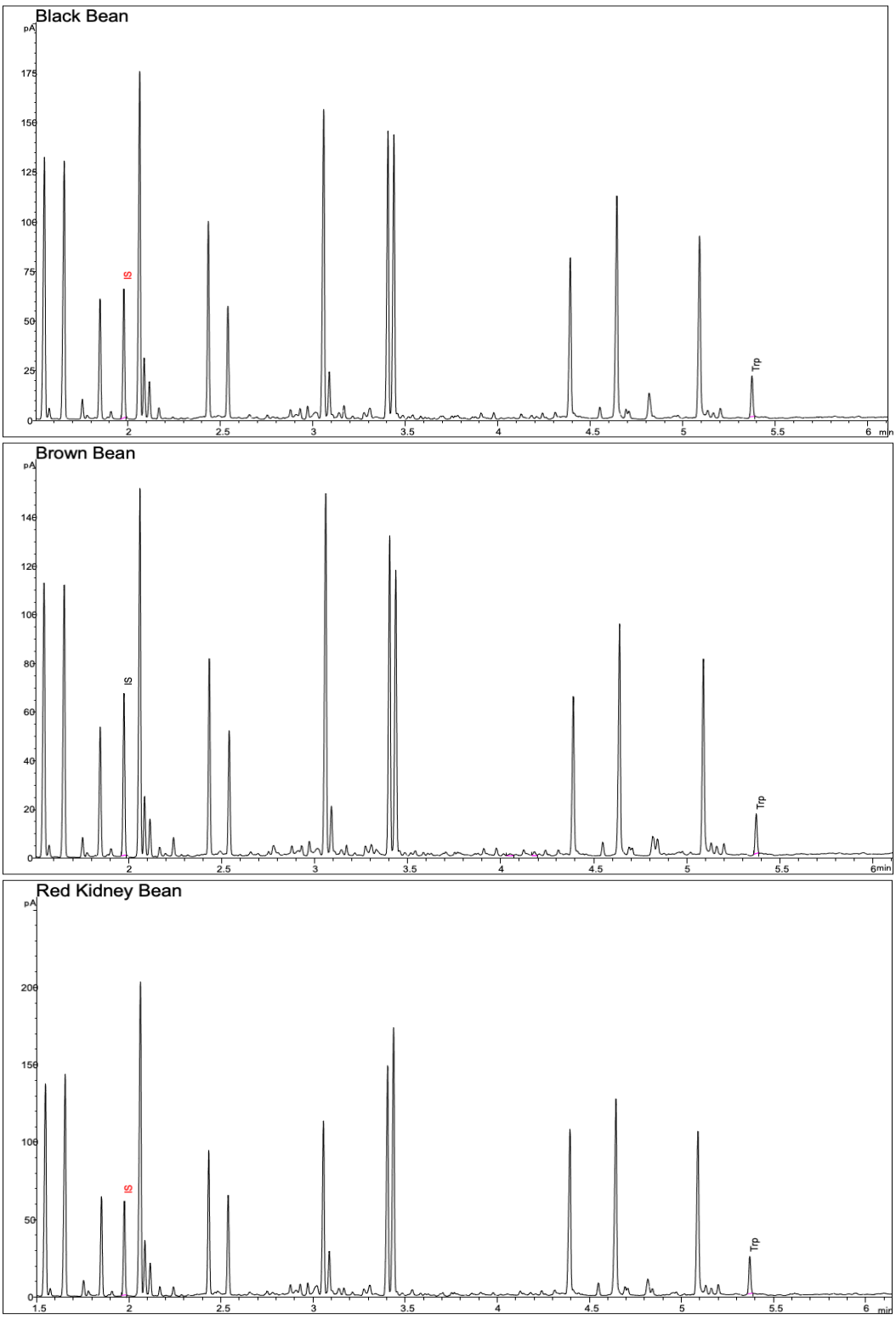
Appendix



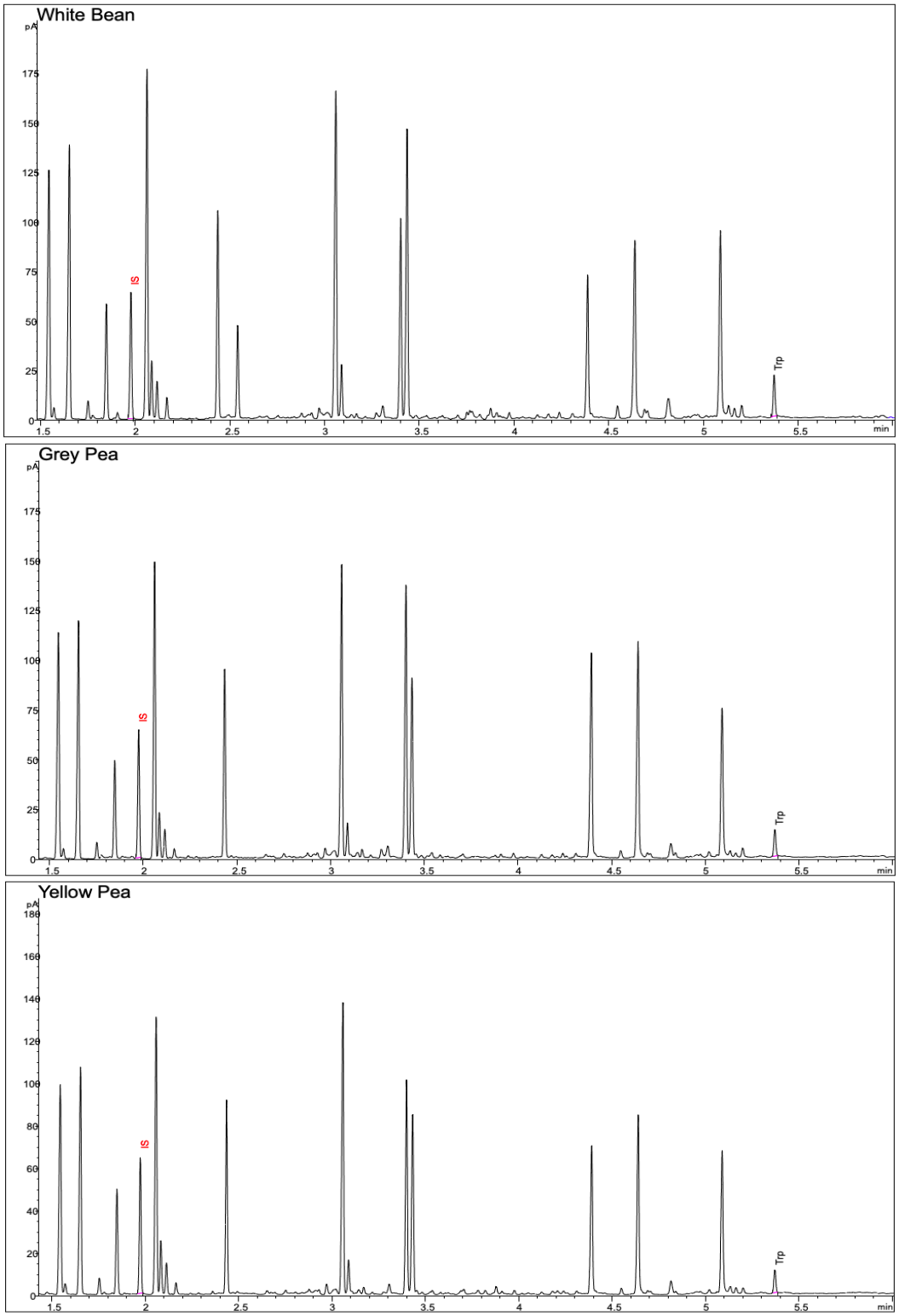
Supplementary Figure 1a. Chromatograms for black bean (top), brown bean (middle) and red kidney bean (bottom) prepared with acid hydrolysis. All peaks used for quantification are marked with the associated amino acid and IS marked in red.



Supplementary Figure 1b. Chromatograms for white bean (top), grey pea (middle) and yellow pea (bottom) prepared with acid hydrolysis. All peaks used for quantification are marked with the associated amino acid and IS marked in red.



Supplementary Figure 2a. Chromatograms for black bean (top), brown pea (middle) and red kidney bean (bottom) prepared with alkaline hydrolysis. Trp peaks used for quantification are marked and IS marked in red.



Supplementary Figure 2b. Chromatograms for white bean (top), grey pea (middle) and yellow pea (bottom) prepared with alkaline hydrolysis. Trp peaks used for quantification are marked and IS marked in red.