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DETERMINATION OF AMINO ACIDS IN BEE PRODUCTS BY DIETHYL ETHOXYMETHYLENEMALONATE DERIVATIZATION USING LC-ESI-MS/MS

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ABBREVIATIONS

AA	Amino Acid
ACN	Acetonitrile
CE	Collision Energy
CID	Collision induced dissociation
DEEMM	Diethyl ethoxymethylenemalonate
DNS	1-dimethyaminonaphthalene-5-sulfonyl chloride, Dansyl chloride
EtOH	Ethanol
ESI	Electrospray ionization
FA	Formic Acid
FMOC-Cl	9-fluorenylmethyl chloroformate
FOSF	2,5-dioxopyrrolidin-1-yl N
HCl	Hydro chloride Acid
HPLC	High performance liquid chromatography
LOD	Limit of detection
LOQ	Limit of quantitation
MeOH	Methanol
M	Mass of amino acid
M/DEEMM+H^+	Derivatized protonated amino acid
min	Minute
MS	Mass spectrometry
MS/MS	Tandem Mass Spectrometry
m/z	Mass to charge ratio
NA	Not Applicable
ND	Not detected
PVDF	Polyvinylidene difluoride
R^2	Regression Coefficient
RJ	Royal Jelly
RSD	Relative standard deviation

Rt	Retention time
SCX	Strong cation exchange
SPE	Solid phase extraction
UPLC	Ultrahigh pressure liquid chromatography
OPA	Ortho - phthalaldehyde
v/v	Volume/ volume

Amino Acids

Asn	L-Asparagine
Asp	L-Aspartic Acid
Cys	L-Cysteine
GABA	γ-Aminobutyric acid
Gln	L-Glutamine
Glu	L-Glutamic Acid
Gly	Glycine
His	L-Histidine
Ile	L-Isoleucine
Leu	L-Leucine
Lys	L-Lysine
Met	L-Methionine
Orn	L-Ornithine
Phe	L-Phenylalanine
Pro	L-Proline
Ser	L-Serine
Thr	L-Threonine
Trp	L-Tryptophan
Tyr	L-Tyrosine
Val	L-Valine
α-Ala	L-Alanine (Alpha-Alanine)
β-Ala	Beta-Alanine

1. INTRODUCTION

Amino acids are well-known compounds which are widely distributed in natural products. They have played a crucial role in many biological processes in human body, such as the synthesis of proteins, fatty acids and ketone bodies. For this reason, many analytical methods have been developed to determine the concentrations of amino acids in variable categories of foods to estimate the quality of consumption.

One of the best candidate methods for this purpose is high performance liquid chromatography (HPLC) compatible with electrospray ionization (ESI), coupled with tandem mass spectrometer (MS/MS) which allows to determinate low concentrations of analytes in complex matrices during short period with reliable results. In addition to develop the method, derivatization have been concerned to improve chromatographic retention in reversed phase, enhancing ionization efficiency as well as giving more information through fragmentation of derivatized compounds with MS.

A considerable amount of literature has been published on bee products, especially honey. Several methodologies have been examined to understand the relationship between free amino acids content in honey and its authenticity. Honey from different geographical and botanical origins has been investigated so far; however, no study has examined sample from coffee nectar. In addition, the case of free amino acids in royal jelly has not been received great attention by the researchers in the past and this motivated the present study.

The aim of this thesis is:

- To study the derivatization of free Amino Acids (AAs) using Diethyl ethoxymethylenemalonate (DEEMM) reagent.
- To development an analytical method and implement it in LC-ESI-MS/MS positive mode.
- To apply the method to Vietnamese bee products: Royal Jelly and honey which are collected from *Apis mellifera* bee in Robusta coffee farm.

2. REVIEW OF LITERATURE

2.1. Amino acids and their classifications

Amino acids are organic compounds containing two functional groups are amine ($-NH_2$) and carboxyl (-COOH), side chain group (R) is an organic substituent. Structure of α-amino acid are characterized by having both the amine and the carboxylic acid groups attached to the α-Carbon, except Gly, β-Ala and GABA. Based on functionality of the side chain group attached to the α-carbon, amino acids were classified into 4 groups: acidic and basic side chain, polar (uncharged) side chain and hydrophobic side chain. The structure of 23 amino acids in Annex 1.

In terms of protein and peptide, they include 22 proteinogenic amino acids which are polymerized together to form various peptides and proteins which can be found in all living organisms. Asn was first discovered in 1806, the last of 22 was Thr which was identified in 1938. Among 22 genetically encoded amino acids, there are 20 standard genetic codes and 2 additions (Selenocysteine and Pyrrolysine) that can be combined through particular mechanisms. Otherwise, non-proteinogenic AAs are not generally incorporated into proteins, for example Orn or GABA. Some will take part in synthesis of non-ribosomal peptide.[1]

Research has found that His, Thr, Met, Trp, Val, Phe, Ile, Leu and Lys are nine essential (indispensable) amino acids which are not synthesized in the human body, thus must be taken from external source following WHO [2]. The other six amino acids: Arg, Cys, Gly, Gln, Pro and Tyr were considered as semi essential in human diet due to their biosynthesis pathway are limited under some special conditions. Non-essential amino acids fall to the group of Ala, Asp, Asn, Glu, Ser and Selenocysteine, meaning they are dispensable and can be synthesized internal from other materials of intermediary metabolism.

According to the product of catabolism, amino acids can be classified into glucogenic amino acids which glucose will be formed or ketogenic amino acids which can be ultimately degraded to carbon dioxide. Because of the tetrahedral arrangement around the *α*-carbon atom, it is possible that chiral center has D and L isomers. However, it is remarkable observation of

L-Amino acid residues occur in proteins and peptides in living system. For stabilize and repeat the structure of proteins, one stereochemical series are generally required [3].

2.2.Amino acid analysis and DEEMM derivatization

For higher sensitivity and selectivity of method, chemical derivatization of analytes has been utilized in the past decades. An increase in molecular mass will allow the method to distinguish better between background and analyte signals, since the background is from lower mass range. Other advantages of analyzing derivatized compounds with LC-MS/MS include enhancement of ESI response by sensitive moiety, improvement of structural elucidation during fragmentation and facilitation of isomer separation [4]. Their structures are placed in Annex 2.

To derivatize amino acids, there are two options: with the carbonyl group or with the amine group. There are several papers reported about conventional reagents with reaction time depends on the type of reaction and functional group involved. Some of first derivatization reagents are Ortho -phthalaldehyde (OPA) which was applied in seawater [5], honey and wine samples [6] and 6-aminoqui-nolyl-N-hydroxy-succinimidyl carbamate (AQC) in food sample [7]. Besides, reagent which reacted with amine group will help to remain carboxylic acid group in the derivatized amino acids. Thus, the derivatized molecules could be analyzed with MS detection under negative ion mode. Generally, the analytical sensitivity of the derivatized amino acids was higher than that using the ninhydrin method [8].

In addition to increase the ionization efficiency in ESI, (5-nsuccinimidoxy-5-oxopentyl) triphenylphosphonium bromide (SPTPP) was used to form a positively-charged quaternary phosphonium ion with amino acids under mild condition [9]. Another positive charge derivatization reagents are p-N,N,Ntrimethylammonioanilyl N´-hydroxysuccinimidyl carbamate iodide (TAHS) and 2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino)-phosphoranyl-ideneamino carbamate (FOSF). The modified analytes will be ionized better in positive mode ESI, together with the larger molecule volume that helps for converting to gas phase ions easier, therefore enhance the sensitivity. APDS (3-aminopyridyl-*N*-hydroxysuccimidyl carbamate) was also designed based on TAHS but without charged and more than 100 compounds with amino group in biological fluid were analyzed with this reagent.

There still exist many challenges in development of derivatization reagents for LC MS/MS, including harsh reaction with toxic chemical condition, long reaction time, matrix effect of by-products and derivatized product stability. Thus, one of models for derivatization reagent is a compound which could react with functional group of target analytes. It also includes a modifying group which have specific fragmentation in MS and good separation in LC. Reaction time, condition and yield are prerequisite criteria as well as stability of derivatized products. For applicable purpose, the reagent should be synthesized easily or be commercially available.

When comparing TAHS, 9-fluorenylmethyl chloroformate (FMOC-Cl), 1-dimethyaminonaphthalene-5-sulfonyl chloride (DNS) and DEEMM derivatizations in honey and tea sample, DEEMM was found as less affected by matrix effect. Some benefit of DEEMM that over other reagents is ending of the reaction is not essential even though the large excess amount of reagent was added. Reaction was carried out at room temperature following the mechanism of nucleophilic substitution in borate buffer (0.75 M, pH = 9). DEEMM reagent did not react with matrix substances usually present in food samples, such as cheese and beer [10,11].

Furthermore, derivatization yield of Gly, β-Ala and Phe with DEEMM has showed that it closes to 100% with RSD below 11% for 3 cases. This proof gave a benefit for DEEMM reagent to assume that other amino acids could share the same behavior. Reaction took place immediately, except unstable signal of Proline due to secondary amine group. Therefore. it is recommended to analyze the derivatized AAs within 24 – 48 hours.

2.3.Royal Jelly, honey and their origin

2.3.1. Royal Jelly

Royal Jelly is a milky white to yellowish complex substance which is mainly secreted by young worker nurse honeybees (*Apis mellifera*) between the sixth and twelfth days of their life. It is an essential food for all young larvae, no more than 3 days for the development of workers and drones in bee colony. After that, worker larvae are fed a mixture of RJ, pollen and honey [12]. Meanwhile, larva of queen bee will be fed intensively, directly with RJ throughout her larval period. Basically, fresh RJ contains water (50 – 70%), proteins (9 – 18%), carbohydrates

(7 – 18%), fatty acids and lipids (3 – 8%), polyphenols, amino acids, mineral and vitamins [13]. Because this nutrition contributed to the mature of ovaries for reproduction and longer life span of the queen bee, it has drawn attention to be used as cosmetic and diet supplement in Asia.

Recently investigators have examined the effects of RJ on human health who consumed it thank to the promotion of its pharmacological activities [14] such as anti-tumor, anti-oxidant, anti-inflammatory, antibacterial and anti-aging during 6 months [15]. At latest published information, RJ helped to improve life quality of postmenopausal woman, lipid metabolism and fertility. It also enhanced glucose tolerance and mental health.

RJ is naturally inhomogeneous and often presents undissolved granules from varying size with a distinctively sharp odor and taste. Major RJ proteins (MRJPs) are related to numerous essential amino acids [16]. Fu-Liang Hu et al. [17] suggested standard methods for RJ, has mentioned the freshness in quality control RJ that it consists low amounts of free amino acids. However, specific values have not given yet neither the method to determinate the concentration of them even in Royal Jelly specification ISO 12824:2016 [18]. As a product of Maillard reaction between amino acid and reducing sugar to form furosine over time with temperature effect. Thus, quantitation of furosine is an index to assess the quality of RJ as well as its appearance could become to a different darker color if degradation happens [19].

2.3.2. Honey

Chemically, honey is a supersaturated mixture which mainly comprises 60 – 85% carbohydrate (38.5% fructose, 31% glucose, 12.9% other sugars). Other minor compounds include proteins (0.15%), organic acids, minerals, amino acids, vitamins, phenols. In addition, honey consists of minor amounts of bioactive components [13] were reviewed by Pasupuleti et al. to summarize the potential health benefits of bee products.

In the authentic aspect, the descriptions about geographical, botanical origin, natural, organic have gained the interest of regulatory authorities as well as consumer [20]. European directive 2001/110/EC has defined that honey is the natural sweet substance produced by *Apis mellifera* bees [21] and produced country should be named on the label for geographical origin.

In general classification, there are 4 general types of honey which are blended honey, monofloral and polyfloral honey, honeydew honey [22].

Water content following Codex Alimentarius Standard for Honey should be less than 20% except for heather honey up to 23%. According to International Honey Commission (IHC) suggestion [23], Pro content could be considered as a criterion in honey quality assessment and depends honey type, the minimum value of Pro is 180 mg kg^{-1} honey for acceptance in control laboratories.

From 1980, amino acid contents have been proposed for the determination of botanical origin of bee products. The overall amino acid profile could differentiate various type of nectar origin. However, the content of single amino acid or a group of them could not give enough information for characterization of honey. After Pro, Phe and Leu, Tyr are the significant amino acid which were detected in majority of different honey samples. In case of lavender honey, the predominant amino acid is Phe and Tyr, not Pro. Trp and Glu were the key elements to distinguish honeydew honeys and nectar honeys [20]. In case of chestnut honey and acacia honey, Arg and Trp are important discriminating factors useful, respectively [24]. Therefore, attempts should be added to improve the database of free amino acids in various types of honey, to specify their botanical origin.

2.3.3. Sample origin

In Vietnam, the honey bee (*Apis mellifera*) is an introduced species which has been managed and widely naturalized by beekeepers. The combination of growing coffee farm together with beekeeping has brought the mutual benefit for the agriculture, since Robusta coffee has been known as cross pollinated flower. Gia Lai province is the location where sampling was taken thanks to the highest growing area (96% coffee farm is Robusta coffee). In this thesis, honey and RJ were collected in December and March 2018 respectively (Figure1). Honey is stored at room temperature, while RJ needs to be stored at -20 ^{o}C.

Figure 1: Royal Jelly (left) and Honey (right) were used in this thesis

3. EXPERIMENTAL

3.1.Chemicals (The preparation is mentioned detail in Annex 3).

- Amino acids: L-Amino Acids Kit (Sigma) includes 21 AAS: L-Histidine hydrochloride (His.HCl), L-Arginine hydrochloride (Arg.HCl), L-Asparagine (Asn), L-Glutamine (Gln), L-serine (Ser), L-aspartic acid (Asp), glycine (Gly), L-glutamic acid (Glu), L-threonine (Thr), γ-Aminobutyric acid (GABA), α-Alanine (α-Ala), L-proline (Pro), L-Tyrosine (Tyr), L-Methionine (Met), L-Valine (Val), L-Tryptophan (Trp), L-Cysteine.HCl (Cys.HCl), L-Phenylalanine (Phe), L-Isoleucine (Ile), L-Leucine (Leu), L-Lysine hydrochloride (Lys,HCl) and β-Alanine (β-Ala) (Fluka), L-Ornithine hydrochloride (Orn.HCl) (Fluka)
- Derivatization reagents: DEEMM (Sigma)
- Other chemicals: hydrochloric acid (HCl) (Sigma), phosphoric acid (H_3PO_4) (Sigma), Ammonium hydroxide (NH_4OH) (Merck), boric acid (Reakhim), potassium hydroxide (KOH) (Sigma).
- LC eluents: HPLC grade Acetonitril (ACN) (Sigma), Methanol (MeOH) (Sigma) and Formic Acid (FA) (Sigma).
- Aqueous solutions were prepared with ultrapure water purified by Millipore Milli-Q Advantage A10 (Milli-Q water).
- All buffers were adjusted pH with KOH saturated solution.
- All reagents were of analytical grade if not stated.
- All concentrations of AAs which were mentioned in this thesis were expressed as the concentration of AA not derivatived.

- Concentration of 3 AAs: His.HCl, Arg.HCl, Cys.HCl, Orn.HCl and Lys.HCl were converted to His, Arg, Cys, Orn and Lys before calculation.

3.2.Instrument

- Agilent 1290 UHPLC with Agilent 6495 Triple Quad LC/MS equipped with Jet Stream ion source.
- Vortex mixer VWR and Centrifuge Eppendorf 5702.
- Reacti-therm III # TS-18824 Heating Module – Thermo Scientific evaporator.
- All the dilution factor and concentrations were determined by weighing mass with Sartorious GENIUS analytical balance (0.00001 g readability).

3.3.Preparation of standard and sample solutions

- Dilutions of all standard solutions were made with 0.1 M HCl containing 30% MeOH and were stored at –20 ºC. Stock solutions of individual AAs were made in the concentrations range around 5 mg g^{-1}.
- An intermediate stock solution of 23 AAs (**W1**) in the concentrations range 70 – 90 µg g^{-1} for 21 AAs, Lys and Pro have the concentrations are 200 µg g^{-1}. **W1** solution was prepared by taking an amount of stock solution (0.1 – 0.3 mL) individually into 10 mL of 0.1 M HCl containing 30% MeOH.
- A final stock solution of 23 AAs (**W2**) were prepared by taking the 0.4 mL of the intermediate stock solution (**W1**) into 10 mL of 0.1 M HCl containing 30% MeOH to obtain the concentrations range 2000 - 3000 ng g^{-1} for 21 AAs, Lys and Pro have the concentrations range 6000 - 8000 ng g^{-1}.
- Working standard solutions were prepared before derivatization by diluting the final stock solution of 23 AAs (**W2**) with different dilution factors to obtain the concentrations range 1 – 250 ng g^{-1} for 21 AAs and 3 – 700 ng g^{-1} for Lys and Pro.
- 23 individual AA solutions ($\mathbf{W_{AA}}$) with the concentration around 250 µg g^{-1} were prepared for optimize LC MS/MS parameters in section 3.5.

3.4.Sample preparation

Solid phase extraction (SPE) were chosen for sample preparation following the paper [25]. As strong cation exchange process had been demonstrated more efficient than weak cation exchange, then styrene-divinylbenzene polymetric, 500 mg (Alltech, USA) SPE cartridges were used for treatment. However, in the last step, there was modified in the volume of dilute solution from 1 to 10 mL, and 0.1 M HCl containing 30% MeOH was used instead of Milli-Q water.

The SPE cartridges needs to be conditioned with 10 mL HCl 0.1 M before use, adjust the vacuum to around 70 mbar, flow rate approximately 4.0 mL min^{-1}.

3.4.1. Sample dilution approach

- Step 1: Weight accurately 1 g of honey or RJ (± 0.1 g) into 50 mL tube, add 25 mL phosphate buffer, mix well. Centrifuge for 10 min with 4000 rpm, then apply the supernatant to SPE cartridge, adjust the vacuum to around 50 mbar, flow rate ~1.0 mL min^{-1}. Maintain a low flow rate while applying buffered sample to the cartridge for a good recovery.
- Step 2: Rinse the 50 mL tube with 3 mL of phosphate buffer then applying to the cartridge. In the end of this step, increasing flow rate to remove all phosphate from the system (about 20 s).
- Step 3: Elution by applying 15 mL of 2.5 M NH_4OH containing 10% ACN to the cartridge. The flow rate could be increased up to 5 mL min^{-1}, which will not affect the recovery.
- Step 4: Dry 15 mL eluent with nitrogen flow in Thermo evaporator at 50 ^oC. After dryness, let the sample cool down to room temperature, reconstitute in 10 mL of 0.1 M HCl containing 30% MeOH.

The collected sample after SPE was considered as diluted 1:10 solution. Thereafter, the post extracted solution was diluted 2 – 5 – 10 – 20 – 50 times. Meanwhile, RJ sample was tested with dilution factors are 20 – 40 – 50 – 100 times before derivatization. The diluted samples were used to check Matrix effect (ME) and discussed results are at section 4.2.

Together with this preparation, a standard solution of 23 AAs (**W**$_{SPE}$) with concentration around 200 ng g^{-1} was prepared and carried out SPE to estimate the recovery after extraction (n = 2) and discussed in section 4.5.2.

3.4.2. Derivatization procedure

The derivatization procedure (Figure 2) followed the conditions stated in the paper [26]: Take 250 µL of diluted post-extracted sample or standard solutions into 1.5 mL vial, add 375 µL of DEEMM: MeOH (1:50), add 875 µL of Borate buffer (0.75 M, pH 9.0), vortex to mix the solution. Let the derivatized solution stand at room temperature, avoid from direct light for 24 - 48 hours. Mix and filter through 0.2 µm cellulose acetate syringe filter to vial before MS analyzing.

Blank reagent sample was also prepared with this procedure and 250 µL of 0.1 M HCl containing 30% MeOH was used to be derivatized. The result is discussed in section 4.4.

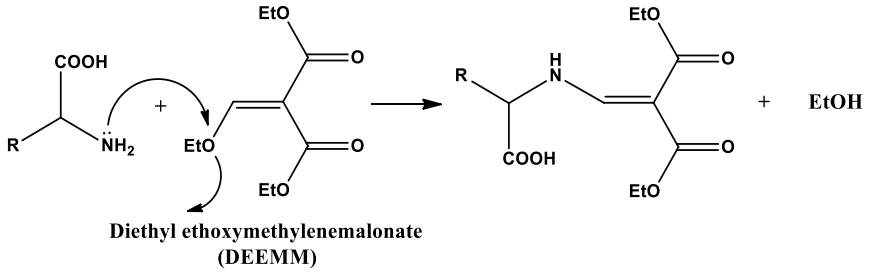

Figure 2: Chemical reaction to obtain derivatized Amino Acid with DEEMM

3.5. LC-ESI-MS/MS parameters

3.5.1. LC parameters

Analysis of 23 AAs derivative was carried out using analytical column Agilent Zorbax RRHD Eclipse Plus C18 (2.1 × 50 mm, 1.8 µm) with guard column Agilent Zorbax Eclipse Plus C18 (2.1 × 5 mm, 1.8 µm). Eluent components were (A) 0.1% FA in water and (B) ACN with 4% Milli-Q water. Column temperature was kept at 40 °C. Autosampler was at 20 °C to avoid precipitation. Injection volume was 2 µL and post time was 4 min. The gradient program presented in Table 1 was used for analysis. Eluent flow rate was 0.4 ml min^{-1}.

Table 1. The LC gradient for analysis of derivatized Amino Acids

Time (minute)	0	1	1.5	4.5	6.5	12	13	16	17
Eluent A (%)	90	90	85	85	65	65	0	0	100
Eluent B (%)	10	10	15	15	35	35	100	100	10

The gradient program was referenced from [26] with the extended time to equilibrate the column before running new injection. It is necessary to separate between Ile and Leu isomers, as well as α-Ala and β-Ala in mixture of AAs. However, with the current LC parameters, Ile and Leu are partly separated (Figure 3).

When new column was used, it was conditioned to activate the sorbent with ACN with 4% Milli-Q water and Milli-Q water in 24h before use. Washing column after use in at least 2 hours with FA 0.1% and ACN (with 4% Milli-Q water) with flow rate 0.1 mL min^{-1} and column temperature 40 ^{o}C following the gradient in table 2.

Table 2: The LC gradient for washing column

Time (minute)	0	30	60	120	180	240
Eluent A (%)	0	0	25	50	75	90
Eluent B (%)	100	100	75	50	25	10

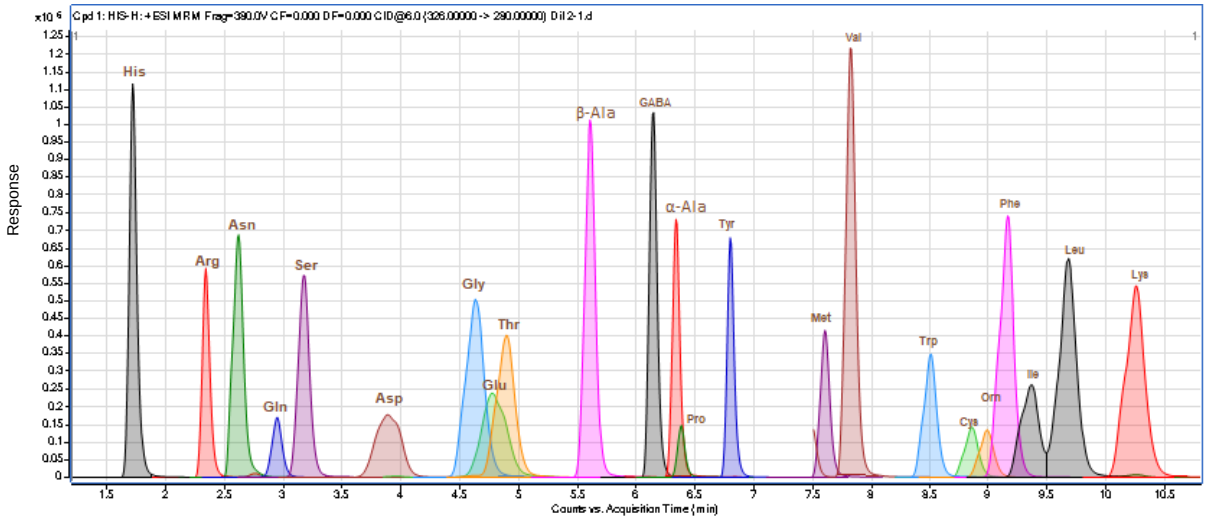

Figure 3: Dynamic MRM chromatogram of standard solution used in calibration curve

3.5.2. ESI-MS/MS parameters

The following ionization source parameters were used: iFunnel parameters in positive mode: high pressure RF 130 V and low pressure RF 60 V; nebulizer gas (nitrogen) pressure 45 psi (310 kPa); sheath gas (nitrogen) flow rate was 12 L min^{-1} and temperature 400 ^{o}C; drying gas (nitrogen) flow rate 11 L min^{-1}, temperature 220 ^{o}C. Capillary voltage was 3500 V, nozzle voltage 500 V. Cell accelerator voltage was 5 V and collision energy was optimized for each transition. At first, Product ion scan mode for individual standard derivatized AAs (**W_{AA}**) was used for identification. Both protonated, Na adducts were compared and only Lys-Na adduct was chosen due to higher intensity than the protonated form.

After optimization, dynamic multiple reaction monitoring (MRM) mode was set with retention times and m/z values of the precursor and product ions of AA derivatives. Mass axis calibration should be checked before analysis. From 0 to 1 minute and after 13 minutes, the flow was switched to waste instead of to MS to decrease the number of unknown compounds introduced to ionization source.

The transitions which were used in quantitation in all cases are loss of one ethanol group to form $[M/DEEMM+H-46]^+$ from the derivatized AA protonated molecule $[M/DEEMM+H]^+$. except for Cys, Orn and Lys, which corresponded to the $[M/2DEEMM+H]^+$.

These are the characterized fragmentations of DEEMM derivatives which brings the highest intensity. In case of qualifier, there is losing one of another group such as: ethanol, ammonia, CO, CO_2 or H_2O (table 3)

Table 3: MRM amino acid derivative transitions and corresponding collision energies in the order of retention times (one molecule of AA reacted with 2 molecules of DEEMM) - NA: Not Applicable*

Amino acid	Rt (minute)	RSD of Rt	Precursor Ion	Product ion		Collision energy (V)	
				Quantifier	Qualifier	Quantifier	Qualifier
His	1.71	0.3%	326	280	236	6	10
Arg	2.33	0.2%	345	299	253	10	10
Asn	2.60	0.3%	303	257	240	10	20
Gln	2.93	0.3%	317	271	254	10	15
Ser	3.16	0.3%	276	230	202	10	15
Asp	3.92	0.2%	304	258	212	10	20
Gly	4.60	0.3%	246	200	156	10	15
Glu	4.73	0.9%	318	272	254	10	15
Thr	4.87	0.3%	290	244	198	10	20
β-Ala	5.60	0.1%	260	214	170	6	20
GABA	6.14	0.1%	274	228	210	6	15
α-Ala	6.34	0.1%	260	214	170	10	15
Pro	6.38	0.1%	286	240	166	8	15
Tyr	6.80	0.1%	352	306	262	6	20
Met	7.60	0.1%	320	274	200	8	15
Val	7.82	0.1%	288	242	214	10	20
Trp	8.49	0.1%	375	329	NA	8	NA
Cys*	8.85	0.1%	462	416	370	10	10
Orn*	8.99	0.1%	473	427	381	4	10
Phe	9.15	0.1%	336	290	262	10	20
Ile	9.35	0.1%	302	256	228	10	20
Leu	9.66	0.1%	302	256	228	10	20
Lys*	10.26	0.1%	509	463	419	18	20

4. RESULTS AND DISCUSSTION

4.1. Linearity – LOD – LOQ

To estimate the linearity and linear range, the mixture of final stock solution of 23 AAs was diluted and derivatized from the concentration range approximately 1 – 250 ng g^{-1} for 21 AAs, with Lys from 3 – 526 ng g^{-1} and Pro from 19 – 733 ng g^{-1} based on the obtained concentration of from samples, to make sure they fit in linear range.

The derivatized standard solutions were injected 3 times for each concentration. As table 4 and table 5 below have shown, there are two linear ranges, the low concentration is approximately from 1.0 – 20 ng g^{-1} and from 20 – 250 ng g^{-1} for 21 AAs except Pro and Cys.

Different linear ranges could be explained that the ionization of derivatized standard AAs are various and depends on concentration. The purpose of using 2 ranges which helps to estimate the concentration in sample and trueness test more accurate. To estimation of LOD and LOQ, linearity results in low concentration range were used to calculate according to equation (1) and (2):

$$\boldsymbol{LOD = 3.3 * \frac{SD_{(b)}}{a}} \qquad \textbf{(1)}$$

$$\boldsymbol{LOQ = 10 * \frac{SD_{(b)}}{a}} \qquad \textbf{(2)}$$

Where $\mathbf{SD_{(b)}}$ is the standard deviation of the residuals and **a** is the slope of the calibration curve. Table 4 shows the obtained LOD and LOQ values and calibration curves in low concentration range, when table 5 indicates the linearity in higher concentrations.

Table 4: Linearity in the low concentration range approximately 1.0 – 20 ng g^{-1} for 21 AAs except Pro, Cys and the estimated values of LOD, LOQ

Amino Acid	Calibration curve	Linear calibration range (ng g^{-1})	Regression coefficient	LOD (ng g^{-1})	LOQ (ng g^{-1})
His	y = 19736x + 3466	1.3 - 19.2	0.9987	0.8	2.3
Arg	y = 10882x + 444	1.3 - 19.5	0.9978	1.0	3.1
Asn	y = 18561x + 6796	1.3 - 19.5	0.9995	0.5	1.5
Gln	y = 4215x + 1779	1.3 - 19.2	0.9863	2.5	7.7
Ser	y = 15575x + 18124	1.4 - 20.8	0.9977	1.1	3.4
Asp	y = 11844x + 12395	1.3 - 18.7	0.9995	0.5	1.5
Gly	y = 14417x + 23780	1.6 - 23.1	0.9992	0.9	2.6
Glu	y = 20051x + 22753	1.4 - 21.0	0.9992	0.7	2.0
Thr	y = 17299x + 9053	1.4 - 20.3	0.9781	3.4	10.3
β-Ala	y = 23293x + 8273	1.7 - 24.2	0.9993	0.7	2.1
GABA	y = 20570x + 10146	1.6 - 23.2	0.9971	1.4	4.2
α-Ala	y = 15641x + 12005	1.4 - 19.8	0.9976	1.1	3.3
Pro	y = 967x + 6528	19.4 - 733.0	0.9972	41.5	125.8
Tyr	y = 14261x + 5948	1.3 - 19.2	0.9991	0.7	2.0
Met	y = 9826x + 1104	1.4 - 20.4	0.9983	1.0	2.9
Val	y = 32368x + 25004	1.4 - 20.7	0.9980	1.0	3.2
Trp	y = 12169x + 6888	1.3 - 19.0	0.9972	1.1	3.4
Cys	y = 4673x - 39891	22.1 - 269.2	0.9975	15.0	45.3
Orn	y = 4563x + 1893	1.4 - 20.6	0.9939	1.8	5.5
Phe	y = 28231x + 10269	1.4 - 20.2	0.9984	0.9	2.8
Ile	y = 10943x + 19277	1.4 - 20.6	0.9819	3.1	9.5
Leu	y = 30783x + 27529	1.3 - 18.6	0.9948	1.5	4.6
Lys	y = 12069x + 3519	3.0 - 43.1	0.9985	1.9	5.7

In the low concentration range, most of R^2 values ≥ 0.99 except Gln and Ile (0.98), Thr (0.97). The signal of derivatized Cys in low concentration were not detected and Pro were observed in the high concentration range due to it has been known that is the highest AA content in both samples. With higher concentration range, the obtained R^2 values ≥ 0.99 for 23 AAs.

The average LOQ value was estimated as 5 ng g^{-1} for 21 AAs, except Pro and Cys.

Table 5: Linearity in the concentration range approximately 20 – 250 µg g^{-1} for 21 AAs.

Amino Acid	Calibration curve	Linear calibration range (ng g^{-1})	Regression coefficient
His	y = 22184x - 34914	19.2 - 234.6	0.9997
Arg	y = 11496x - 642.22	19.5 - 237.9	0.9997
Asn	y = 19352x + 55842	19.5 - 237.9	0.9982
Gln	y = 4600x + 1529	19.2 - 233.8	0.9989
Ser	y = 15822x + 32365	20.8 - 254.0	0.9990
Asp	y = 12213x + 17775	18.7 - 228.4	0.9992
Gly	y = 21564x - 10223	23.1 - 191.8	0.9984
Glu	y = 14505x + 45664	21.0 - 255.6	0.9984
Thr	y = 18697x + 40261	20.3 - 247.7	0.9985
β-Ala	y = 23244x + 33285	24.2 - 200.7	0.9995
GABA	y = 18877x + 72518	23.2 - 192.9	0.9992
α-Ala	y = 14779x + 62429	19.8 - 164.1	0.9982
Tyr	y = 13042x + 70004	19.2 - 159.7	0.9927
Met	y = 10101x + 21729	20.4 - 249.1	0.9976
Val	y = 31085x + 71043	20.7 - 253.0	0.9996
Trp	y = 12389x + 9508	19.0 - 231.2	0.9982
Orn	y = 4717x + 10101	20.6 - 251.2	0.9993
Phe	y = 26712x + 66894	20.2 - 246.6	0.9976
Ile	y = 11652x + 29419	20.6 - 251.3	0.9986
Leu	y = 29146x + 149934	18.6 - 226.6	0.9978
Lys	y = 11713x - 17438	43.1 - 526.0	0.9989

4.2.Sample dilution and matrix effect evaluation

As described in section 3.4.1, the concentration of AAs in honey (table 6) and RJ (table 7) were back-calculated based on their dilution factors according to equation (3) in suitable linear range. Thereafter, using relative standard deviation (RSD) according to equation (4) of these concentrations to estimate the matrix effect. Following SANTE guideline [27], the acceptable range for ME is 80 – 120%, the suggested RSD value was at 20%, which counted the additional error from sample preparation and derivatization yield.

$$\textbf{dilution factor} = \frac{\textbf{mass}_{\textbf{sample}}}{\textbf{mass}_{\textbf{solution}}} \qquad \textbf{(3)}$$

$$\mathbf{RSD} = \frac{\sqrt{\frac{(C_{dil\ a} - C_{mean})^2 + \cdots + (C_{dil\ z} - C_{mean})^2}{n}}}{C_{mean}} \qquad \mathbf{(4)}$$

Where $\mathbf{C_{dil\ a..z}}$ are the back-calculated concentrations of AAs in sample at different dilution factors (ng g^{-1}), $\mathbf{C_{mean}}$ is the average concentration of each AA (ng g^{-1}) and n is the number of dilutions.

Table 6: Concentration of AAs in Honey using dilution approach to estimate ME.

(NA: Not Applicable, ND: Not Detected)

Amino acid	Concentration of AA in Honey sample (µg g^{-1})						RSD
	1:10	1:20	1:50	1:100	1:200	1:500	(< 20%)
His	11.1	12.1	13.7	14.9	16.9	22.6	27%
Arg	19.8	22.2	23.4	23.3	24.4	27.5	11%
Asn	49.2	50.4	34.9	41.0	51.6	85.6	34%
Gln	78.2	86.0	100.9	93.1	88.3	81.3	9%
Ser	35.1	40.5	38.0	38.7	40.3	42.3	6%
Asp	33.7	33.6	44.1	44.4	43.8	46.7	14%
Gly	5.7	73.0	182.5	7.0	60.2	150.4	92%
Glu	1.2	10.2	10.1	10.5	10.5	10.3	42%
Thr	1.4	13.2	15.3	14.8	13.6	11.0	45%
β-Ala	13.3	13.6	14.7	14.0	14.1	14.4	4%
GABA	11.4	11.4	11.8	12.0	11.9	12.4	3%
α-Ala	28.8	33.5	37.0	40.7	40.2	40.9	13%
Pro	420.0	513.2	614.8	670.9	688.5	736.2	20%
Tyr	103.1	148.0	187.0	212.0	202.3	209.1	24%
Met	ND	ND	ND	ND	ND	ND	NA
Val	21.1	20.9	21.7	24.1	23.1	24.5	7%
Trp	ND	ND	ND	ND	ND	ND	NA
Cys	ND	ND	ND	ND	ND	ND	NA
Orn	14.7	17.2	17.4	18.5	18.2	21.4	12%
Phe	125.8	171.1	196.9	201.1	197.8	197.0	16%
Ile	15.6	13.9	14.2	14.9	14.7	16.7	7%
Leu	23.2	24.3	25.2	26.1	25.1	27.6	6%
Lys	14.8	18.0	19.0	15.0	21.9	28.2	26%

The RSD values were equal or greater than 20% included His, Asn, Glu, Gly, Thr, Pro, Tyr, Lys in honey sample. It was a clear signal suppression at dilution 1:10 for Glu, Gly and Thr. In case of Trp, Met and Cys, the concentrations were below LOD in all dilutions which means they were not detected in honey sample. To eliminate ME by dilution, honey sample preparation in section 4.3 was carried out.

Table 7: Concentration of AAs in RJ using dilution approach to estimate ME.
(NA: Not Applicable, ND: Not Detected)

Amino acid	**Concentration of AA in RJ sample (µg g^{-1})**				**RSD**
	1:200	**1:400**	**1:500**	**1:1000**	**(< 20%)**
His	135.3	136.0	135.1	138.1	1.0%
Arg	421.0	406.8	417.3	418.9	1.5%
Asn	20.0	22.5	23.9	32.3	21.7%
Gln	38.4	35.6	37.5	38.8	3.8%
Ser	15.0	14.5	16.0	16.5	5.9%
Asp	274.7	269.3	232.4	257.3	7.3%
Gly	25.5	25.3	24.3	23.9	3.0%
Glu	17.8	110.8	260.1	369.2	82.3%
Thr	8.5	7.2	7.9	7.5	7.1%
β-Ala	406.6	412.5	408.5	416.2	1.0%
GABA	87.0	87.7	84.4	86.1	1.7%
α-Ala	24.0	25.5	25.1	24.4	2.7%
Pro	2864.2	3134.0	3173.5	3347.5	6.4%
Tyr	41.3	40.7	37.6	35.8	6.7%
Met	ND	ND	ND	ND	NA
Val	36.6	37.6	35.0	36.5	3.0%
Trp	ND	ND	ND	ND	NA
Cys	ND	ND	ND	ND	NA
Orn	79.6	78.3	73.2	87.3	7.4%
Phe	42.6	43.6	42.0	44.3	2.4%
Ile	25.0	27.5	25.8	28.6	6.2%
Leu	23.3	24.0	22.3	23.6	3.1%
Lys	1670.9	2173.6	2234.6	2443.5	15.4%

The signal suppression of the derivatized RJ has shown especially at dilution 1:200 for Glu in table 7. The RSD values were higher than 20% included Asn, Glu. Similar to honey sample,

Trp, Met and Cys were not detected in RJ. Therefore, RJ sample was carried out the next preparation in section 4.3 to eliminate ME.

4.3.Sample preparation modification and results

Based on the evaluation of matrix influence, there is a modification in step 1 to introduce less amount of matrix to SPE cartridge.

- Modified Step 1: Weight accurately 1 g of honey or RJ (± 0.1 g) into 15 mL centrifugal tube, add 10 mL Phosphate buffer (0.03 M, pH 2.12). Shake or vortex around 5 minutes to dissolve completely. Centrifuge for 10 min with 4000 rpm, take **1 mL** of the supernatant into 50 mL tube, add 25 mL phosphate buffer and carry out as describe in section 3.4.1 in other steps.

The post extracted solution was diluted 10 – 20 – 25 – 40 – 50 – 60 – 80 – 100 times. Meanwhile, RJ sample was tested with dilution factors are 20 – 40 – 80 – 100 times before derivatization. The RSD values of derivatized AAs after SPE modification for honey and RJ are smaller than 20%, this means that ME was eliminated.

The target of choosing dilution factor to obtain the concentrations of AAs in vial were above LOQ value for most of AAs and inside of linear ranges. Therefore, honey and RJ sample were diluted 500 and 800 times respectively before derivatization.

To shorten the drying time, the final step of SPE (step 4) was changed by taking 3 mL from 15 mL eluent with 2.5 M NH_4OH containing 10% ACN to dry and reconstitute in 10 mL of 0.1 M HCl containing 30% MeOH.

4.4.Carryover

Blank reagent sample were analyzed after injection of standard solutions to estimate the carryover. The concentrations in vial were calculated from the calibration curves. They are below of the estimated LOD values from section 4.1 (table 8), meaning carryover is neglectable.

Table 8: Concentration of AAs in blank reagent sample to estimate carryover.

Amino Acid	His	Arg	Asn	Gln	Ser	Asp	Glu	Gly	Thr	β-Ala	GABA
Average concentration in vial (ng g^{-1})	0.0	0.4	0.5	1.1	0.0	0.0	0.6	0.9	0.5	0.0	0.0

Amino Acid	α-Ala	Pro	Tyr	Met	Val	Trp	Orn	Phe	Ile	Leu	Lys
Average concentration in vial (ng g^{-1})	0.0	7.1	0.1	0.6	0.0	0.5	0.0	0.1	0.4	0.0	0.0

4.5. Accuracy

4.5.1. Repeatability

The RSD of LC retention time for 23 derivatized AA standard solutions were calculated in table 3. The repeatability is very good when the maximum value is smaller than 1.0%.

Honey and RJ unspiked samples were extracted with SPE in section 4.3 for 6 individual replicants. The dilution factors before derivatization for honey and RJ are 500 and 800 times respectively. The derivatization of samples followed procedure in section 3.4.2. Each vial was injected 2 times.

The concentration in both samples were back-calculated with RSD to estimate the repeatability in table 9. The RSD values are less than 11% in all case which is acceptable, except Ser in honey and α-Ala in RJ when comparing with the precision criteria of AOAC method of analysis at 10 and 100 µg g^{-1} are 7.3% and 5.3% respectively [28].

Table 9: Concentration of AAs in Honey and RJ unspiked sample (n=6) – Date 1.

Amino Acid	DATE 1 - Concentration in sample (µg g^{-1})					
	Honey	STD	RSD Date 1	RJ	STD	RSD Date 1
His	20.2	0.8	4.1%	112.4	4.7	4.2%
Arg	21.2	0.6	3.0%	344.2	10.7	3.1%
Asn	67.1	2.0	3.0%	16.0	0.6	3.7%
Gln	61.7	2.9	4.8%	39.8	1.8	4.5%
Ser	37.4	2.9	7.8%	12.6	0.6	5.1%
Asp	46.9	1.6	3.4%	297.5	10.4	3.5%
Gly	14.9	0.8	2.9%	29.3	1.4	3.1%
Glu	45.9	1.4	5.1%	558.2	17.3	4.8%
Thr	14.8	0.3	2.0%	15.6	0.7	4.5%
β-Ala	12.9	0.8	5.9%	426.1	17.2	4.0%
GABA	11.3	0.6	5.1%	89.5	5.1	5.7%
α-Ala	39.6	1.3	5.5%	17.0	1.7	10%
Pro	613.9	33.7	3.4%	3201.5	131.5	4.1%
Tyr	188.9	10.1	5.4%	38.3	2.1	5.5%
Val	25.9	0.6	2.4%	36.7	2.4	6.7%
Orn	17.9	1.0	5.4%	61.6	2.3	3.7%
Phe	198.3	6.2	3.1%	43.9	1.6	3.6%
Ile	15.4	0.5	3.4%	26.0	1.7	6.4%
Leu	31.4	1.0	3.3%	29.9	1.7	5.7%
Lys	14.1	0.8	6.0%	1887.0	91.8	4.9%

4.5.2. Recovery

Honey and RJ sample were spiked at 2 different concentrations. Six replicants of each concentration levels were studied in 2 different days. The average estimated LOQ values (table 4) were chosen to spike on sample. Therefore, with 21AAs, an amount of stock solution was spiked in sample to obtain the added concentration approximately 5 and 10 ng g^{-1} (as 2 times of LOQ values) in vial. With Lys, Pro and Cys, the added concentration approximately 60 and 80 ng g^{-1} in vial. Recoveries were determined according to equation (5).

$$\textbf{Recovery}\ (\%) = \frac{\mathbf{C_{spiked\ sample} - C_{unspiked\ sample}}}{\mathbf{C_{added}}} * \mathbf{100}\% \quad \textbf{(5)}$$

Where $C_{spiked\ sample}$ and $C_{unspiked\ sample}$ are the concentrations (ng g^{-1}) in spiked and unspiked sample and C_{added} is the added concentration (ng g^{-1}) to sample.

The standard solution ($\mathbf{W_{SPE}}$) which were prepared for SPE in section 3.4.1 were checked the extraction yield in table 10, according to Equation (6)

$$\textbf{SPE Recovery}\ (\%) = \frac{\mathbf{C_{STD\ found}}}{\mathbf{C_{STD\ added}}} * \mathbf{100}\% \qquad \textbf{(6)}$$

Where $C_{STD\ added}$ and $C_{STD\ found}$ are the concentrations (ng g^{-1}) which were added and found of standard solutions ($\mathbf{W_{SPE}}$)

Table 10: Recovery of SPE standard solution of 23 AAs. (ND: Not detected)

Amino Acid	His	Arg	Asn	Gln	Ser	Asp	Glu	Gly	Thr	β-Ala	GABA	α-Ala
SPE recovery	87%	96%	86%	80%	101%	108%	99%	91%	90%	92%	91%	93%

Amino Acid	Pro	Tyr	Met	Val	Trp	Cys	Orn	Phe	Ile	Leu	Lys
SPE recovery	66%	90%	73%	95%	80%	ND	105%	103%	103%	64%	89%

Recovery at 10 ng g^{-1} following AOAC guideline [28] is in the range 60 – 115% were used as a reference. Most of AAs have good recoveries with values are higher than 80%, except Pro (66%), Met (73%) and Leu (64%), Cys added concentration was below the LOD value. The recoveries of Pro and Met also were found as the lowest values in this paper [25]. Generally, at the concentration around 20 ng g^{-1}, the SPE process has acceptable recoveries.

The criteria for RSD of recovery of 5, 10, 60 and 80 ng g^{-1} are approximately at 35%, 33%, 24% and 23% respectively [28]. With 21 AAs, the recovery range is within 40 – 120% at 5 ng g^{-1}, 60 – 115% at 10 ng g^{-1} and 80 – 110% at 60 and 80 ng g^{-1} following FDA quantitative method acceptability criteria [30].

In honey sample, the recovery of Cys in table 11 was not determined due to its signal did not increase through spiking as expectation as Trp and Met. Therefore, it is required to be investigated further. At 5 ng g^{-1} spiked concentrations, there are poor recovery of Glu, Tyr, Phe and Ile. While Gln and Leu have low repeatability. In general, the recovery has been improved when increase the spiked concentration in honey sample from 5 to 10 ng g^{-1} for 20 AAs as well as the intra-day and inter-day repeatability, except Gln, Tyr, Trp and Phe. Pro has been seen with unstable signals which was confirmed in the paper [26], but acceptable results have been found for Lys.

Table 11: Recovery of Honey sample (n=6) spiked at 5, 10, 60 and 80 ng g^{-1}

Amino Acid	Honey sample spiked at 5 ng g^{-1} Recovery within 40 – 120%, RSD < 35%				Honey sample spiked at 10 ng g^{-1} Recovery within 60 – 115%, RSD < 33%			
	Intra-day		Inter-day		Intra-day		Inter-day	
	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD
His	87%	10%	89%	18%	74%	9%	77%	11%
Arg	118%	11%	112%	17%	95%	12%	97%	12%
Asn	62%	6%	65%	22%	60%	15%	60%	12%
Gln	71%	43%	97%	59%	**223%**	10%	**169%**	37%
Ser	64%	20%	80%	33%	74%	7%	72%	13%
Asp	54%	24%	67%	26%	73%	11%	68%	16%
Gly	101%	13%	101%	29%	110%	10%	96%	19%
Glu	**121%**	10%	**134%**	17%	113%	10%	103%	15%
Thr	70%	16%	84%	24%	77%	10%	78%	12%
β-Ala	95%	12%	103%	17%	86%	9%	87%	11%
GABA	114%	10%	116%	12%	97%	9%	100%	11%
α-Ala	99%	13%	96%	23%	78%	21%	82%	18%
Tyr	**195%**	19%	**147%**	87%	98%	40%	103%	36%
Met	82%	12%	87%	11%	76%	6%	77%	10%
Val	106%	11%	116%	18%	88%	13%	91%	13%
Trp	96%	10%	99%	17%	68%	50%	77%	33%
Orn	105%	12%	117%	18%	102%	14%	93%	17%
Phe	**269%**	9%	**248%**	64%	102%	59%	**119%**	44%
Ile	**172%**	8%	**130%**	35%	103%	12%	106%	12%
Leu	52%	38%	82%	42%	84%	16%	82%	20%

Amino Acid	Honey sample spiked at 60 ng g^{-1} Recovery within 80 – 110%, RSD < 24%				Honey sample spiked at 80 ng g^{-1} Recovery within 80 – 110%, RSD < 23%			
	Intra-day		Inter-day		Intra-day		Inter-day	
	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD
Pro	**160%**	20%	**136%**	39%	**38%**	53%	59%	56%
Lys	89%	11%	92%	10%	82%	9%	85%	14%

In table 12, Cys in RJ sample has shown the same trend in both spiked. Thus, further tests need to be carried out. Expectedly, Pro has shown low recovery as well as intra-day and inter-day repeatability. This amino acid also could be lost in SPE preparation since its recovery is lowest (table 10 section 4.5.2). Lys in this case did not provide acceptable results.

At spiking 5 and 10 ng g^{-1}, the high recovery showed in case of Gln, Glu, Gly, GABA and Phe. In the other hand, Arg, β-Ala and Orn were obtained better recovery but low repeatability.

Table 12: Recovery of RJ sample (n=6) spiked at 5, 10, 60 and 80 ng g^{-1}

Amino Acid	RJ sample spiked at 5 ng g^{-1} Recovery within 40 – 120%, RSD < 35%				RJ sample spiked at 10 ng g^{-1} Recovery within 60 – 115%, RSD < 33%			
	Intra-day		Inter-day		Intra-day		Inter-day	
	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD
His	98%	17%	102%	13%	97%	5%	84%	20%
Arg	69%	34%	56%	40%	111%	18%	91%	36%
Asn	80%	6%	68%	19%	61%	5%	61%	6%
Gln	**183%**	11%	**202%**	18%	**205%**	10%	**183%**	19%
Ser	117%	21%	101%	25%	85%	2%	83%	5%
Asp	117%	10%	108%	14%	94%	14%	90%	13%
Gly	**406%**	6%	**270%**	54%	**141%**	22%	**159%**	31%
Glu	**154%**	21%	102%	18%	94%	3%	91%	9%
Thr	110%	13%	90%	30%	90%	10%	90%	8%
β-Ala	77%	27%	60%	44%	97%	12%	88%	30%
GABA	**159%**	14%	**130%**	27%	104%	2%	**111%**	14%
α-Ala	101%	18%	102%	24%	93%	1%	88%	10%
Tyr	105%	17%	96%	16%	95%	4%	91%	8%
Met	82%	3%	80%	4%	90%	3%	85%	7%
Val	102%	11%	87%	20%	82%	2%	82%	7%
Trp	102%	4%	97%	8%	98%	4%	91%	10%
Orn	103%	9%	76%	39%	89%	5%	82%	18%
Phe	**126%**	8%	100%	29%	85%	3%	88%	9%
Ile	87%	23%	87%	16%	109%	24%	103%	19%
Leu	90%	13%	78%	19%	67%	19%	70%	16%

Amino Acid	RJ sample spiked at 60 ng g^{-1} Recovery within 80 – 110%, RSD < 24%				RJ sample spiked at 80 ng g^{-1} Recovery within 80 – 110%, RSD < 23%			
	Intra-day		Inter-day		Intra-day		Inter-day	
	Recovery	RSD	Recovery	RSD	Recovery	RSD	Recovery	RSD
Pro	**-187%**	12%	**-131%**	57%	**65%**	22%	**51%**	38%
Lys	**73%**	15%	**47%**	58%	**55%**	9%	**62%**	28%

In general, after excluded the out of range values, the acceptable recovery range which was obtained for honey sample 52 - 118% (intra-day). and 60 – 117% (inter-day). While in RJ sample, same scale for acceptable recovery 61 – 117% (intra-day) and 60 – 108% (inter-day)

4.6.Sample solution stability

The posted extracted solutions of Honey and RJ samples (Date 1) which were used in section 4.5.1 (Date 1) were kept in -20 ^{o}C and re-derivatized in another days (Date 2). The RSD between concentrations obtained in 2 days was used to assess the stability in table 13.

Table 13: Concentration of AAs in Honey and RJ (n=6) unspiked sample – Date 2 and Stability Comparison.

Amino Acid	DATE 2 - Concentration in sample (µg g^{-1})							
	Honey	STD	RSD Date 2	RSD 2 days	RJ	STD	RSD Date 2	RSD 2 days
His	18.5	1.8	10%	6%	133.5	3.6	3%	12%
Arg	21.7	2.0	9%	2%	373.8	9.0	2%	6%
Asn	73.4	3.3	4%	6%	19.2	0.3	2%	13%
Gln	59.9	0.8	1%	2%	34.1	0.8	2%	11%
Ser	36.4	0.8	2%	2%	11.6	0.8	7%	6%
Asp	45.8	3.5	8%	2%	245.3	2.5	1%	14%
Gly	14.0	1.4	10%	4%	27.3	0.5	2%	5%
Glu	38.1	6.2	16%	13%	496.6	8.6	2%	8%
Thr	11.1	1.1	10%	20%	10.2	0.8	8%	30%
β-Ala	10.1	0.5	5%	18%	388.8	4.8	1%	6%
GABA	9.2	0.4	4%	14%	86.2	1.6	2%	3%
α-Ala	41.7	1.0	2%	4%	24.9	1.2	5%	27%
Pro	756.9	64.3	8%	15%	4068.7	265.6	7%	17%
Tyr	222.4	6.8	3%	12%	38.2	0.3	1%	0%
Val	25.5	0.4	2%	1%	35.3	0.5	1%	3%
Orn	14.2	0.6	4%	17%	60.2	1.8	3%	2%
Phe	205.4	6.5	3%	2%	44.4	0.5	1%	1%
Ile	15.9	2.2	14%	2%	27.6	1.4	5%	4%
Leu	27.3	1.1	4%	10%	26.6	0.3	1%	8%
Lys	18.3	0.6	3%	18%	2417.4	45.0	2%	17%

The reproducibility at 10 and 100 µg g^{-1} following AOAC [28] are 11% and 8% respectively. After 2 weeks, the RSD values of the re-derivatized in honey and RJ sample showed good stability of Arg, Ser, Gly, Val and Phe and Ile. In contrast, the stability of Thr, Pro and Lys were changed (RSD ≥ 15%).

In honey sample, there is high consistency in concentrations of His, Asn, Gln, Asp, α-Ala whereas in RJ sample, Gly, Glu, β-Ala, GABA, Tyr, Orn and Leu concentrations remained stable.

4.7.Comparison results with literature

4.7.1. With Japanese honey and RJ

A recent study in 2013 by Shigeki [30] developed method using capillary electrophoresis MS/MS to determine simultaneously free AAs in RJ raw material and honey in Japan using Cys as an internal standard. The results were expressed as dry weight in table 14.

Compared with literature, the AA concentrations in RJ and honey are in good agreement. Pro has been found as a major AA in both sample; however, the obtained content in honey coffee was higher than in literature around 2 times (both cases > 180 mg kg^{-1}). Similarly, there were 3 undetectable AAs in the article samples which were Met, Trp and Cys.

Following, the second greatest AAs in coffee RJ sample were Lys, Arg, Asp and Glu. While in coffee honey, Phe contributed significantly in the free AAs contents. According to the study, Ala concentration were around 30 µg g^{-1} in RJ, revealing this could be α-Ala when compared to coffee RJ, β-Ala content was comprised remarkably.

In case of honey in the literature, one sample has the highest AA concentration belongs to Phe, not to Pro. Additionally, there was an inconsistency in Tyr and Thr concentrations which were found in coffee honey and the studied results. Those differences could be explained that the botanical and geographical origin of honey affected the free AAs components.

The concentrations of 16 AAs in the document of RJ were greater than 3 times of those in honey, the ratio was calculated in dried forms. While in coffee honey and RJ, the total of 23 free AAs were 1499.7 and 7283.1 µg g^{-1} respectively, meaning the AAs ratio less than a fifth when comparing coffee honey and coffee RJ, both samples are in as is forms. This proved in general there is noteworthy difference in RJ and honey from coffee flower.

The equation (7) can be used to convert the concentration in dried weight or as is forms.

$$\mathbf{C_{dried\ form}} = \frac{\mathbf{C_{as\ is} * 100}}{\mathbf{100 - W}} \qquad \mathbf{(7)}$$

Where $C_{dried\ form}$ and $C_{as\ is}$ are the concentrations of AAs in dried from and as is form. W is water content (%).

4.7.2. With Estonian honey

The results in this work also were compared with the data in Estonian honeys (table 14) following the article [31] since the used method in this thesis were developed from that research. The honeys were collected from heather, dandelion, linden, rape and willow flowers and presented as "as is" form. There is general agreement about Pro is the highest AA content in Estonian honeys. Meanwhile, Met were not detected in all cases and Trp was not found in heather and linden honey. In general, AA concentrations in Estonian honey are lower than in Coffee honey.

It has been seen that Phe is the second highest AA concentration in all type of Estonian honeys (39.2 µg g^{-1} in Willow honey) which was consistent with the finding in Coffee honey components; however, the Phe content in Coffee honey is higher than Estonian honeys approximately one order of magnitude, Orn concentration has shown the same trend.

In case of Lys, the found content in Coffee honey agrees relatively well with finding in Estonian honeys as well as Gly. The dominant AAs in Estonian honeys are Asp, Glu and Gly revealing the similarity between honeys.

To confirm that the differences of AA contents in Coffee honey and RJ with others could be used to characterize the origin of honey, the larger number of honey and RJ Coffee samples should be analyzed. This way, the representativeness will be assured.

Table 14: Concentration of AAs in Honey and RJ of Coffee flower (n=6) and in literature.

** In Japanese sample, Ile and Leu were determined together. NA: Not Applicable, ND: Not Detected.*

Amino Acid	Concentration (μg g^{-1}) in											
	Vietnamese sample – as is form		**Japanese sample – dried form** [30]					**Estonian honey – as is form** [31]				
	Coffee Honey	**Coffee RJ**	**RJ 1**	**RJ 2**	**Honey 1**	**Honey 2**	**Honey 3**	**Heather**	**Dandelion**	**Linden**	**Rape**	**Willow**
His	20.2	112.4	116	110	3	3.8	2.7	3.8	3.8	2.7	3.9	4.2
Arg	21.2	344.2	378	342	5.3	4.8	3.9	9.8	7.4	8.2	7.7	7.2
Asn	67.1	16.0	NA	NA	NA	NA	NA	7.9	6.7	4.5	8.4	11.7
Gln	61.7	39.8	NA	NA	NA	NA	NA	11.2	16.3	8.3	14.4	17.2
Ser	37.4	12.6	24	23	8	8.9	7.2	9.9	6.7	6.0	6.9	7.7
Asp	46.9	297.5	231	226	16	61	12	12.2	7.6	8.7	8.6	9.1
Gly	14.9	29.3	32	27	4.3	4.9	3.6	17.3	11.1	11.8	13.7	14.0
Glu	45.9	558.2	595	594	8.5	12	7.2	5.5	3.1	3.9	3.6	3.5
Thr	14.8	15.6	67	32	210	663	193	5.3	3.5	2.7	3.5	3.8
β-Ala	12.9	426.1	NA	NA	NA	NA	NA	6.9	5.6	5.1	6.3	7.0
GABA	11.3	89.5	101	86	4.9	4.4	3.5	4.1	3.2	2.3	3.3	3.9
α-Ala	39.6	17.0	30	29	9.9	12	7.3	13.8	6.6	8.4	7.6	8.6
Pro	613.9	3201.5	3500	3210	275	312	213	487.4	246.4	345.3	327.0	281.9
Tyr	188.9	38.3	34	27	19	41	14	8.7	6.8	5.2	6.7	7.5
Val	25.9	36.7	35	32	8.9	8.5	6.1	8.0	6.3	5.2	5.7	6.5
Trp	ND	ND	ND	ND	ND	ND	ND	ND	0.6	ND	0.4	0.4
Orn	17.9	61.6	NA	NA	NA	NA	NA	ND	0.6	ND	0.4	0.4
Phe	198.3	43.9	92	47	293	956	261	19.7	32.6	17.6	33.4	39.2
Ile	15.4	26.0	27*	27*	8.5*	7*	5*	5.5	3.8	3.4	4.1	4.7
Leu	31.4	29.9						7.0	5.0	3.9	5.6	6.8
Lys	14.1	1887.0	2210	2380	18	14	ND	12.0	12.7	7.2	12.6	13.5

5. SUMMARY

The derivatization of AAs with DEEMM has shown an improvement of the sensitivity of its analysis with LC ESI MS/MS in low concentrations, obtaining a fast analysis time (17 min) with good repeatability (RSD < 11%). The findings provide a reliable data which can be used to compare the performance between derivatization reagents with AAs.

In this study, the aim was to assess the concentration of 23 AAs in honey and RJ from monofloral (coffee nectar) in the same region could be confirmed as a successful method to broaden the database about free AAs in bee products. Now, pollen which is from the same origin with the analyzed sample could be a candidate for this method to understand the relationship of AAs components. Other choice could be honey from Arabica coffee flower.

SPE together with sample pre-treatment have brought a crucial benefit for this method as well as in routine using LC-ESI-MS/MS. One of the core values that must be remembered is to avoid the introduction of unknown compounds to SPE and ion source as much as possible. Definitely, other extraction methods are worthy to try to shorten the preparation period.

It is promising to be carried out full validation with robustness and spiking higher concentrations (medium and high levels) for trace AAs to confirm the determination range. Since the obtained recoveries at low concentration have shown good results in honey (15 out of 23 AAs with recovery 60 – 117%) and in RJ (14 out of 23 AAs with recovery 60 – 108%).

Using isotopic labelling was recommended to examine the matrix effect in different AAs; however, thanks to the recovery test at low concentration, there pointed which AAs need to put more effort to be analyzed with isotopic internal standards.

Lately, most of analytical research has focused on other components in honey as well as RJ to test the freshness, not in free AAs content. This study could be a stepping stone for taking the sample to ng g^{-1} concentration level to observe the signal behaviors. Thereafter, the AAs content are suggested as one of indices to be used in cleaning validation when RJ or honey were used as an active ingredient in dietary supplements, pharmaceutical products. In other case, it could be used to check the authenticity, quality of the product.

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8. ANNEX

ANNEX 1: STRUCTURE OF AMINO ACID.

ANNEX 2: STRUCTURE OF SOME DERIVATIZATION REAGENTS.

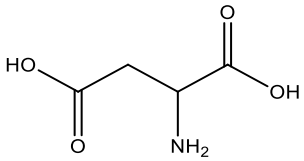
ANNEX 3: PREPARATION OF CHEMICAL SOLUTIONS.

ANNEX 4: GRAPHS OF AMINO ACIDS IN COFFEE HONEY AND RJ SAMPLE.

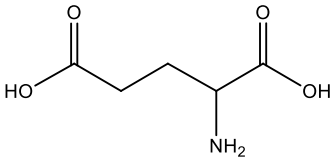
ANNEX 1: STRUCTURE OF AMINO ACID

Acidic Side Group

Aspartic Acid

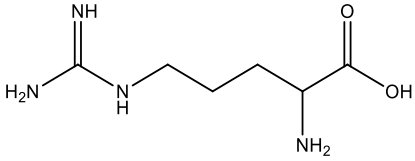


Glutamic Acid

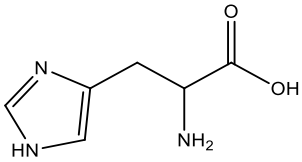


Basic Side Group

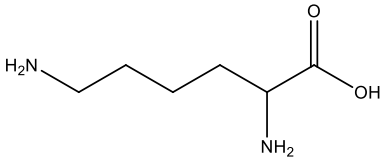
Arginine



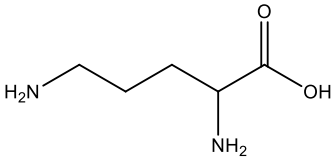
Histidine



Lysine

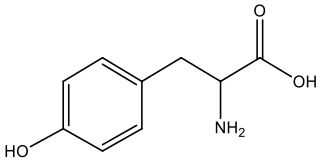


Ornithine

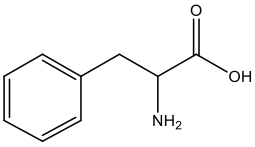


Non Polar Group

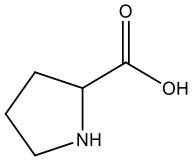
Tyrosine



Phenylalanine



Proline



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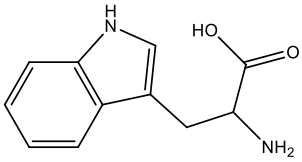
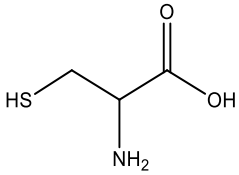
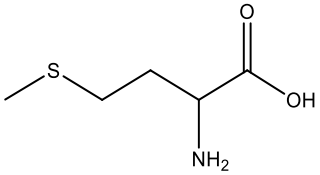


Sulfur Group

Cysteine



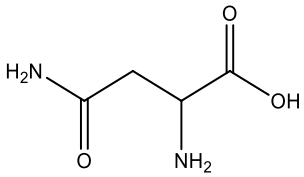
Methionine



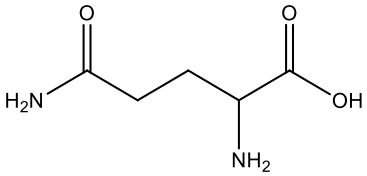
ANNEX 1: STRUCTURE OF AMINO ACID (continue)

Polar Group

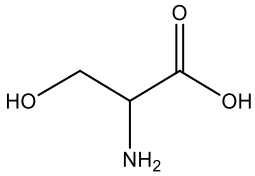
Asparagine



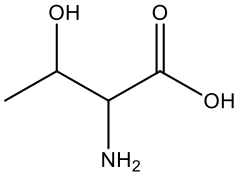
Glutamine



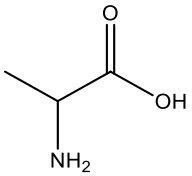
Serine



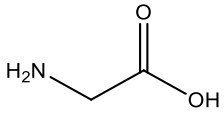
Threonine



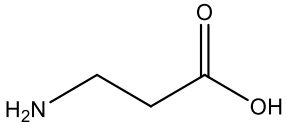
α-Alanine



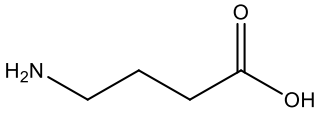
Glycine



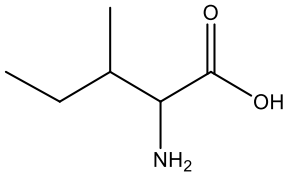
β-Alanine



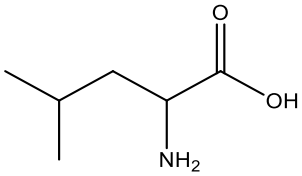
γ-Aminobutyric Acid



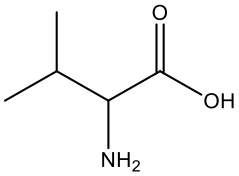
Isoleucine



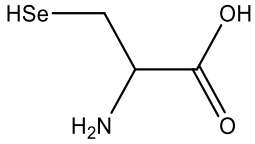
Leucine



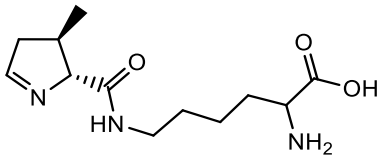
Valine



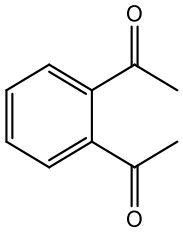
Selenocysteine



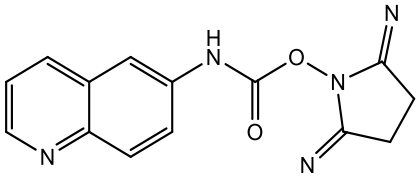
Pyrrolysine



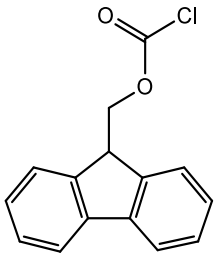
ANNEX 2: STRUCTURE OF SOME DERIVATIZATION REAGENTS.



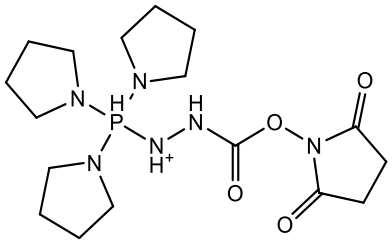
Ortho phthalaldehyde (OPA)



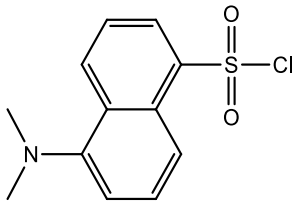
6-aminoqui-nolyl-N-hydroxy-succinimidyl carbamate (AQC)



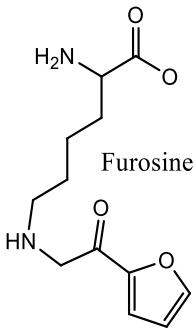
9-fluorenylmethyl chloroformate (FMOC-Cl)



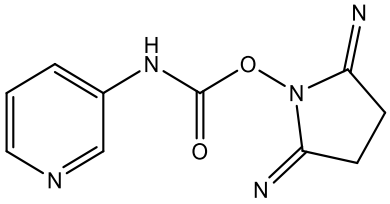
2,5-dioxopyrrolidin-1-yl N-tri(pyrrolidino) -phosphoranyl-ideneamino carbamate (FOSF)



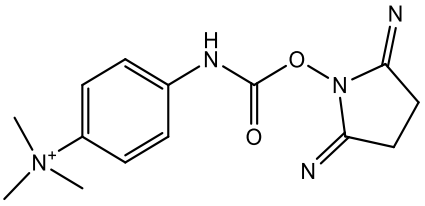
1-dimethyaminonaphthalene-5-sulfonyl chloride Dansyl chloride (DNS)



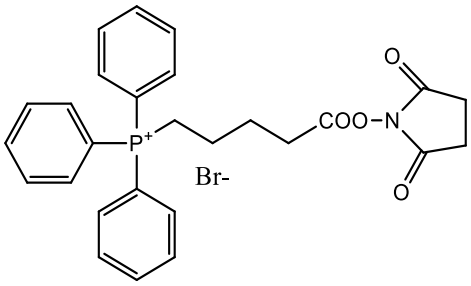
Furosine



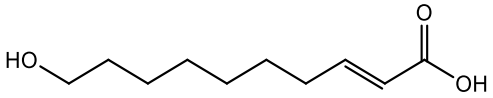
3-aminopyridyl-N-hydroxy succimidyl carbamate (APDS)



p-N,N,Ntrimethylammonioanilyl N´-hydroxysuccinimidyl carbamate iodide (TAHS)



5-N,succinimidoxy-5-oxopentyl triphenylphosphonium bromide (SPTPP)



10-hydroxy-2-decenoic acid (10-HDA)

ANNEX 3: PREPARATION OF CHEMICAL SOLUTIONS.

0.1M HCl containing 30% MeOH: Pipet 4.165 mL HCl concentrated into a beaker which contained 100 mL Milli-Q water, add Milli-Q water up to 350 mL, add 150 mL of MeOH, mix well.

Phosphate buffer (0.03M pH 2.12): Pipet 2.05 mL of H_3PO_4 concentrated (14.6M) into 1L of Milli Q water, adjust pH to 2.12 with KOH saturated.

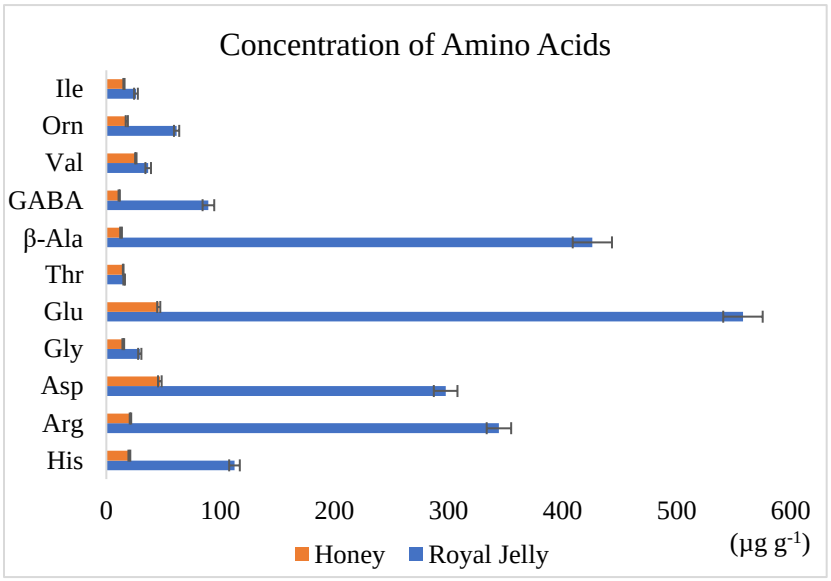
2.5M NH_4OH containing 10% Acetonitrile: Fill 154.8 mL of NH_4OH concentrated (56.6% w/w) into 1L cylinder, add Milli Q water up to 900 mL, add 100 mL of Acetonitrile, mix well.

Borate Buffer (0.75M pH 9.0): Weigh 11.59g of H_3BO_4 in beaker 250mL, add 250mL Milli-Q water, the mixture could be warmed up to dissolve faster with magnetic stirrer, adjust pH with KOH saturated.

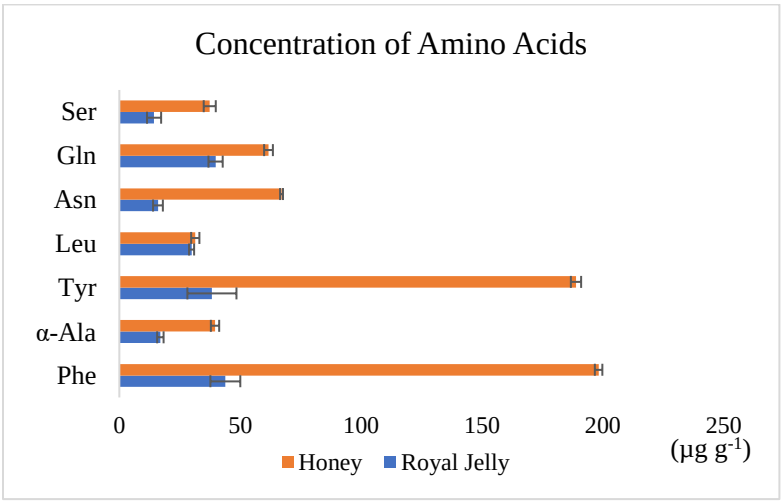
Mixture of DEEMM:Methanol (1:50) (v/v) – prepared by volume

Formic Acid (0.1%) (v/v) – prepared by volume and filtered through 0.2 µm PVDF membrane filter.

ANNEX 4: CHARTS OF AMINO ACIDS IN COFFEE HONEY AND RJ SAMPLE.



Graph 1: Concentration of Amino Acids in Honey and Royal Jelly, in order that dominant AAs in Honey



Graph 2: Concentration of Amino Acids in Honey and Royal Jelly, in order that dominant AAs in RJ

Determination of Amino Acids in Bee Products by Diethyl ethoxymethylenemalonate Derivatization Using LC-ESI-MS/MS

Amino acid content of honey and royal jelly has been used for the product authentication, geographical and botanical origin evaluation, indication of freshness and nutritional value. In this thesis analytical method was tested and improved for analysis of the concentration of free amino acids in Vietnamese monofloral (Robusta coffee) honey and Royal Jelly by derivatization with diethyl ethoxymethylenemalonate (DEEMM) using liquid chromatography coupled to mass spectrometry (LC-MS/MS). The derivatization proceeds under aqueous conditions and is sufficiently fast reaction for most amino acids. Limits of quantitation at ng g^{-1} level were achieved for most amino acids. The method has good repeatability and recovery in general. Determined amino acid contents agree, in general, well with literature findings in different honey and royal jelly samples. The differences found may be indicative of botanical/geographical origin of these bee products.

Keywords: Amino Acids, Robusta coffee, honey, Royal Jelly, LC-MS/MS
CERCS code: P300

Aminohapete määramine mees ja mesilaspiimas LC-ESI-MS/MS meetodil kasutades derivatiseerimist dietüül-etoksümetüleen malonaadiga

Mee ja mesilaspiima aminohapete sisaldusi on kasutatud nende produktide autentsuse ja päritolu tõestamiseks ja värskuse ning toiteväärtuse hindamiseks. Käesolevas töös katsetati ja parendati dietüül-etoksümetüleen malonaadiga (DEEMM) derivatiseerimisega vedelikkromatograafia-massispektromeetria (LC-MS/MS) metoodikat aminohapete määramiseks Vietnami päritolu monofloorsest (Robusta kohv) meest ja mesilaspiimast. Derivatiseerimine kulgeb vesikeskkonnas enamiku aminohapetega piisava kiirusega. Enamiku aminohapete jaoks saavutati ng g^{-1} tasemel määramispiir. Samuti on metoodikal hea korratavus ning saagis. Leitud aminohapete sisalduses olid üldiselt kooskõlas kirjanduses toodud erinevate mete ja mesilaspiima aminohapete sisaldustega. Leitud erinevused võivad osutuda botaanilise/geograafilise päritolu indikaatoriteks.

Keywords: aminohapped, Robusta kohv, mesi, mesilaspiim, LC-MS/MS
CERCS kood: P300

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