Review Article

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Amino Acids Profiling in Fruit Juices by High Performance Liquid Chromatography: A Review

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Abstract

In the preservation of normal physiological functions, the building blocks of the body called amino acids play a crucial role. A number of valuable and nutritional phytoconstituents are contained in fruit juices, such as vitamins, minerals, microelements, organic acids, antioxidants, flavonoids, amino acids and other components. Due to the growing population and demand, the quality of fruit juices is decreasing. One of the unethical and harmful practices called adulteration or food fraudulence has been adopted by most food and beverage industries. The amino acids which is one of the most important phytochemicals of fruit and fruit juices which affects the organoleptic properties like color, odor, and taste of juices and also helps in authenticity process from governing bodies by providing total amino acid content. Consequently, the main aim of the present review work is to provide information regarding the importance of amino acids, how they are adulterated, the potential analytical approach to detected amino acids and which methods are generally accepted method by the food industries. According to the literature review, we presume that reverse phased high-performance liquid chromatography with pre-column derivatization was the most adopted method for quality checking due to its advantages over other old and recent analytical approaches like simple, rapid, cost-effective nature, less / no sample matrix effect with high sensitivity, accuracy, and precision.

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INTRODUCTION

Amino acids are one of the essential molecules in all the fundamental and well-known biological processes of the body (Acquaviva *et al.*, 2014; Otter, 2012). The food products based on plants and animal are high in amino acids. All vital amino acids are only reached by food products to the body. There are so many important elements in fruit and fruit juices, including sugars, proteins, amino acids, vitamins, flavonoids and others. This is the rationale behind the demand for juices (Figure 1). The following are 8 essential amino acids, typically found in various fruit juices with the exception of papaya and banana, such as proline, aspartic acid, serine, asparagine, glutamic acid, alanine, aminobutyric acid and arginine, which make up about 90-95 percent of the free amino acids. Glycine is abundant in papaya, while leucine and histidine are high in banana valine. But the concentration of these 8 amino acids in different fruit types through varying. Although the fruits are seasonal, they are made available throughout the year with the help of processing technology. For commercial processing of fruit juices, various fruit varieties such as apple, orange, apricot, grapes, pomegranate, pineapple are used. The quality of these juices is tested by using numerous chemicals, physical and microbiological techniques. These are now adulterated with other chemicals, such as colorants and the addition of inexpensive juice from other less expensive fruit varieties, which are not naturally present in the juice, which is depicted in Table 1 and Figure 2 respectively. In some cases, Low-cost amino acids (glycine, glutamic acid) or protein hydrolytes are often used to increase the total content of amino acids in fruit juices (Dasenaki and Thomaidis, 2019). By the characterization of amino acids profile in the juices, adulterations can be detected and also variation in the amino acid concentration from the standard values is an indication of adulteration. Amino acids are relatively weak chromophores in their native form, and they fail to absorb UV light. Quite a few amino acids contain chromophore groups, but several are non-fluorescence amino acids, they must also be chemically modified/derivatized for analytical purposes for this reason. In human tissues, body fluids, dietary supplements and drugs, several free amino acids are found, Liquid chromatography has been the preferred technique for separating these compounds. The amount and structure of amino acids influence the consistency of foods, such as taste, aroma and colour, using amino acids as markers, the genuineness of fruit juices is checked by. There is a clear trend in the development of an inexpensive, easy and reliable analysis system to determine the quality of foods for dietary and regulatory purposes.

So many analytical techniques for the study of amino acids have been suggested, including spectrophotometer gas chromatography, high-performance liquid chromatography (HPLC), ion-exchange chromatography, flow injection analysis and capillary electrophoresis. In particularly they are analysed after derivatization especially, precolumn derivatization with reversed-phase HPLC due to the short time, simple instrumentation, and low-cost required (Sun *et al.*, 2012; Kaspar *et al.*, 2009).

In the global market, orange juice products have been the major segment and nearly accounts for 42%.

Objectives Of This Review Work

Now -a- days as the population increases the quality of the food materials, including fruit juices, also decreases. Safe, useful and expensive amino acids are substituted with low-grade, cheaper amino acids in fruit juices in order to increase the amino acid content. In fruit juices, amino acids play an important role and also affect the various organoleptic properties, such as taste, smell and consistency (Antolovich *et al.*, 2001). Therefore, it is important to research amino acids in fruit juices and the goals of this current review paper are

1. How can we detect amino acids in fruit juices?
2. What are the various possible methods to detect amino acids and which is mostly accepted method?
3. What is the actual amount of amino acids in fruit juices and how much adulteration is taken place?

Materials Required

The following Table 2 gives brief information regarding various chemicals useful in amino acid profiling in fruit juices.

Derivatization

Most of the amino acids are inactive towards UV absorbance and non- fluorescence in nature. In order to convert into an active molecule, derivatization is necessary prior to analysis (Botoran *et al.*, 2019), which represented in Figure 3. It is one of the important steps in the analysis of amino acids and it is necessary to make sure that the

1. Reaction is complete
2. Controlling of various factors affecting the derivatization process (temperature, pH, reaction time etc.)
3. A product which is formed should be stable.

The derivatization of amino acids is done in 2 ways.

1. Pre-column derivatization
2. Post-column derivatization

The following Table 3. summarizes some of the advantages and disadvantages and most commonly used reagent examples (Munir and Badri, 2020).

Chemical reaction

Derivatization free methods for detection of amino acids

Mostly derivatization method is preferred for analysis of amino acids, but it has few drawbacks like lengthy reaction procedure; unstability of the derivatized products; the necessity of removal of excess reagent before analysis.

Table 1: Adulteration types, examples, methods and overall adverse effects.

Type of adulteration	Example	Possible methods to detect adulteration	Comment	Overall adverse effects of adulterated juices	Reference
Simple dilution	water	ºBrix ratio by refractometer, Isotopic methods	The ratio of 18 O isotope is used for detection of water content	The adulterated juices may harm to the digestive system and also causes 1. Ulceration 2. Gastric problems 3. Allergic reactions Juice to adulteration affects the drug transport mechanism and reduces the bioavailability	(Li *et al.*, 2012)
Sugars	Cane or corn, Beet sugars Invert sucrose	Stable isotope ratio analysis (SIRA), Chromatographic techniques	The added sugars can be detected by using 13 C isotope ratios.		
Cheaper juices	Grape, apple, pear juices	Phytochemicals like sorbitol, anthocyanins, polyphenols are used as a marker for detection of juice to juice adulteration.	Due to the demand for costly juices like pomegranate, citrus is frequently mixed with cheaper juices.		
Pulp wash & peel extract	Citrus and other fruits	Isotope analysis, Total flavonoid content analysis by chromatographic techniques	15 N isotope ratio and high flavonoids is an indication of added pulp wash to increase juice content		
Substitution with cheaper amino acids & organic acids	Glycine, glutamic acids, malic & citric acids	DNA based techniques And chromatographic methods like capillary electrophoresis HPLC, GC, NMR, Mass spectroscopy.	These 2 amino acid s are very cheap compared to other amino acids. So, they are frequently used to raise the overall amino acid content of any particular juice. Mostly natural fruit juices contain L- amino acids only. The presence of D- amino acids are indicative of low graded juice.		

Table 2: Most commonly used chemicals for amino acid profiling

S.no	Chemical	Most commonly used examples
1.	Buffers	sodium hydroxide, sodium acetate, sodium borate, carbonate buffers, Hydrochloric acid.
2.	Derivatizing agents	6-aminoquinolyl-N-hydroxy succinimidyl carbamate (AQC), phenyl isothiocyanate (PITC), o-phthalaldehyde (OPA), 9-fluorenylmethyl-chloroformate (FMOC-Cl), 1-fluoro-2,4-dinitrobenzene, 1-fluoro-2,4-dinitrophenyl-5-L-alanine amide, Dansyl-chloride, 4-fluoro-7-nitro-benzo-2-oxa-1,3-diazole, 1,2-naphthoquinone-4 sulphonate, acridone-N-acetyl chloride (ARC-C1), carbazole-9-acetyl chloride (CRA-C1) and carbazole-9-propionyl chloride (CRP-C1).
3.	Columns	C18 column Octadecyl sillyl column
4.	Mobile phase (HPLC grade solvents)	Acetonitrile: water (40:10%) Acetate buffer: Acetonitrile
5.	Detector	Fluorescence detector Photodiode array detector

Table 3: Advantages and Disadvantages of the derivatization process

	Advantages	Disadvantages	Examples	Mode of detection
Pre-column Derivatization	Reduces Consumption of reagent. Sensitivity gets increases. Reagents are freely available.	Sample matrix effect. Unstability of reaction products.	O-Phthalaldehyde phenyl Isothiocyanate Dansyl chloride	Fluorescence detector UV detectors
Post-column Derivatization	Less/ no sample matrix effect during the derivatization process	Needs a substantial amount of reagent. Reversed-phase chromatography is not useful. Very few reagents are available.	Ninhydrin O-Phthalaldehyde Fluorescamine	Colorimetry Fluorescence detector

Table 4: Important parameters with optimum conditions

S.no	Parameter	Example/ condition
1	Derivatizing agent	6-aminoquinolyl-N-hydroxy succinimidyl carbamate (AQC)
2	Borate buffer	pH - 8.0 Concentration - 20mM
3	Reaction time	10-15 min
4	Temperature	55

Table 5: The findings of adulteration in samples of fruit juice

Name of Fruit Juice	No of samples	Adulterated samples- N (%)
Orange	13	7 (53.8%)
Sure Cherry	13	4 (30.7%)
Grape	8	3 (37.5%)
Apple	4	2 (50%)
Pomegranate	3	1 (33.3%)
Pineapple	6	2 (33.3%)
Peach	11	2 (40%)
Apricot	3	1 (33.3%)
Total	66	26 (39.4%)

Table 6: Profile of amino acids in some fruit juices in mg/L

A/A fruit	Pineapple	Orange	Mango	Peach	Sour Cherry	Apple	Grape	Pomegranate	Apricot
Asp	51	35	370	353	195	155	77	171	387
Glu	106	172	96	223	168	15	98	19	7
Ser	83	28	147	182	27	17	57	198	49
His	22	11	8	28	40	12	50	40	49
Gly	13	1	12	16	3	1	4	59	15
Arg	17	366	358	1	2	1	592	121	57
Ala	111	171	86	132	7	14	172	42	83
Tyr	26	5	50	9	1	1	26	30	66
Met	11	2	6	0.6	0.1	0.3	12	27	24
Val	24	15	16	42	4	5	47	33	25
Phe	20	6	19	27	1	1	21	5	9
Ile	12	6	10	30	ND	16	33	12	10
Luc	13	10	9.84	14	21	1	51	8	21
Lys	14	20	73	3	1	1	4	77	60

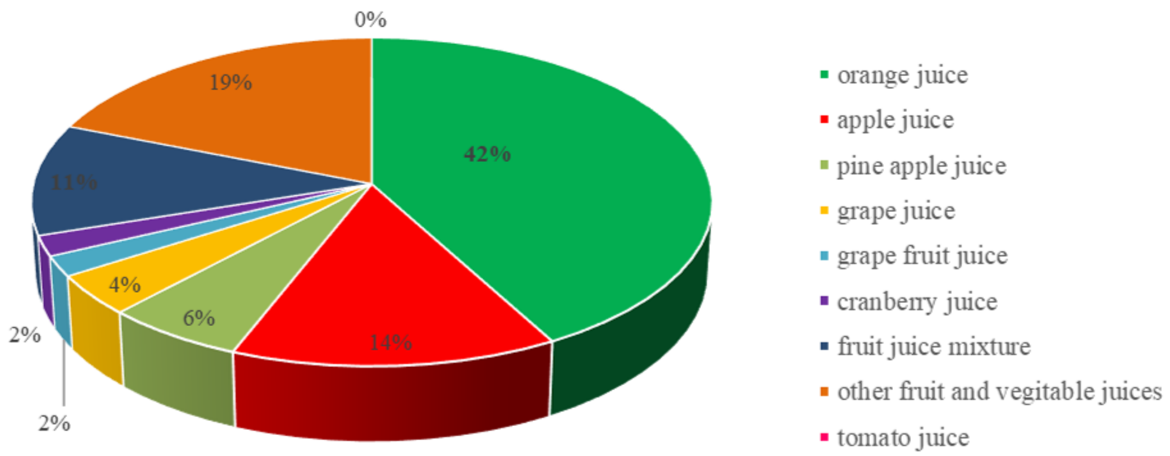


Figure 1: An overview of annually traded fruit juice products around the globe

Table 7: Examples of fruit juices with its amino acids profiling by HPLC method

Amino acids found	Type of juice	Separation method	Conditions	Derivatizing agent	Reference
Asp, Glu, Asn, His, Arg, Gly, Thr, Ala, Tyr, Met, Val, Trp, Phe, Ile, Leu, Lys	Water melon& lime	HPLC	M/P (A) sodium acetate (B) ACN: methanol: water (50:32:18) FLD	OPA	(Asadpoor *et al.*, 2014)
Asp, Glu, Asn, His, Arg, Gly, Ala, Met, Trp, Phe, Ile, Leu, Lys, Ser, Gln	Orange	HPLC	Solvent A: 0.01M sodium acetate+0.01M acetic acid in H2O: methanol: acetonitrile (8:1:1) Solvent B water: methanol: acetonitrile (1:2:2) Fluorescence detector	OPA	(Corleto *et al.*, 2019)
Asp, Glu, Asn, Ser, Gln, Ala, His, Hse, Gly, Thr, Arg, β-Ala, Ala, Tyr, Val, Phe, Ile, Leu	Apple juice	HPLC	Solvent A: dihydrogen orthophosphate treated with sodium nitrate& tetrahydrofuran Solvent B: methanol	OPA/ 2-mercaptoethanol	(Peter, 1986)
L-Asp, L-Glu, L-Asn, L-Arg, L-Glu, L-Ala, L-Trp, L-Leu, D- Pro, L-Pro L-Ser, L-Gln	Pomegranate juice	MEKC-LIF	Sodium dodecylbenzene sulphonate as a surfactant	Fluorescein isothiocyanate	(Gomis *et al.*, 1990)
Proline	Grape juice	HPLC	M/P A: 30mM sodium acetate trihydrate, 0.1 M triplex III and 0.25% tetrahydrofuran in water. M/P B: 100mM sodium acetate trihydrate, 0.1 M triplex III, in water and acetonitrile (20:80%) Fluorescence detector	FMOC	(Tezcan *et al.*, 2013)

Table 8: Examples of fruit juices with its amino acids profiling by HPLC method

Amino acids found	Type of juice	Separation method	Conditions	Derivatizing agent	reference
Asn, Ser, Asp+Arg, Glu, Pro, GABA	Pear, apricot, strawberry raspberry, blackberry	HPLC	Acetonitrile containing tri-ethanolamine phosphate buffer (pH 3.0) UV-detector	FDAA	(Long *et al.*, 2012)
Asp, Glu, Asn, His, Arg, Gly, Ala, Met, Trp, Phe, Ile, Leu, Lys, Thr, GABA, Pro, Cys, Tyr, Val, Ser, Gln	Strawberry, Longan, Apple, Pear, Litchi, Orange	HPLC	20 mM sodium acetate trihydrate with 0.04% TEA and acetonitrile. Fluorescence detector	AQC	(Kuneman *et al.*, 1988)
Asp, Glu, Asn, Hys, Arg, Gly, Ala, Met, Thr, Phe, Ile, Lys, Tyr, Val, Ser, Pro	Orange, grape, pear, pineapple, peach, apricot	HPLC	50 mM acetate buffer and acetonitrile. Photodiode array detector	FMOC	(Zeng *et al.*, 2015)
Tyr, Phe	Grape, watermelon	EME followed by HPLC	Acetonitrile and HPLC water (5:95% v/v) UV- detector	-	(Fabiani *et al.*, 2002)
Ile, His, Trp, Pro, Phe, Tyr, Glu, Asp	Apple juice	Open tubular nano liquid chromatography	Acetonitrile: methanol: water (85:10:5% V/V)	-	(Sedehi *et al.*, 2018; Aydogan, 2018)

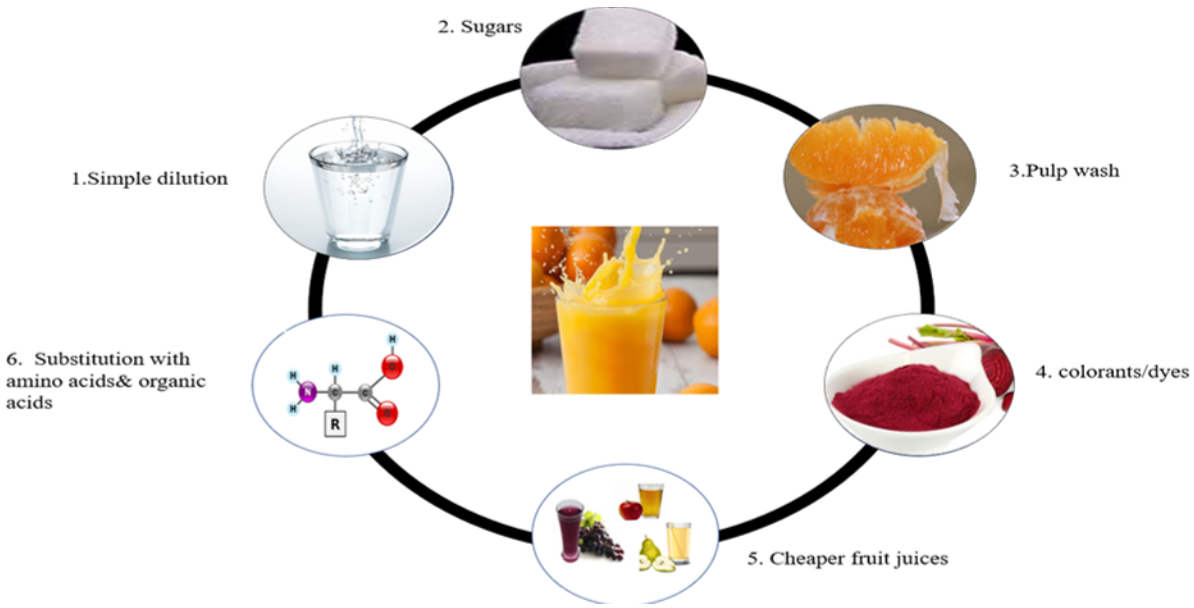


Figure 2: Possible modes of adulteration of fruit juices

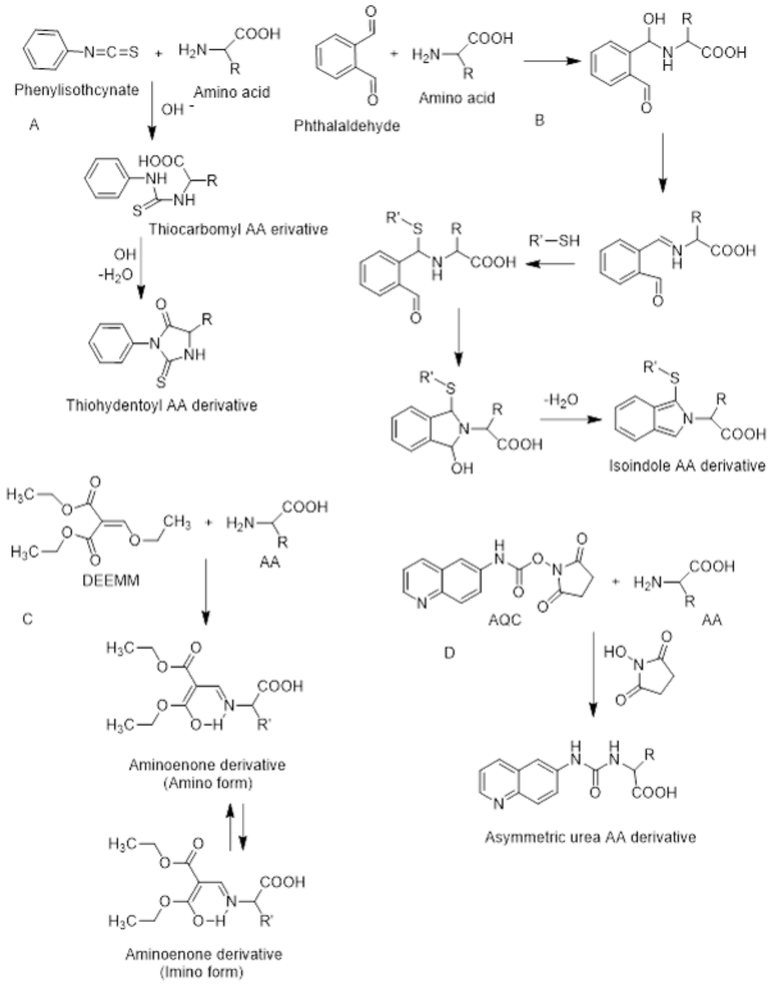


Figure 3: Derivatization reaction of amino acid with derivatizing reagent

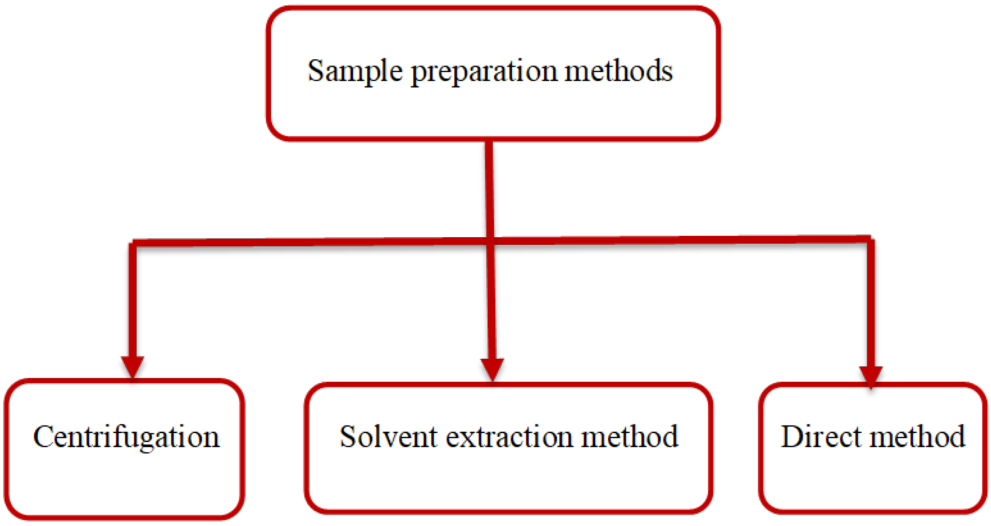


Figure 4: Separation methods for amino acids analysis

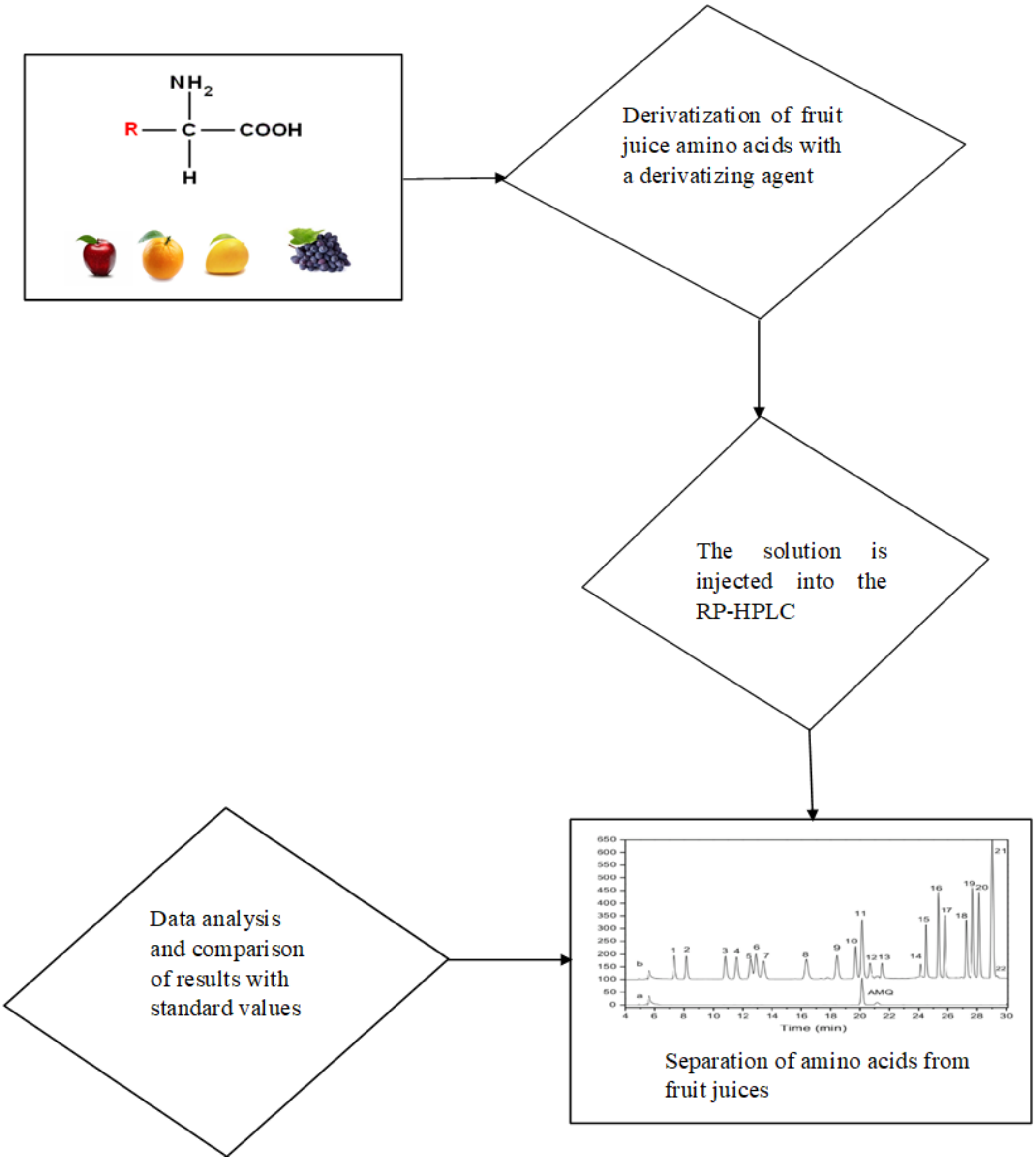


Figure 5: General flow chart of amino acid profiling by RP-HPLC method

Due to that drawbacks, sometimes analysis is carried out without derivatization (Tyler, 2001). Separation of amino acids without derivatization was accomplished by reverse-phase ion-pair chromatography using UV- detector. The method was developed for the determination of free amino acids and vitamins in intravenous solutions and beverages without derivatization by Schuster and separation was mainly performed on an NH_2 column.

Sample preparation for the analysis of amino acid profiling in fruit juices

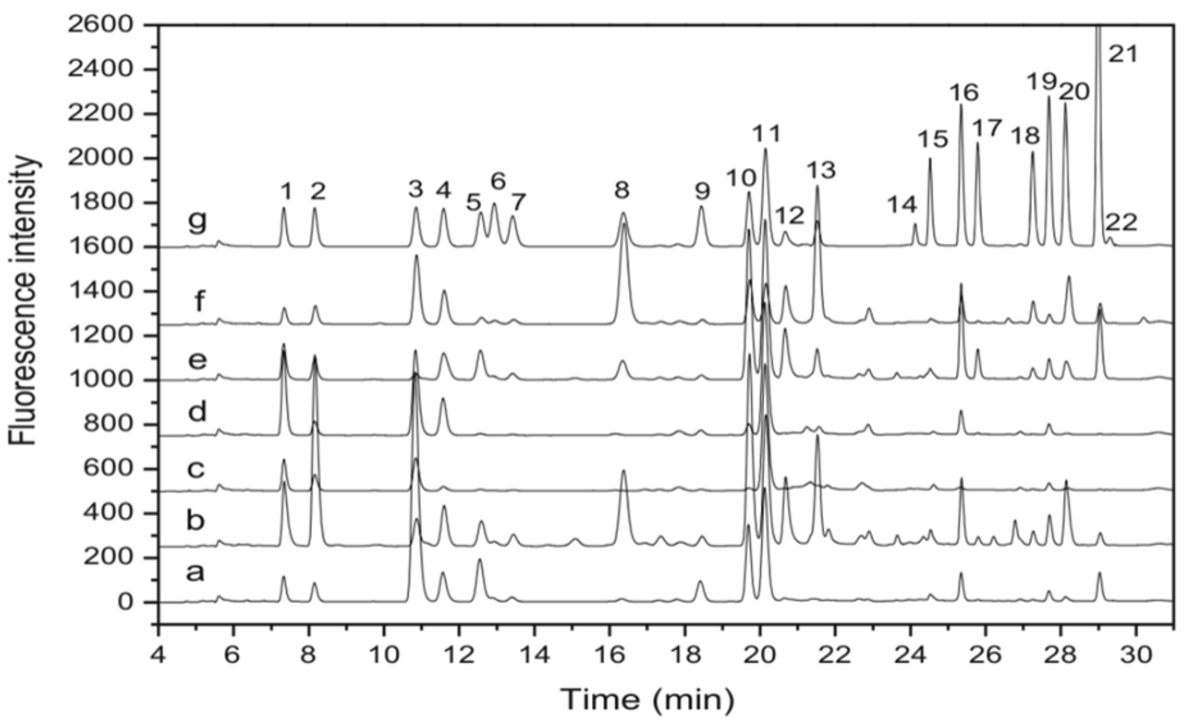
The commonly used methods for amino acid analysis in various food samples are mentioned in Figure 4.

Centrifugation

It is commonly preferred to separate, insoluble solids from the process of centrifugation of fruit juices and it is also the easiest method for sample preparation. The extracted juice is usually centrifuged at 10000 rpm in a high-speed refrigerated centrifuge for 15 mins. The supernatant extracted from the centrifuge is loaded into a test tube and stored at 0°C for further study.

Method of solvent extraction

In this method, the solvents like methanol: chloroform: water (12: 5: 3 V/V) are used to extract free amino acids from fruit juices. Again, we need to mix the clear part of the solution with chloroform: water solution in the ratio of 2:3 V/V. The supernatant obtained after centrifugation is condensed by a rotary vaccine evaporator. For high viscous fruit juices, this is an appropriate method.



a. Strawberries **b**. Longan **c.** Apple **d.** Pear, **e.** Litchi **f.** Orange **g.** Amino acid Standard

Figure 6: Standard and sample amino acid chromatogram of fruit juices

Direct method

Here the fruit juices are combined directly with a filtering agent like celite and filtered on a Buchner funnel by whatmann filter paper. The filtrate can be used specifically for the study of amino acids.

Among those three methods, the centrifugation method is widely used due to its simplicity, easy to handle, cost-effective and quicker results. It doesn't require any additional technical skills.

Methodology

The C18 column with a guard column was primarily used to isolate amino acids from fruit juices. Here the guard column plays a crucial role in shielding of the main parent/ analytical column from various interfering ions/particles and increases the life span of the column because the fruit juices contain a number of phytochemicals (Elfakir, 2005). The separated amino acids were detected by fluorescence type of detectors. We need to set optimum conditions like column temperature, flow rate, injection volume, mobile phase ratio and detection wavelength etc. A brief methodology of amino acid profiling was mentioned in Figure 5. The standard amino acid solutions and sample solutions are injected into the HPLC instrument and chromatograms were developed at optimum conditions.

Chromatographic conditions

Stationary phase - RP- C_{18} column (250- × 4-mm, 5-μm)

Guard column - same material as that of the analytical column

Mobile phase - 50mM acetate buffer (pH 4.2) as eluent A and acetonitrile as eluent B.

Flow rate - 1.0 mL/min

Flow volume - 20-μL

Detector - photo diode-array

RESULTS AND DISCUSSION

In the present review paper, we mentioned about various optimum conditions in order to get the best results like good retention, resolution and shape of peaks in a chromatogram. As per the literature review, even though a number of buffers available, borate buffer (20 mM, pH 8.0) is best and offers maximum peak area. The proper separation of amino acids depends on derivatization efficiency, which is affected by a number of parameters like concentration of the derivatizing agent, buffer pH, temperature and reaction time. The few parameters with optimized conditions are mentioned in the Table 4 where most of the amino acid derivatives are analyzed. The standard and sample amino acid chromatogram, as shown in Figure 6.

In apricot juices, asparagine was the major amino acid accounting for up to 78% of the total amino acid content (versari et al.). But the available apricot samples are showing amino acid content less than the total amino acid content (Mena *et al.*, 2012). In pomegranate juice, proline was the main amino acid and is representative of certain added grape products if its content is greater than 25 mg / L (Versari *et al.*, 2008).

As per literature review, a complex amino acid profile was found in fruit juices and most of the fruit juices are adulterated with other cheap components, and they contain less concentration of amino acids compare to the labelled information and it is shown in Table 5 (Zhang *et al.*, 2009).

Grape juice

Out of eight grape juice samples, 3 samples are Froude and contains (37.5%) lower concentration than labelled.

Apple juice

Out of four samples, 2 samples contain (50%) lower concentration of amino acid than the labelled information.

Orange juice

Seven samples contain (53.8%) less concentration of amino acids out of 13 samples than the labelled information.

Pineapple juice

The two samples are froude out of 4 tested samples and contain (40%) lower concentration of amino acids than the original content.

Mango juice

Out of 3 tested mango juice samples, one sample contains (33.3%) lower concentration levels of amino acid.

Among various analytical methods for amino acid profiling in fruit juices, RP-HPLC is a most accurate, reliable method and it is adopted by most of the food industries as a tool for the quality checker. The actual amount of each amino acids in some fruit juices was represented in the Table 6 as per available scientific information. We found that the high-performance liquid chromatography is one of the most important and commonly employed technique through the literature survey which is mentioned in Tables 7 and 8 for all the fruits by the food industries to check its quality for authenticity.

CONCLUSIONS

The amino acid profiling in fruit juices and the comparison of the sample amino acid profile with the regular values serve as a quality control indicator. The derivatives which are formed through pre-column derivatization are more stable than post-column derivatization. So mostly reverse phased High-performance liquid chromatography is preferred for amino acid profiling in fruit juices because of its simplicity, accurate and reliability. The different detection systems have been employed for the analysis of amino acids in the following order, UV and diode array detection, Fluorescence detection, Electrochemical detection (rarely used), Mass spectrometry detection.

Conflict of Interest

The authors declare that they have no conflict of interest for this study.

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