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Development of investigation method for *Citrus reticulata* Blanco *cv.* Sai-Nam-Phueng juice in bottle packaging by applying amino acid profiles

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ABSTRACT

This research aimed to develop investigation method for real orange juice products in the market to appropriately obtain a tax exemption. The existing formol titration method is unable to fully identify nutrients or chemicals in the products, resulting in inaccurate tax exemption decisions. The present study applied the orange juice (*Citrus reticulata* Blanco *cv.* Sai-Nam-Phueng) amino acid profile as a reference profile, evaluated the orange juice products based on the bottle label information, and compared the results to the previous technique. To assess the amino acid profile of squeezed orange juice, high-performance liquid chromatography (HPLC) was used. Thirty orange juice samples were collected from market products around Bangkok and its vicinity in hermetically sealed packaging. The HPLC chromatograms demonstrated that six amino acids; glutamic acid, asparagine, serine, arginine, alanine, and valine, were substantially distinguishable at a confidence level of 95%. Aspartic acid was not found to be similarly distinguishable by HPLC. The amino acid profile could be used to inspect marketed orange juice products, distinguish real from fake orange juice, and determine the tax exemption.

Keywords: orange juice; amino acid; bottle packaging; HPLC; tax exemption

1. INTRODUCTION

Beverage products are a kind of commodity subject to tax collection by the Excise Department under the Excise Tax Act in Thailand, B.E. 2558 (2015), which covers fruit and vegetable juice. To support Thai farmers, the Excise Department has issued a notification for tax exemption or reduction. If fruit juice entrepreneurs can produce or import their products with a mixture ratio that meets the specified criteria, they can get a tax privilege of 0% (The Excise Department, 2022; The Excise Department, 2019a). Orange juice must contain at least 20% real fruit juice to be eligible for tax reduction, and fruit juices submitted for tax exemption must be inspected by the Excise Department to ensure that the percentage of fruit juice is as declared. Formol titration is commonly used to test fruit and vegetable juice products to determine the total amino acid concentration in samples (FAO, 1986; CEN, 1994). However, the percentage of the fruit juice identified by formol titration does not offer adequate information in terms of distinguishing fruit varieties or other compounds added, like high-performance liquid chromatography (HPLC) (Subroto et al., 2020). The procedure results in some samples gaining tax benefits in an incorrect manner, particularly those containing a mixture of ingredients besides real juice.

In general, there are two types of fruit and vegetable juices on the market: pure juice and flavored juice with added nutrients or other substances. The former is largely composed of concentrated fruit or pure fruit juice combined with water and other ingredients, whereas the latter is mostly composed of concentrated fruit or pure fruit mixed with water and other additives, with the volume of real juice being less than 10%. The ingredient information for the mixture as well as the type of fruit is normally listed on the fruit juice packaging label to let customers decide whether they prefer real or flavored juice. In 2021, Thailand's fruit juice market had a total domestic sales and export value of about 421.34 million US dollars, or roughly 16,065.39 million Thai baht, an increase of 8.99% from the previous year, and orange juice is one of the top 5 export fruit juice market products, with a value of about 19 million US dollars, or roughly 724.46 million Thai baht (Trade Strengthening Policy Division, 2022). Tax advantages can reduce costs, thereby increasing market competition among operators. However, as previously stated, the method of determining the proportion of fruit juice by formol titration did not reveal the kind and other components blended in the juice; rather, it just provided quantitative findings of amino acids, which had an effect on the tax privilege decision.

According to Nelson and Cox (2005), amino acids are organic molecules that include both amino and carboxylic acid functional groups. They can be identified using HPLC (Asadpoor et al., 2014; Subroto et al., 2020). In accordance with Asadpoor et al. (2014), the quantity of amino acids and comparison of sample amino acid profiles with the standard values could be applied for quality control and decision-making, regarding the truth of fruit juice (Asadpoor et al., 2014; Dasenaki and Thomaidis, 2019). Consequently, to detect fruit juice adulteration and to obtain tax benefits, the present study utilized the formol titration in conjunction with the chromatographic characterization of amino acids. Orange juice is the most popular consumed fruit juice beverage, and *Citrus reticulata* Blanco cv. Sai-Nam-Phueng (SNP), one of the most widely grown cultivars of tangerine (Kwangjai et al., 2021; Navarattara et al., 2022), is frequently used in production of orange juice in Thailand (Kwangjai et al., 2021). Therefore, the amino acid profiles of SNP orange juice were determined using HPLC as a standard for the SNP amino acids, and compared to marketed orange juice profiles.

2. MATERIALS AND METHODS

2.1 Materials

SNP oranges were from, three areas, which were Fang district in Chiang Mai province, Phu Ruea district in Loei province, and Phop Phra district in Tak province. The standard amino acids for the study comprised aspartic acid (Asp), glutamic acid (Glu), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), Alanine (Ala), tyrosine (Tyr), cysteine (Cys), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), lysine (Lys), and proline (Pro), were purchased from Agilent Technologies (Thailand) Ltd. (Bangkok, Thailand). The marketed orange juice samples collected from several locations throughout Bangkok and its vicinity, such as grocery stores, supermarkets, and department stores were used.

2.2 SNP orange juice preparation

To find out the SNP standard amino acid profile, SNP oranges with a circumference of around 5-6 cm and a greenish-yellowish peel color after harvesting were chosen at random from markets in the three areas. Two oranges from each area were cleaned, peeled, and squashed with a manual juice squeezer (Figure 1). Before analysis, each acquired juice was filtered through a syringe filter (0.45 µm) before being poured into a 2-mL vial with a tight cap and stored in a refrigerator at 0-4°C (Long, 2015). Sufficient samples from each area were prepared to provide three replicates.

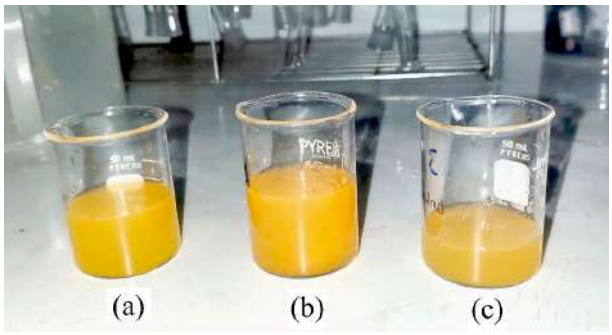

Figure 1. Squeezed Sai-Nam-Phueng orange juice from a) Fang district in Chiang Mai province, b) Phu Ruea district in Loei province, and c) Phop Phra district in Tak province

2.3 Orange juice sample preparation from the marketed products

To examine the amino acid profiles of orange juice from the market, thirty orange juice products packaged in both glass and plastic bottles and distributed in groceries, supermarkets, and department stores were examined. The samples were selected based on the package ingredient information, which included the name "Sai-Nam-Phueng" orange juice on the label and covered both real (natural) and flavored (added nutrients or other substances) orange juices. Each sample juice was passed through a 0.45-µm syringe filter, put into a 2-mL vial with a tight lid and stored in the same manner as SNP orange juice preparation (Figure 2).

Figure 2. Some marketed orange juice samples

2.4 Amino acids of the SNP orange determination

Amino acids of the SNP orange juice from the three areas were analyzed by HPLC (Agilent Technologies, Santa Clara, CA, USA) at 338 nm and 262 nm wavelengths for primary and secondary amino acid types, respectively, and chemicals employed as the mobile phase following the methods of Long (2015). The stationary phase or column was ZORBAX Eclipse Plus C18 (Agilent Technologies, Santa clara, CA, USA). Prior to scrutinizing the SNP orange juices, seventeen standard amino acids were first analyzed for use as comparators, or amino acid identification. The standard amino acids were confirmed by chromatographic conditions from Long (2015). The orange juice amino acid peaks from each area were compared and considered as indicators for the SNP orange juice identification. Three replications for each area were conducted.

2.5 Marketed orange juice samples examination

The amino acids of the 30 orange juice samples from the market were analyzed by the HPLC in the same way that the SNP orange juice was. The amino acid peaks were compared to the SNP profile and the corresponding packaging ingredient information of each bottle was taken into account. At the same time, the percentage of orange in the 30 samples was examined using formol titration as well. The formol titration method involved neutralizing the solution with sodium hydroxide (NaOH) added with formaldehyde, which does not affect the acid-base reaction of NaOH to form dimethylol (Subroto et al., 2020). Equation 1 was used to calculate the percentage of fruit juice (The Excise Department, 2019b).

Orange juice has the specified criteria percentage of 20 and the formol number of 9.1. The percentage of orange juice could be calculated once the volume was titrated by NaOH is known.

Aside from the SNP amino acid peaks, another detected peak, glycine, primarily used as one of the additives to increase protein content, was found in the sample products (Asadpoor et al., 2014; Dasenaki and Thomaidis, 2019). As a result, using Equation 2, the actual percentage of the real orange juice mixture was calculated in order to determine the actual percentage for the tax exemption decision.

$$\text{The percentage of fruit juice} = \frac{\textit{volume of titrated by NaOH x the specified criteria percentages each kind of fruit}}{\textit{the formal number each kind of fruit}} \quad (1)$$

$$\text{The actual percentage} = \left(A - \frac{A \times B}{C}\right) \text{x} \ \frac{\textit{the specified criteria percentages each kind of fruit}}{\textit{the formal number each kind of fruit}} \quad (2)$$

where A is the volume of titrated by 0.1 N NaOH in mL, B is the overall chromatogram areas in milli absorbance unit seconds (mAU*s), C is the chromatogram areas of the other peak in milli absorbance unit seconds (mAU*s).

2.6 Statistical analysis

The mean area and standard deviation of the amino acids from all three orange areas were calculated and used as the amino acid profiles of the SNP orange juices. In addition, in this study, one-way multivariate analysis of variance (MANOVA) (Phusee-Orn, 2018) and K-means cluster analysis were applied for statistical analysis.

3. RESULTS AND DISCUSSION

3.1 Identifying the amino acid profile of the SNP orange juice

The chromatogram of the seventeen standard amino acids was analyzed by the HPLC, which was applied for amino acid type identification, as shown in Figure 3. The chromatogram results of the SNP orange juice from the three regions of Fang district in Chiang Mai province, Phu Ruea district in Loei province, and Phop Phra district in Tak province all indicated the same seven peaks of the standard amino acids; Asp, Glu, asparagine (Asn), Ser, Arg, Ala, and Val, as presented in Figure 4. Because the seventeen standard amino acids used for identifying amino acids did not contain Asn, the Asn peak in Figure 4 was confirmed by using twenty-three standard amino acids that did contain Asn under the same chromatographic conditions as Long (2015). Furthermore, Wistaff et al. (2021) discovered that Asn is an important amino acid in classifying orange juices from blond orange juices and blood orange juices.

HPLC was used to determine the area of each amino acid peak (mAU*s) and the retention time. The area served as the basis for statistical analysis. A profile comprised of the means of the SNP orange juice amino acids from all three areas, with three representatives from each area, was used as the reference for the examination of the 30 marketed orange juice samples, as presented in Table 1. Only one independent variable, province area, and seven dependent variables, amino acid peak areas, were present. A one-way MANOVA was applied to find out whether or not the location factor had an impact. The three different areas from which the SNP orange juice samples were obtained had a significant influence on the majority of the SNP orange juice amino acid profiles, with a confidence level of 99%, by using MANOVA tests [F (12, 2) = 4431.456 (Wilks' Lambda), p<0.01]. In addition, ANOVA results indicated that only Asp had no significant impact on the orange location, with a confidence level of 95%.

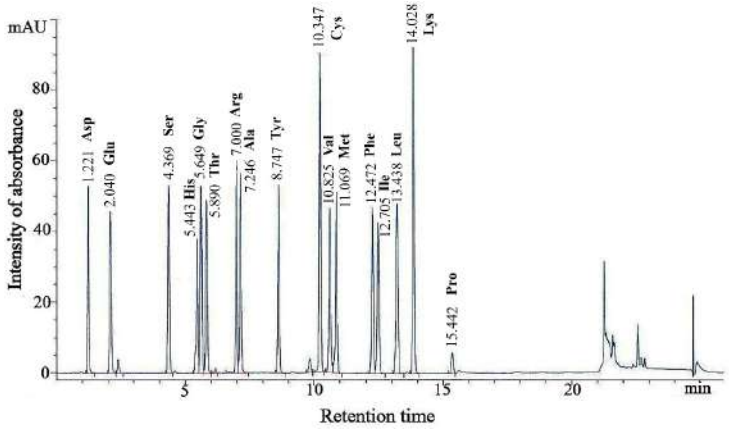

Figure 3. Chromatogram of standard amino acids used in the study; aspartic acid (Asp), glutamic (Glu), serine (Ser), histidine (His), glycine (Gly), threonine (Thr), arginine (Arg), alanine (Ala), tyrosine (Tyr), cysteine (Cys), valine (Val), methionine (Met), phenylalanine (Phe), isoleucine (Ile), leucine (Leu), lysine (Lys), and proline (Pro)

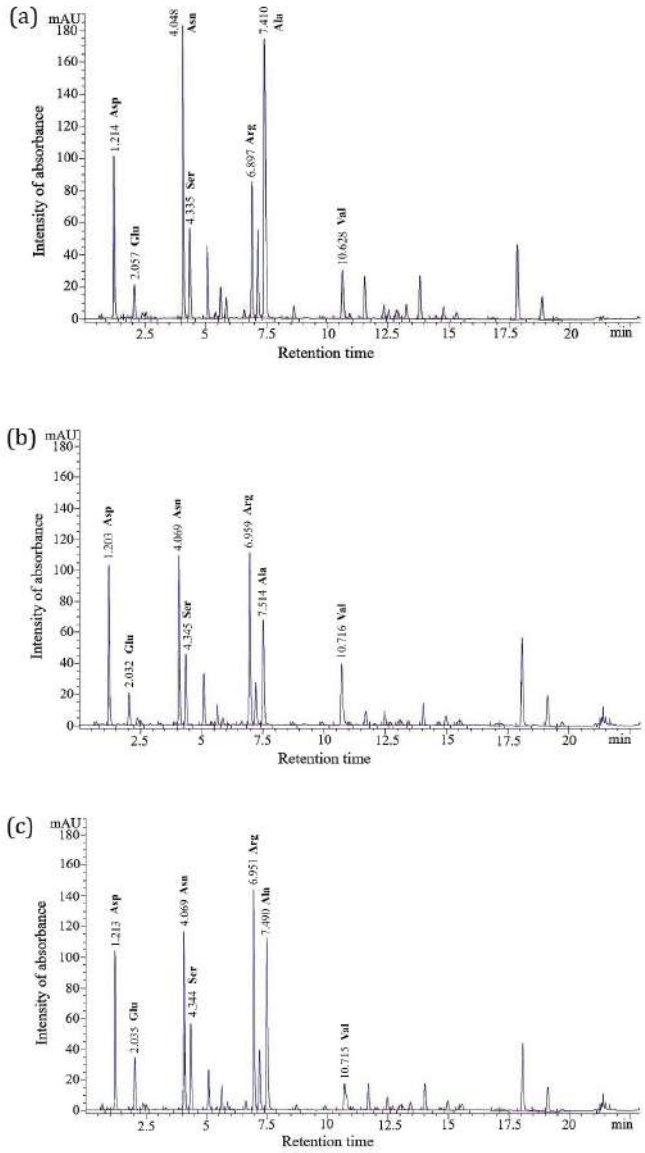

Figure 4. The chromatogram results of the standard amino acids of SNP orange juice from the three areas, (a) Fang district in Chiang Mai province, (b) Phu Ruea district in Loei province, and (c) Phop Phra district in Tak province, show the 7 peaks of aspartic acid (Asp), glutamic acid (Glu), asparagine (Asn), serine (Ser), arginin (Arg), Alanine (Ala), and valine (Val)

Table 1. Peak area of 7 amino acids of Sai-Nam-Phueng orange juice

Amino acid type	Peak area (mAU*s) (mean±S.D.)
Aspartic acid	299.00±36.34
Glutamic	102.45±19.94
Asparagine	484.53±160.92
Serine	202.18±24.91
Arginine	417.80±83.04
Alanine	591.29±303.31
Valine	183.76±45.28

3.2 Examining the quality of orange juice from the marketed products

Table 2 summarizes the cluster analysis results of the amino acid areas of the 30 orange juice samples, as well as the SNP orange juice profile for comparison (no. 31). The majority of the 30 orange juice samples in the packaging bottles omitted information on the orange type. The findings showed that the 30 samples were classified into three groups based on the seven amino acids. Orange juice samples Nos. 2, 13, 16, 19, 20, and 21 from the market had peak areas of seven amino acids that were similar to the reference of the SNP orange juice (No. 31) and were categorized in the second group in Table 2, which comprised real orange juice. The formol titration results also agreed with the percent as declared. As a result, the orange juices in the second group were considered exempt from taxation.

Orange juice samples Nos. 22 and 27-29, categorized in the first group in Table 2, contained no peaks of the seven SNP amino acids, as shown in Figure 4, indicating that there was no actual or flavored orange juice as stated on the label since the amino acid peak should appear even when other types of orange juice are involved (Correa et al., 2018). Furthermore, despite the absence of the seven SNP amino acids, another prominent amino acid, as indicated by the arrows in Figure 5, was found in each of the four samples, but it could not be identified. As a result, all four samples failed to qualify for the fruit and vegetable beverage tax exemption.

Moreover, certain samples from the first group were mixed with other substances, such as coconut juice jelly, collagen, carrageenan, and konjac powder, for which there is no tax exemption. However, if the investigation results show that the threshold of 10% real orange juice is surpassed, a tax privilege of three percent is granted. The chromatogram yielded a Gly peak in the first group, which was added from the seven SNP amino acid profile (No. 15), as shown in Figure 6. Gly, an amino acid found in natural orange juice from other species such as *Citrus sinensis*, is normally present in a consistent 1:1 ratio to Val (Keng et al., 2015). According to the findings, the Gly peak of the amino acid profile of Sample No. 15 in Figure 6 showed more than a 1:1 ratio to Val. Furthermore, the formol titration results of sample No.15 revealed that the total amino acid concentration exceeded the regulatory limit of 20%. Adding one or more amino acids may result in an increase in protein content, resulting in a false positive in the formol index test (Asadpoor et al., 2014; Dasenaki and Thomaidis, 2019). Therefore, Gly was labeled as "other amino acid peak found" in Table 2; the actual percentage for the tax exemption decision of sample No. 15 was calculated using Equation 2.

Apart from the SNP amino acid profile, the lone sample of flavored orange juice obtained by blending orange concentration (No. 8) was detected in the third group due to seven SNP amino acid peaks with a similar area of Asp and Glu, including the Gly peak. However, because the SNP amino acid profile in Figure 4 shows a Glu peak ratio of 1:3 to Asp, the increased peak could indicate an addition intended to increase the protein content (Dasenaki and Thomaidis, 2019) or that the orange juice was blended with other kinds of fruit, such as lemon, which has a similar amino acid profile but with higher levels than those of orange juice (Bi et al., 2005). Like this study, Asadpoor et al. (2014) and Dasenaki and Thomaidis (2019) found Glu and Gly as inexpensive amino acid additions to increase the total amino acid content of juice through adulteration.

The results of HPLC and formol titration were utilized to make a final determination on tax privilege for the thirty samples. The samples containing additional amino acids, particularly Gly, which is commonly found in orange juice, were calculated using Equation 2 to determine the exact percentage of orange juice. If the actual percentage remains within the threshold, the sample will qualify for tax exemption. Sample No. 15, for example, had 20% real orange juice, vitamin C, and less sugar. The computed Gly amino acid chromatogram area was 236.07 mAU*s, with a total chromatogram area of 2695.74 mAU*s. The volume of 0.1 N NaOH was 9.05 mL. The actual percentage of orange juice was 18.17%, which was lower than the label's claim of 20%. Sample No. 15 was chosen for tax privilege because it surpassed the 10% threshold. Therefore, only eighteen out of the thirty samples were tax-free, as can be seen in Table 2. The tax privilege rate of 3% was applied to eight samples (Nos. 3, 7, 9, 10, 14, 15, 23, and 30), while four samples (Nos. 22, 27, 28, and 29) were all false orange juices whose profiles did not correspond to the seven amino acid identification peaks.

Table 2. The results of cluster analysis, formol titration, and final tax exemption of 30 marketed samples

Case number	Orange type	Volume (%)	Other substances	Cluster*	Other amino acids peak found	Formol titration Percent criteria**	results	Tax rate (%)
1	n.a.	25	-	1	-	>20	pass	0
2	n.a.	25	-	2	-	>20	pass	0
3	n.a.	25	Coconut juice jelly added	1	-	>10	pass	3
4	n.a.	25	Orange Pulp added	1	-	>20	pass	0
5	n.a.	25	-	1	-	>20	pass	0
6	n.a.	25	-	1	-	>20	pass	0
7	n.a.	25	Coconut juice jelly added	1	-	>10	pass	3
8	n.a.	25	From concentration	3	glycine	>20	pass	0
9	n.a.	25	Carrageenan and konjac powder added	1	-	>10	pass	3
10	n.a.	25	From Concentrate plus coconut juice jelly added	1	-	>10	pass	3
11	n.a.	25		1	-	>20	pass	0
12	n.a.	25		1	-	>20	pass	0
13	Sai-Nam-Phueng	25		2	-	>20	pass	0
14	n.a.	15	Orange flavored from concentration	1	-	>10	pass	3
15	n.a.	20	Vitamin C plus less sugar	1	glycine	>20	pass	3
16	n.a.	25		2	-	>20	pass	0
17	n.a.	15	Orange Pulp added	1	-	>20	pass	0
18	Sai-Nam-Phueng	25		1	-	>20	pass	0
19	n.a.	25		2	-	>20	pass	0
20	n.a.	100	Pulp add mix with steviol glycosides 0.01%	2	-	>20	pass	0
21	n.a.	100		2	-	>20	pass	0
22	n.a.	25	Coconut juice jelly added	1	n.a.	>10	reject	-
23	n.a.	n.a.	Vitamin C and collagen added	1	-	>10	pass	3
24	n.a.	35		1	-	>20	pass	0
25	n.a.	100	Orange Pulp	1	-	>20	pass	0
26	n.a.	75		1	-	>20	pass	0
27	n.a.	-	Coconut juice jelly added	1	n.a.	>10	reject	-
28	Sai-Nam-Phueng	20		1	n.a.	>20	reject	-
29	n.a.	20		1	n.a.	>20	reject	-
30	n.a.	25	Collagen added	1	-	>10	pass	3
31	Sai-Nam-Phueng	100	Reference	2	-	control		

Note: n.d. = not applicable, *similarity of the 7 amino acid areas to SNP profile, 1 = no similarity, 2 = similarity, and 3 = others besides from 1. ** percent criteria of the Excise Department under the Excise Tax Act in Thailand.

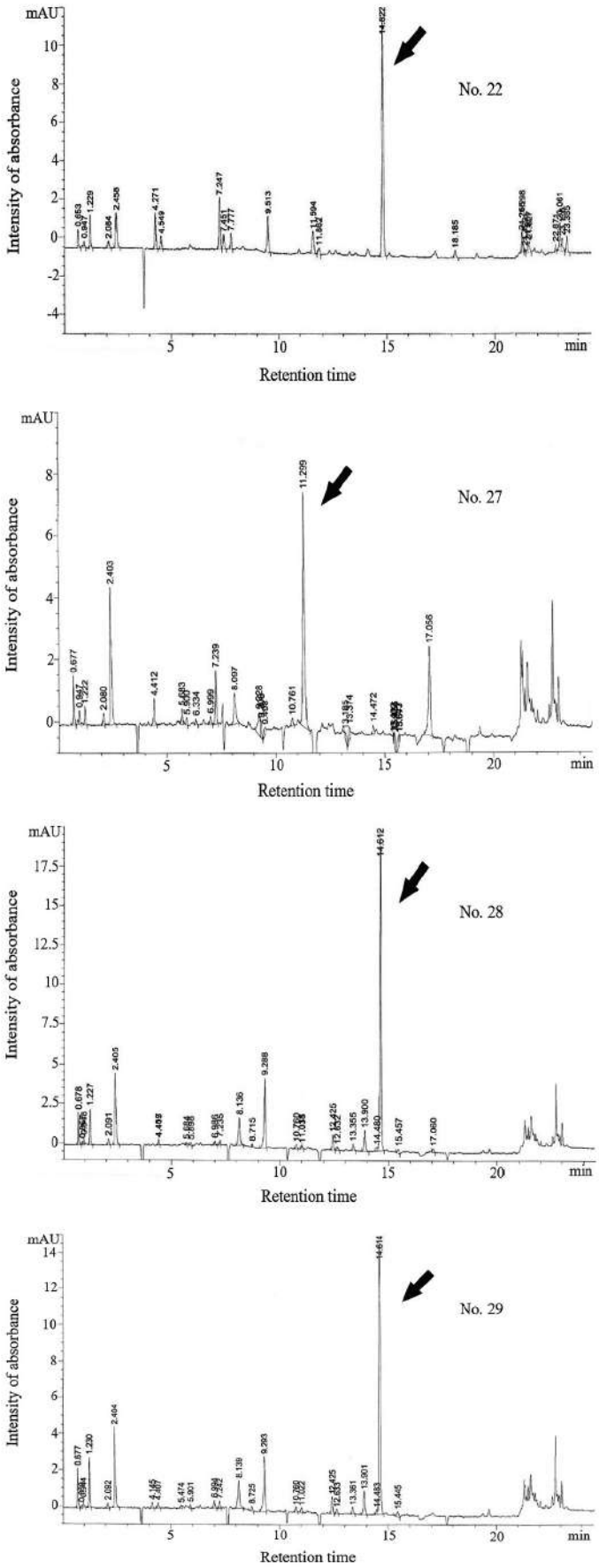

Figure 5. Amino acid chromatograms of orange juice sample Nos. 22, 27, 28, and 29 (as marked by arrows)

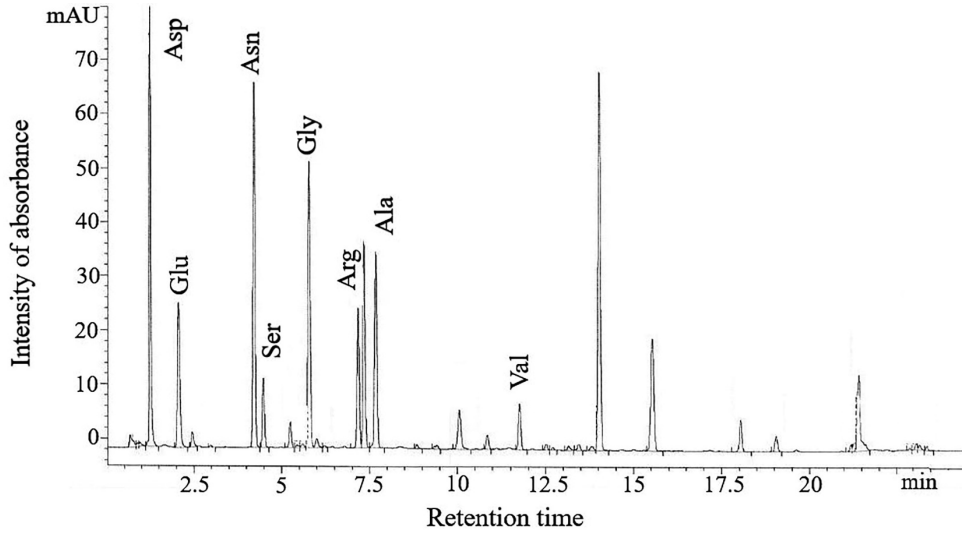

Figure 6. Chromatograms of orange juice sample No. 15, showing seven amino acids and another glycine (Gly)-containing amino acid

4. CONCLUSION

Seven amino acids (Asp, Glu, Asn, Ser, Arg, Ala, and Val) were identified in the SNP orange juice in this investigation. The profile could be applied in order to examine orange juice samples for tax exemption as well as formol titration, in terms of actual or flavored orange juice, including the orange juice percentage as labeled on the packaging. However, more research should be done on other kinds of orange juice, other types of fruit juice, and other substances mentioned on the packaging.

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