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Application of stepwise discriminant analysis to classify commercial orange juices using chiral micellar electrokinetic chromatography-laser induced fluorescence data of amino acids

The use of chiral amino acids content and stepwise discriminant analysis to classify three types of commercial orange juices (*i.e.*, nectars, orange juices reconstituted from concentrates, and pasteurized orange juices not from concentrates) is presented. Micellar electrokinetic chromatography with laser-induced fluorescence (MEKC-LIF) and β-cyclodextrins are used to determine L- and D-amino acids previously derivatized with fluorescein isothiocyanate (FITC). This chiral MEKC-LIF procedure is easy to implement and provides information about the main amino acids content in orange juices (*i.e.*, L-proline; L-aspartic acid, D-Asp, L-serine, L-asparagine, L-glutamic acid, D-Glu, L-alanine, L-.arginine, D-Arg, and the non-chiral γ-amino-*n*-butyric acid (GABA), *i.e.*, γ-aminobutyric acid). From these results, it is clearly demonstrated that some D-amino acids occur naturally in orange juices. Application of stepwise discriminant analysis to 26 standard samples showed that the amino acids L-Arg, L-Asp and GABA were the most important variables to differentiate the three groups of samples. With these three selected amino acids a 100% correct classification of the samples was obtained either by standard or by leave-one-out cross-validation procedures. These classification functions based on the content in L-Arg, L-Asp and GABA were also applied to nine test samples and provided an adequate classification and/or interesting information on these samples. It is concluded that chiral MEKC-LIF analysis of amino acids and stepwise discriminant analysis can be used as a consistent procedure to classify commercial orange juices providing useful information about their quality and processing. To our knowledge, this is the first report about the combined use of chiral capillary electrophoresis and discriminant techniques to classify foods.

Keywords: Capillary electrophoresis / Chemometrics / Enantiomers / Fluorescein isothiocyanate / Fruits / Juice / Laser-induced fluorescence / Micellar electrokinetic chromatography
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1 Introduction

Nowadays, the fruit juice industry has become one of the most important agricultural businesses in the world with trades exceeding $10 billion per year [1]. Since the fruit juice industry is dominated by orange juice, adulteration and quality assessment of such juices are important issues that demand the development of new analytical procedures. These new analytical methods should be able to both detect the everyday more sophisticated adulteration strategies tailored to defeat detection methods and provide in a fast way objective data to assess the quality of juices.

Enantiomer-selective analysis, of, *e.g.*, amino acids, is a powerful detection method that can provide important information on adulteration and quality of orange juices [1–3]. Gas chromatography (GC) and high-performance liquid chromatography (HPLC) have been the techniques of choice to carry out this type of enantiomeric separations [2, 3], since they provide in some cases unequivocal results. However, the procedures for sample preparation prior to GC or HPLC are frequently laborious and time-consuming [2], separations may be lengthy [4] and these techniques generally use expensive chiral columns. Also,

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Abbreviation: GABA, γ-amino-*n*-butyric acid

in some GC procedures the derivatizing procedure does not work for some basic amino acids such as Arg [5]. In order to overcome some of the aforementioned drawbacks and taking into account the economic impact of orange juice, other new procedures have been developed based on, *e.g.*, the use of micellar electrokinetic chromatography (MEKC) [6].

For the fruit juice industry and consumers, pasteurized juice not from concentrate is considered to have a superior flavor when compared with juice reconstituted from concentrate or nectars (*i.e.*, orange juices containing a minimum of 50% of fruit), and thus commands a higher price [7]. Chilled juices continue to increase in popularity, and pasteurized juice, not from concentrate, is one of the fastest-growing segments of the orange juice industry [7]. Since consumers perceive that orange juice not from concentrate has a better flavor than orange juice from concentrate and nectars, there is a need to demonstrate this difference objectively. Such an objective method would be useful to juice processors as an alternative to subjective sensory judgments for product evaluation and quality control.

Evaluation of quality in commercial citrus juice products relies on basic measurements such as total soluble solids, acidity, color score, total oil, and pulp content, which are official standards of identity [8]. Sensory evaluation is also used, but it can be a time-consuming and subjective measurement requiring significant expertise. Recently, the combination of instrumental and discriminant techniques have been reported as a good alternative to evaluate the quality of orange juices allowing their classification in a fast, reliable and more objective way. Thus, the instrumental procedure mainly used up to now is GC [7, 9–14] and it has been mostly applied to the determination of the volatile fraction. Following similar idea, some other procedures based on NMR [15], or near-infrared (NIR) spectroscopy [16] have also been employed. Recently, a cluster of electronic sensors (electronic nose), has been used together with discriminant techniques to evaluate and classify processed orange juices [9]. However, GC methods frequently require laborious sample preparation procedures and long analysis times, while spectroscopic techniques and electronic sensors can often suffer the undesired influence of additional constituents on their measurements since no separation is involved.

It can be concluded that there is a large necessity and economical concern in the development of new analytical strategies that can help to assess the quality of citrus juices. The goal of this work was, therefore, to study the possibilities of the use of chiral MEKC-LIF of amino acids and discriminant techniques to classify commercial orange juices. Namely, nectars, pasteurized juices not from concentrate and reconstituted orange juices from concentrates were studied. To do this, a MEKC-LIF method previously developed by our group [6] has been modified. These modifications were applied in order to: (i) reduce the analysis time required for each sample; (ii) make the method compatible with different CE instruments; (iii) improve the reproducibility of the derivatization procedure. The main changes are thoroughly described under Section 2.

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical reagent grade and used as received. β-Cyclodextrin (β-CD) from Fluka (Buchs, Switzerland) was used as chiral selector. This compound together with sodium dodecyl sulfate (SDS) from Acros Organics (Morris Plains, NJ, USA) and boric acid from Riedel-De Haën (Seelze, Germany) were used for the MEKC running buffer. A water solution containing 1 M sodium hydroxide from Panreac Quimica (Barcelona, Spain) was used to adjust the pH of the buffer. The buffer was stored at 4°C and warmed at room temperature before use. Distilled water was deionized by using a Milli-Q system (Millipore, Bedford, MA, USA). Fluorescein isothiocyanate (FITC, from Fluka), dissolved in acetone HPLC grade (Scharlau, Spain) was used to derivatize the amino acids at the different concentrations indicated. L- and D-amino acids and GABA (γ-aminobutyric acid) (all from Sigma, St. Louis, MO, USA) were directly dissolved in Milli-Q water at the concentrations indicated and derivatized as indicated below.

2.2 Samples

Thirty-five different commercial orange juices were studied in this work and they were bought at different local markets. Table 1 shows the identity of the 35 juices samples analyzed. Groups A, B and C correspond to nectars (group A), reconstituted orange juices from concentrates (group B) and pasteurized orange juices not from concentrates (group C, these juices have to be stored at low temperature). Group D included nine juices samples, namely, six orange juices from concentrates of which two were sensorially classified as off-flavor; two pasteurized orange juices not from concentrate and one orange juice without indication on its origin (*i.e.*, pasteurized, from concentrate, nectar, *etc.*). All samples were centrifuged for 5 min at 10 000 rpm and the supernatant used for derivatization.

Table 1. Description of samples analyzed by duplicate in this work

	Group	Number of samples	Type
Standard set	A	10	Nectar
	B	9	Reconstituted from concentrate
	C	7	Pasteurized not from concentrate
Test set	D	9	Diverse (see Section 2.2)

2.3 Derivatization procedure

The test sample containing L- and D-amino acids plus GABA was prepared dissolving in water each amino acid to obtain a final concentration similar to that expected in the real samples (*i.e.*, from 1.5 to 5.7 mM) [1]. The optimum FITC derivatization procedure consisted of mixing an aliquot of 625 μL of the supernatant from orange juice (or the amino acid standard solution) with 1375 μL of water and 7 mL of 355 mM borate buffer at pH 10. This mixture was adjusted to pH 10 by adding 1 M sodium hydroxide. Water was added till a final volume equal to 10 mL. An aliquot of 100 μL of this final solution was mixed with 200 μL of a 3.75 mM FITC solution in acetone instead of 50 μL of the 30 mM (saturated) FITC dissolution used in our previous work [6]. This change has been observed to improve the reproducibility of our derivatization procedure without modifying the derivatization yield. The reaction took place overnight in darkness at room temperature. After derivatization, test samples, and samples from orange concentrates and juices were diluted with water (1:83 v/v) prior to their injection in the MEKC-LIF instrument. All the measurements were done by duplicate.

2.4 MEKC-LIF conditions

Analyses were carried out in a P/ACE 2050 CE apparatus (Beckman Instruments, Fullerton, CA, USA) equipped with an Ar+ laser at 488 nm (excitation wavelength) and 520 nm (emission wavelength) from Beckman Instruments (Fullerton, CA, USA) to detect FITC-amino acids. Bare fused-silica capillaries with 50 μm ID were purchased from Composite Metal Services (Worcester, UK). Injections were made at the anodic end using N_2 pressure of 0.5 psi for a given time (1 psi = 6894.76 Pa). The P/ACE 2050 CE instrument was controlled by a PC running the System GOLD software from Beckman. In our previous work [6], a modern P/ACE-MDQ instrument was used; therefore, different modifications have now to be included. Besides, we also wanted to reduce the analysis time needed (20 min in the old procedure [6]). Firstly, we could observe some problems with the temperature control of the old P/ACE 2050 used in this work (*i.e.*, it was not able to go down 15°C). Moreover, the different design of both instruments made necessary to use different capillary lengths. These facts took us to introduce the next modifications: the temperature was fixed at 25°C (instead of the 15°C used in the old procedure); the capillary lengths used in the new procedure were 50 cm for detection and 57 cm as total length (*versus* 40 cm and 50 cm in the old procedure, respectively); in order to increase the analysis speed, a similar electric field was obtained (*i.e.*, the effect of the new temperature on the viscosity was not compensated) and 23 kV was used as running voltage (electric field equal to 403 V/cm) against the 20 kV used in the old method (electric field equal to 400 V/cm) [6]. The injection was increased from 5 s·0.5 psi in the old procedure to 6 s·0.5 psi in the new procedure. Under these conditions the MEKC-LIF analysis time could be reduced in a 15% (namely, from 20 min to 17 min), maintaining the sensitivity and without any noticeably efficiency and resolution decrease. Before first use, any new capillary was preconditioned by rinsing with 0.1 M NaOH for 30 min. Between injections, capillaries were rinsed using water for 2 min and the separation buffer for 3 min. The optimum running buffer consisted of 100 mM sodium tetraborate, 20 mM β-CD, 30 mM SDS at pH 9.4. At the end of the day, the capillary was rinsed with deionized water for 5 min and stored overnight with water inside.

2.5 Statistical analysis

The statistical methods used for the data analysis were: principal component analysis from standardized variables, applied to discover natural groupings of the juices samples of the study; one-way analysis of variance (ANOVA) to test the effect of the factor studied (processing); Scheffé test for means comparisons; and discriminant analysis techniques (stepwise discriminant analysis to select the variables most useful to differentiate the three groups of samples and to obtain the classification functions, and Fisher's canonical variates analysis in order to obtain a low-dimensional graphical representation of the samples that separates the groups as much as possible). STATISTICA program for Windows (release 5.1; Statsoft, Tulsa, OK) was used for data processing. This program was run on a Pentium III personal computer.

3 Results and discussion

3.1 Chiral MEKC-LIF analysis of orange juices

Figure 1A shows a typical electropherogram obtained after injecting a standard mixture containing 15 L- and D-amino acids (*i.e.*, the most abundant amino acids in

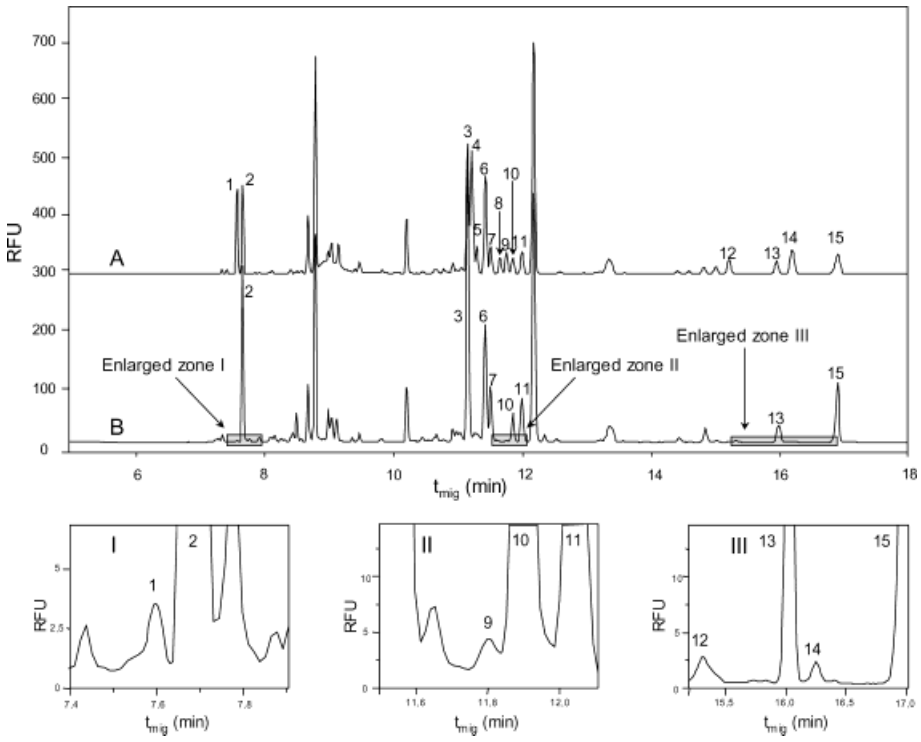

Figure 1. Chiral MEKC-LIF separation of (A) standard FITC-amino acids, (B) orange juice from concentrate. Capillary, l_d = 50 cm, l_t = 57 cm with 50 μm ID running buffer, 100 mM sodium tetraborate, 30 mM SDS, 20 mM β-CD at pH 9.4; capillary temperature, 25°C; run voltage, 23 kV; injection at 0.5 psi for 6 s of FITC-derivatized 1, D-Arg; 2, L-Arg; 3, L-Pro; 4, D-Pro; 5, D-Asn; 6, GABA; 7, L-Asn; 8, D-Ser; 9, D-Ala; 10, L-Ser; 11, L-Ala; 12, D-Glu; 13, L-Glu; 14, D-Asp; 15, L-Asp. Concentration of amino acids injected (μM): DL-Arg, 1.06; DL-Pro, 1.9; GABA, 0.65; Asn, 0.78; Ser, 0.39; Ala, 0.28; Glu, 0.31, and Asp, 0.59. LIF detection at 488 nm (excitation wavelength) and 520 nm (emission wavelength).

orange including the L- and D- forms of Pro, Asp, Ser, Asn, Glu, Ala, and Arg, plus the non chiral amino acid GABA) under the optimized separation conditions. Namely, a 100 mM sodium tetraborate, 30 mM SDS, 20 mM β-CD at pH 9.4 aqueous buffer, was used in this chiral MEKC-LIF method. As can be seen, this procedure can provide a good chiral separation of the main amino acids found in orange juice in *ca.* 17 min. The procedure was demonstrated to be reproducible with %RSD for analysis times better than 0.7% and %RSD for corrected peak areas better than 6.9%, both calculated for three different days (n = 15).

This chiral MEKC method together with the optimized FITC derivatization method allows the easy and straightforward detection of amino acids in orange juices since only centrifugation for 5 min of diluted samples is needed prior to their derivatization. An example is shown in Fig. 1B, where a typical electropherogram obtained from a concentrate orange juice is given. As can be seen, apart of the main L-amino acids (*i.e.*, Pro, Asp, Ser, Asn, Glu, Ala, and Arg, plus the nonchiral amino acid GABA), the D-forms of Ala, Arg, Asp, and Glu are detected by this procedure (see the enlarged zones from Fig. 1B). Interestingly, these D-forms could be detected in all the orange juices studied what seems to indicate that these D-amino acids are naturally occurring in orange juices. To demonstrate this point, an orange juice was freshly prepared at our laboratory and after treating it as indicated in Section 2, this sample was injected in the MEKC-LIF instrument. A similar electropherogram to that given in Fig. 1B was obtained, including the D-forms of Ala, Arg, Asp, and Glu what corroborates the natural origin of these D-amino acids. These results are in good agreement with the findings done by other authors [4, 17] regarding the existence of these four D-amino acids in different fruits including oranges. The use of this chiral MEKC method with the derivatization procedure and a LIF detector provided limits of detection of 0.86 amol (for L-Arg and a signal/noise ratio equal to 3), what demonstrates the possibilities of this procedure to overcome the poor sensitivity normally associated to the use of capillary electrophoresis techniques.

This chiral MEKC-LIF procedure could be used to detect in a fast way certain types of adulteration as, for instance, the addition of inexpensive racemic amino acids (*e.g.*, Glu) or the addition of protein hydrolysates to increase the total amino acid content [1]. Also, the detection of other D-amino acids in orange juice can be indicative of a possible microbial spoilage or related to juices of unsatisfactory quality [5]. The usefulness of this chiral MEKC-LIF procedure together with the use of discriminant techniques was tested in this work to classify different commercial orange juices.

3.2 Classification of orange juices

Table 1 shows the different samples that have been included in this study. As can be seen, from the 26 samples used as standard set, 10 were labeled as nectars (group A), 9 as reconstituted from concentrate (group B) and 7 were pasteurized fresh orange juices (group C). These 26 samples were treated as indicated under Sections 2.2 and 2.3 and then injected into the MEKC-LIF instrument. Each sample was injected twice and the corrected peak areas (*i.e.*, peak area divided by analysis time) of the different L- and D-amino acids were calculated from the electropherogram. The average values were used as the input data for the statistical analysis.

The 26 samples from the standard set (groups A–C) were subjected to principal components analysis using the correlation matrix, and two principal components were selected: the first principal component, which explains 74.8% of the total variance, is highly correlated with all amino acids (loadings $>$ 0.8) with the exception of D-Glu which is more correlated (loading $>$ 0.9) with the second principal component which explains a 12% of the total variance. In Fig. 2 are plotted the scores of the standard samples in the plane defined by the first two principal components. There are three groupings corresponding to the three different juices. The first principal component differentiates the group A from groups B or C, that is, nectars are clearly separated from pasteurized and concentrated juices, what is also correlated to the lower amount of amino acids that are usually found in the first ones (*i.e.*, nectars can contain a minimum of 50% of fruit). As can be seen in Fig. 2, the separation of concentrates and pasteurized based on this procedure is not as clear as the separation of nectars from concentrated and pasteurized juices.

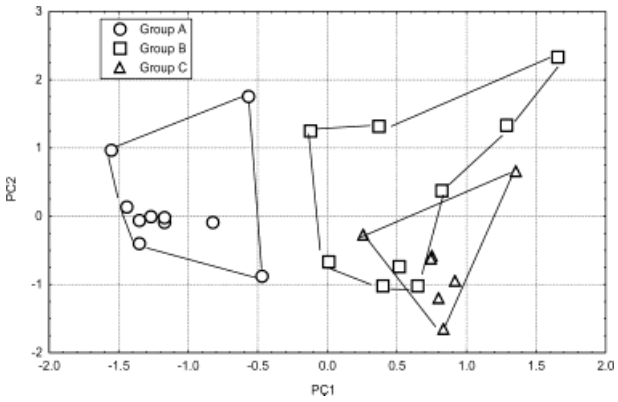

Figure 2. Plot of the standard samples (A–C groups) in the plane defined by the first two principal components.

Table 2 contains the means and standard deviations of the variables analyzed (corrected peak areas) for groups A–C. The results of the application of the Scheffé test for means comparison are also included in the table. All analyzed variables, with the exception of D-Glu, have significantly lower mean values in group A than in groups B or C, in good agreement with the results aforementioned (nectars can be clearly distinguished from concentrates and pasteurized juices). L-Arg and L-Asn have significantly higher mean values in group C than in the other groups, and these variables could be used in the differentiation between pasteurized and concentrated orange juice.

Table 2. Distribution of corrected peak areas of the main chiral amino acids in the three standard groups of juices, and results of the application of the Scheffé test for means comparisons

	Group A ($n = 10$)		Group B ($n = 9$)		Group C ($n = 7$)	
Variable	Mean	SD	Mean	SD	Mean	SD
D-Arg	4.75a	2.17	7.95ab	3.54	8.27b	2.11
L-Arg	493.86a	113.83	1131.49b	167.71	1430.61c	178.19
L-Pro	1004.95a	352.75	1953.92b	626.42	2556.51b	459.96
GABA	320.68a	100.29	748.04b	143.17	733.75b	139.73
L-Asn	184.60a	58.49	357.35b	55.86	445.79c	80.88
D-Ala	4.66a	1.34	7.74b	2.52	7.52b	1.85
L-Ser	96.27a	21.85	198.14b	35.46	215.50b	23.38
L-Ala	120.52a	30.07	270.25b	58.93	267.81b	67.67
D-Glu	2.91a	0.45	3.60a	0.98	2.89a	0.65
L-Glu	62.19a	19.08	131.73b	16.65	125.45b	13.91
D-Asp	3.76a	0.87	7.34b	1.07	8.26b	1.39
L-Asp	150.43a	38.68	331.42b	36.30	295.99b	46.87

SD, standard deviation; a-c, rows without a common letter are significantly different ($P < 0.05$).

Table 3. Coefficients of the classification functions obtained from stepwise discriminant analysis

	Group A	Group B	Group C
L-Arg	0.00473	0.0131	0.0761
L-Asp	0.07403	0.1532	0.0195
GABA	0.00396	0.0103	− 0.0279
Constant	−8.32535	−37.7422	−48.4137

The application of forward stepwise discriminant analysis to the 26 standard samples using the 12 variables listed in Table 2 showed that the amino acids L-Arg, L-Asp and GABA were selected as the most important variables for differentiating the three groups of samples. Values of 4.0 and 3.9 were considered for F-statistic to enter and to remove variables, respectively. With only these three selected amino acids it was possible to classify all the samples correctly. Moreover, a 100% correct classification was also obtained when the leave-one-out cross-validation procedure was used. The three obtained classification functions (Table 3), one for each group, were also applied to the nine samples of group D, and the samples were assigned to the group with the largest linear score (seven were assigned to group B and two to the group C).

It is interesting to mention that some of the amino acids selected after applying the Scheffé test or the forward stepwise discriminant analysis, namely, Arg, Asn and GABA, have been demonstrated to give rise to furoylmethyl derivatives, and that the formation of these derivatives increases with temperature [18], what can be correlated to the different processing applied to concentrated and pasteurized orange juices. Fisher's canonical variates analysis was applied to the data of the 26 standard samples of juices using the three selected amino acids (L-Arg, L-Asp and GABA) from stepwise discriminant analysis. Figure 3 shows the 26 samples on the plane defined by the two canonical variables obtained. The canonical ellipses surrounding each group of samples in the figure represents the 95% confidence level and are calculated from the Mahalanobis distance up to the centroid of the group. The nine test samples of group D are also included in Fig. 3 as asterisks.

The assignment of the nine samples of the test set (group D) permits to estimate the good possibilities of our procedure. The group D was formed by six different orange juices labeled as orange juice from concentrates of which two were sensorially classified as "off-flavour". Other two orange juices of the nine studied were labeled as not from concentrate and found in the refrigerated section since they required to be chilled at 5–8°C. The last orange juice

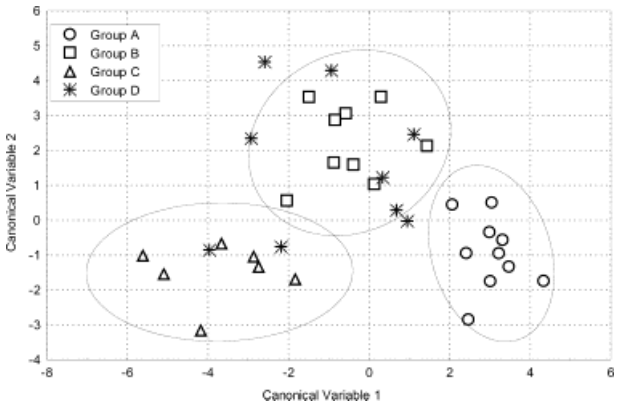

Figure 3. Plot of the standard samples (A–C groups) in the plane defined by the two canonical variables obtained with the selected variables from stepwise discriminant analysis (L-Arg, L-Asp and GABA). The canonical ellipses for the standard groups (95% confidence) and the test samples of group D are also included.

included in the test group did not show in the label any indication regarding its origin (*i.e.*, pasteurized, from concentrate, nectar, *etc.*). This orange juice showed the strange behavior indicated in Fig. 3 (*i.e.*, asterisk with canonical variable 1 equal to −2.5 and canonical variable 2 equal to 4.5), and, therefore, it could not be clearly assigned to any group. Interestingly, the two chilled orange juices labeled as not from concentrate were correctly assigned to group C, while five of the six orange juices labeled as from concentrate were also correctly assigned in this case to group B. However, the classification of one of the orange juices labeled as from concentrate was doubtful (represented in Fig. 3 by the asterisk with a canonical variable 1 equal to 1 and a canonical variable 2 equal to 0). Interestingly, this sample corresponds to an off-flavor orange juice from concentrate. It has been shown that off-flavor in orange juices can be due to many factors (*e.g.*, quality of fruit, contamination of fruit, temperature of storage, microbiological spoilage, *etc.*) that in some cases can alter the amino acids profile obtained [19–21], what would corroborate the usefulness of our chiral approach.

In conclusion, this work shows that the combination of chiral MEKC-LIF analysis of FITC-amino acids and discriminant techniques is an adequate tool to classify orange juices according to the next categories: (i) pasteurized orange juices not from concentrate, (ii) orange juices from concentrate and (iii) nectars. The chiral MEKC-LIF method brings about the separation and detection of 15 enantiomeric forms of amino acids in less than 18 min with reliable analysis time and peak area reproducibility. D-Ala, D-Asp, D-Arg, and D-Glu were determined as D-amino acids naturally occurring in orange juices. To our

knowledge, this work is the first report of the combined use of a chiral CE procedure and chemometric methods to classify foods.

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