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Chiral analysis of derivatized amino acids from kefir by gas chromatography

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ABSTRACT

The amino acids present in the kefir samples were derivatized using different alkyl chloroformates combined with alcohols and chromatographed on a (3-*O*-butanoyl-2,6-di-*O*-pentyl)-γ-cyclodextrin (Lipodex E) chiral gas–liquid chromatographic column. The combination of derivatization reagents that led to better chromatographic performance for standard amino acid solutions was critically compared. All the chloroformate/alchohol mixtures lead to fast and quantitative reactions. However, the ethyl chloroformate/ethanol mixture was selected since the number of enantioseparated amino acid derivatives was higher. The derivatization was applied to samples of kefir grains grown in raw and in skimmed UHT milk. The samples were previously pretreated by elution from a low-pressure ion-exchange columns. Standard addition calibration curves have been used for the quantitative determinations of amino acid derivatives in both samples, and the figs. of merit of the proposed method have been assessed. The L- and D- configuration of D- and L-alanine, D- and L-valine, D-proline, L-threonine, aspartic and glutamic acids, methionine, and cysteine were detected and quantitatively determined.

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1. Introduction

Hundreds of amino acids are known, and twenty of them are constitutive of proteins and peptides of living systems. With the exception of glycine, the other proteinogenic α-amino acids present an asymmetric carbon and, as a consequence, they are chiral and can exist in either of two enantiomeric forms, L- or D-amino acids. Natural proteins are constituted by L-amino acids, but also D-amino acids have been found in different natural samples. Some examples of matrices in which D-amino acids have been detected include *i.* bacterial culture used in the production of food and fermented drinks [1]; *ii.* saliva of human beings where it has been detected the presence of significant levels of D-alanine and D-asparagine [2]; *iii.* biological fluids where their presence were proposed as markers that would allow detection of some metabolic disorder [3–6]; *iv.* food industrial products where amino acids contribute to the food nutritional and biological value. Moreover, it has been found that racemization can occur during manufacturing steps of processed food, such as fermented drinks and dairy products, as well as in biotechnological products [7,8]. Consequently, D-amino acids in the diet deserve systematic controls [9].

Kefir is a fermented milk with demonstrated health benefits including restorative properties of bacterial flora and stomach mucous, reduction of the symptoms of lactose intolerance, immune system stimulation, cholesterol reduction, as well as anti-mutagenic and anti-tumor properties [10–13]. Its consumption as a dairy product has been increasingly recommended for those properties; thus, assessing its nutrient composition is very important.

The determination of enantiomeric amino acids can be carried out by gas chromatography. There are two general approaches to enantiomeric separation; the indirect method, based on the chemical reaction of the racemic mixture with a chiral reagent to obtain a diastereomeric pair and their subsequent chromatographic separation in an achiral stationary phase; and the direct method, based on the use of a chiral stationary phase which can discriminate between enantiomers.

Chiral stationary phases most frequently used in the enantiomeric separation of amino acids by gas chromatography are either, the commercially known as Chirasil-Val (L-valine-*tert.*-butylamide-linked polydimethylsiloxane), introduced firstly by Frank and co-workers [14], which has proved to be capable of resolving most amino acids in short analysis time [15], or modified cyclodextrins such as (3-*O*-butanoyl-2,6-di-*O*-pentyl)-γ-cyclodextrin (Lipodex E), which has been successfully used for separation of trifluoroacetyl methyl esters of amino acids [16-18].

For the analysis by gas chromatography, the amino acids must be derivatized to volatile compounds. A widely used type of derivatization is the preparation of N(O)-trifluoroacetyl alkyl esters amino acid derivatives. This procedure involves laborious and time-consuming multiple steps, including the total removal of water from the sample, esterification under heating, removal of the excess alcohol, followed by acylation also under heating and, finally, removal of the excess reagents. The whole derivatization technique (after drying aqueous samples) takes about 1 h.

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Table 1
Retention factors, separation factors, and enantioresolutions of ECF/EtOH derivatives using isothermal conditions.

Amino acid	T °C	k1	α	Rs
Ala	120	5.5	1.02	0.55
Arg	ne			
Asn	150	11.55	1.04	0.86
Asp	150	10.43	1.05	1.3
Cys	160	24.11	1	–
Gln	ne			
Glu	150	17.73	1	–
His	190	35.28	1	–
Ile	150	3	1.07	1.5
Leu	120	13.99	1.03	0.63
Lys	ne			
Met	150	16.35	1	–
Phe	ne			
Pro	130	11.14	1.05	1.22
Ser	150	10.82	1.06	1.36
Thr	150	8.18	1.12	2.89
Trp	ne			
Tyr	ne			
Val	120	8.74	1.04	0.62

Separation factor $\alpha = k_2/k_1$, and resolution determined at isothermal temperature T (°C); ne = not eluted.

Another type of derivatization involves the treatment of amino acids with a mixture of alcohol–pyridine chloroformate to obtain N-alkyloxycarbonyl alkyl ester amino acids. This technique was introduced by Hušek [19], and then investigations were made to develop quantitative and reproducible methods of amino acids analysis in different matrices [20–22]. It offers many advantages: *i.* it is a one-step reaction; *ii.* it occurs in aqueous medium, so the analysis can be performed directly in the aqueous matrix; *iii.* it is practically instantaneous at room temperature under mild conditions, avoiding possible racemization.

In this study, the enantiomeric characterization of the free amino acids present in kefir samples is proposed. A critical evaluation and comparison of the results obtained by using different combinations of alkyl chloroformates and alcohols to increase the chromatographic signal and to obtain the maximum number of derivatized amino acids that may be enantiomerically resolved at the lowest possible temperature within reasonable periods of time is presented. A commercial Lipodex E (octakis(3-*O*-butanoyl-2,6-di-O-n-pentyl)-γ-cyclodextrin) column was used. To the best of our knowledge, these amino acid derivatives have not been enantioseparated using cyclodextrin-based columns. Derivatization reaction and chromatographic separation conditions were optimized and the method was finally used for the analysis of amino acid composition in kefir.

2. Experimental

2.1. Chemicals

The amino acids alanine (Ala), arginine (Arg), asparagine (Asn), aspartic acid (Asp), cysteine (Cys), glutamine (Gln), glutamic acid (Glu), histidine (His), isoleucine (Ile), leucine (Leu), lysine (Lys), methionine (Met), phenylalanine (Phe), proline (Pro), serine (Ser),

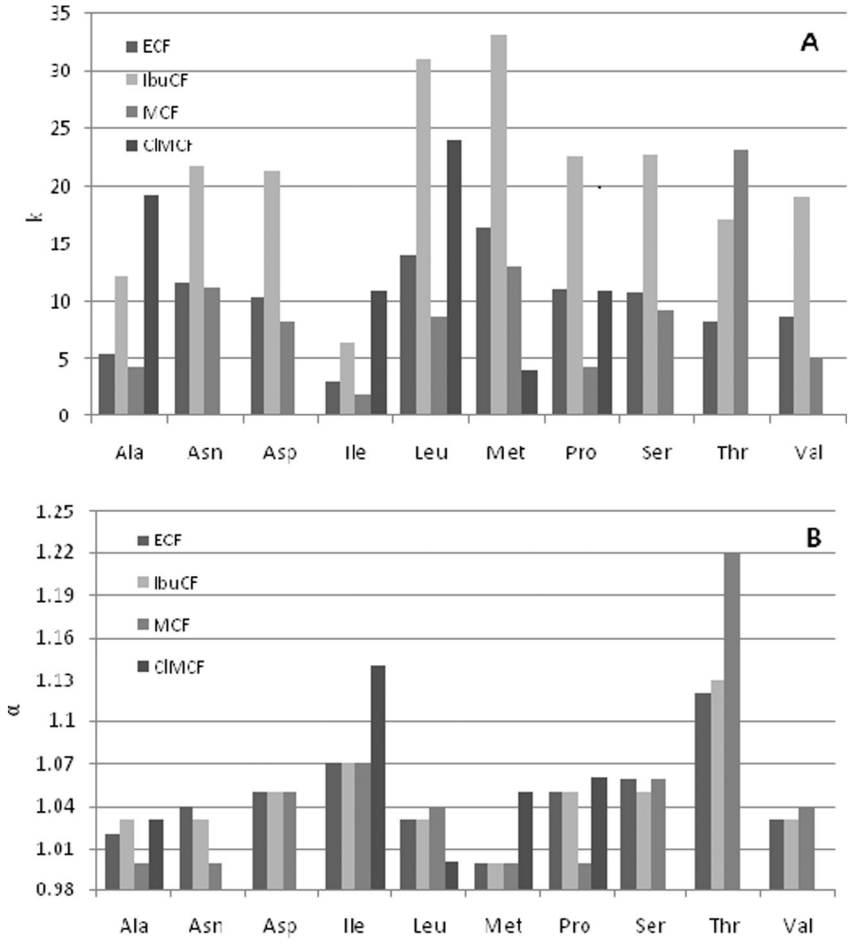

Fig. 1. Comparison of chromatographic retention (A) and enantioseparation factors (B) of amino acids derivatized whit ECF/EtOH, IbuCF/EtOH, MCF/EtOH, and ClMCF/EtOH. Column: Lipodex E, 25 m. Temperature: Ala, Leu, and Val = 120 °C, except for MCF derivatives, run at 110 °C; Asn, Asp, Ile, Met, Ser, and Thr = 150 °C; Pro = 130 °C and ClMCF at 150 °C.

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