The EuroBiotech Journal
RESEARCH ARTICLE
Bioprocess Engineering


# Applying gas chromatography to monitor extracellular free amino acids content in cultivation medium during lactic acid fermentation

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## Abstract

The aim of this work was the adaptation of a Gas Chromatographic-Flame Ionization Detector (GC-FID) method for detection and quantification of extracellular free amino acids in demineralized water, De Mann Rogosa Sharpe (MRS) medium and corn grits (CG) withdrawn during lactic acid fermentation. In order to analyze free amino acids by the GC-FID method it was necessary to convert free amino acids to volatile compounds. This was accomplished by derivatization of free amino acids with ethylchlor formate in aqueous medium followed by extraction of volatile free amino acid esters with chloroform. It was proven that the combination of derivatization and extraction procedure with developed GC-FID method gave accurate, reproducible and sensitive analytical results. Quantification of 15 (Ala, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Asn, Met, Pro, Lys, His, Asp and Glu) out of 20 ethoxycarbonyl-ethyl esters of free amino acids in demineralized water and MRS medium was achieved by established methods. In corn grits medium all of the above mentioned 15 amino acids, except His, were quantified with this GC-FID method. The established method was efficiently verified in monitoring of extracellular free amino acid concentration during lactic acid production with *Lactobacillus rhamnosus* DSM 20021$^T$ in MRS medium and *Lactobacillus amylovorus* DSM 20531$^T$ in corn grits medium.

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Published online: 27 January 2017
doi:10.24190/ISSN2564-615X/2017/01.05

## Introduction

Lactic acid bacteria (LAB) have multiple nutritional requirements for growth, especially amino acids (AA) and peptides which are usually obtained by the proteolysis of proteins or peptides present in yeast extract, meat extract, peptone and other raw materials that are added to the medium (1). Free AA (FAA) are: polar compounds with very low volatility, AA that are not bound in peptides, proteins or any other compounds and, therefore, have free amino and carboxyl groups. Therefore, quantitative determination of LAB requirements for free AA (FAA) is very important for development of sustainable and cheap lactic acid production processes. There are only few scientific works that deal with monitoring of changes in extracellular FAA concentration during growth of the LAB and lactic acid production (2-8). FAAs are an important group of organic compounds that can be detected and quantified by different analytical methods.

Absence of chromophores complicates the possibilities for detection of FAAs. Therefore, most of the methods that are used in analysis of FAA imply derivatization - chemical modification of certain groups of FAA, mostly amino- or carboxyl groups. By means of derivatization FAA are converted to compounds that can be separated and detected by different analytical methods (9). Several methods are usually used in separation and quantification of FAA in different media, like colorimetric methods (10), capillary electrophoresis, high pressure liquid chromatography (ion exchange or reverse phase) and gas chromatography coupled with different detection techniques (ultraviolet, fluorescence, electrochemical, mass spectrometry detection, etc.; 11-14).

Concentration of FAA can be determined by liquid chromatography: directly without derivatization, with derivatization prior to analysis and separation by ion-exchange chromatography followed by post column derivatization (12). Some of the compounds

that are usually used for derivatization of FAA are dansyl chloride, 9-fluorenylmethyl chloroformate, o-phthaldialdehyde, phenylisothiocyanate, bis(trimethylsilyl) trifluoroacetamide, and *N*-methyl-*N*-(tert-butyldimethylsilyl) trifluoroacetamide (15,16). After derivatization with 9-fluorenylmethyl chloroformate, o-phthaldialdehyde, phenylisothiocyanate (11), concentration of FAA can be only determined by gradient HPLC method or reverse phase HPLC method coupled with UV-VIS detection (14, 17). Furthermore, concentration of FAA can be quantified by gas chromatography, but prior to analysis it is essential to convert FAA to volatile compounds (18). Compounds that are often used as derivatizing reagents belong to a trimethylsilyl group [e.g. bis(trimethylsilyl) trifluoroacetamide, and *N*-methyl-*N*-(tert-butyldimethylsilyl) trifluoroacetamide]. These derivatization reactions are performed in organic solutes and these procedures are quite complicated and long lasting, especially when dealing with samples of biological origin. Samples that are used for derivatization can be homogenous or heterogeneous aqueous samples (13). Other reagents, as alkylchlorformates (methyl-, ethyl-, propyl- and iso-butyl chlorformates), are used for esterification of FAA. Advantages of these reagents are: the derivatization procedure can be performed in aqueous medium (19), esterification reaction is fast and the resulting esters (*N*(*O*,*S*)-alkoxycarbonyl alkyl esters) are stable (16, 18). The mechanism of esterification reactions has been described elsewhere (18).

The aim of this work was the adaptation of the Hušek derivatization procedure, development and validation of Gas Chromatographic- Flame Ionization Detector (GC-FID) method for detection and quantification of extracellular FAA in demineralized water and in MRS medium and corn grits (CG) medium during lactic acid production by LAB. One advantage of this method, ahead of other GC or HPLC methods, is extraction of *N*(*O*,*S*)-ethoxycarbonyl ethyl esters of FAA to chloroform phase by which interference of other compounds present in aqueous phase in the samples withdrawn during fermentation experiments is greatly diminished.

## Materials and Methods

### Materials and media

FAAs used for preparation of stock solutions were: Cys, Tyr, Gly, Ala, Val, Leu, Ile, Pro, Lys, Asp, Arg, Glu, Asn, Phe, Trp, Met, Gln, Ser, Thr, His and norleucine (norLeu; Sigma, USA) as internal standard (IS). For derivatization and extraction procedures the following chemicals were used: ethyl chlorformate (ECF), pyridine (Sigma-Aldrich, Germany), chloroform, absolute ethanol (Kemika, Croatia).

For fermentation experiments pre-mixed MRS medium (Biolife, Italy) was used. This MRS medium used for growth of LAB consisted of (g $L^{-1}$): yeast extract, 5.0; peptone 10.0; meat extract 10.0; $KH_2PO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; $MnSO_4 \cdot H_2O$, 0.05; sodium acetate, 2.0; sodium citrate, 5.0; glucose, 20.0 and Tween 80, 1.0 (in mL $L^{-1}$). Chemicals used for preparation of MRS (De Mann, Rogosa, Sharpe) medium without yeast extract, meat extract and peptone were purchased from Merck (Merck, Germany). All chemicals used were of analytical grade. Corn grits (CG) used for preparation of CG medium were purchased from local production company (Podravka d.d., Croatia). All prepared media, MRS medium, MRS medium without yeast extract, meat extract and peptone and CG medium (100 g $L^{-1}$ and 10 g $L^{-1}$) were prepared with demineralized water (conductivity < 1 μS $cm^{-1}$) and sterilized at 121°C for 20 minutes and pressure of 2.5 bars. CG medium with high concentration of CG (100 g $L^{-1}$) could not be used for construction of calibration curves because it has paste-like consistency. Therefore, sterilized CG medium with lower concentration of CG was employed (10 g $L^{-1}$). Due to the heterogeneous consistency of this medium, centrifugation (4000*g* for 20 min at 4°C) was performed to remove CG particles, while resulting supernatant of CG was used for preparation of FAA stock solutions.

### Analysis of extracellular FAA concentration

#### Lactic acid fermentation

Lactic acid production were performed in laboratory-scale stirred tank bioreactor in MRS medium at 40°C by *Lactobacillus rhamnosus* DSM 20021$^T$ (isolated from silage and characterized in the Belgian Co-ordinated Collections of Microorganisms / Laboratory for Microbiology, University of Ghent, Ghent, Belgium) and in CG medium (100 g $L^{-1}$) at 45°C by *Lactobacillus amylovorus* DSM 20531$^T$ (obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany). During bioprocesses conducted in CG medium, for the first 6 h withdrawn samples contained no free flowing water due to the semi-solid consistency of the medium. Therefore, these samples could not be used for determination of FAAs. Bioreactor was loaded with 5 L of the medium which was inoculated with 2.5 % (v/v) of culture suspension. The pH value was maintained at 5.5 by automatic addition of 10 mol $L^{-1}$ NaOH. Samples were taken in regular intervals and afterwards the cells were removed by centrifugation (4000*g* for 20 min at 4°C) and aliquots of resulting supernatants were retained, without further processing, for analysis and stored at -20°C. Samples withdrawn during lactic acid production in the two media were analyzed by the GC-FID method at least in duplicates.

#### Supernatant pretreatment

After defrosting, all supernatants were filtered through 0.22 μm syringe filter (Macherey-Nagel GmbH CoKG, Germany) and diluted with demineralized water. For derivatization procedure 100 μL of diluted supernatant was taken. A volume of 100 μL of prepared mixture of 20 FAA in demineralized water, MRS medium without of yeast extract, peptone and meat extract and supernatant of CG medium (10 g $L^{-1}$) was also taken for derivatization procedure. FAAs from these samples were converted to *N*(O,S)-etohycarbonyl ethyl esters by adapted derivatization procedure (18). In brief, the solution of internal standard (IS; norleucin, $c$ = 10 mmol $L^{-1}$, $V$ = 5 μL) was added to the sample (supernatant, or prepared mixture of 20 FAA). HCl ($c$ = 1

mmol $L^{-1}$, $V$ = 86 µL) and NaOH solution ($c$ = 1 mmol $L^{-1}$, $V$ = 114 µL) were added. The resulting solution was vortexed for 20 sec, and afterwards 100 µL of demineralized water, ethanol and pyridine solution (water: ethanol: pyridine = 60: 32: 8 (v/v)) was added and vigorously mixed for 20 sec. To this solution 5 µL of ethyl-chlorformate (ECF) ($\phi$ > 98 %) was added and vortexed for 20 sec until evolution of carbon dioxide ends, and left for one min at 22°C and again vortexed. The reaction mixture obtained was used for extraction of FAA esters from aqueous phase to chloroform phase.

To the 410 µL of reaction mixture, 100 µL of chloroform containing 1% ECF (v/v) was added and mixed. The sample was left to allow separation of aqueous phase and chloroform phase. For GC analysis the chloroform phase was used.

### Calibration curves

Stock solutions of 20 FAA (Cys and Tyr, $c$ = 2.50 mmol $L^{-1}$; Gly, $c$ = 7.50 mmol $L^{-1}$; Ala, Val, Leu, Ile, Pro, Lys, Asp, Arg, Glu and Asn, $c$ = 8.50 mmol $L^{-1}$; Phe, Trp and Met, $c$ = 9.00 mmol $L^{-1}$; Gln, $c$ = 17.50 mmol $L^{-1}$; Ser, Thr and His, $c$ = 25.00 mmol $L^{-1}$) and internal standard (IS) solution (norLeu, $c$ = 10.00 mmol $L^{-1}$) were prepared by dissolving appropriate amounts of each FAA and IS in 10 mL of demineralized water. The resulting 20 solutions of FAA were used for preparation of mixture of all 20 FAAs. Mixtures of all 20 FAA in demineralized water was prepared by taking an aliquots of 400 µL of Cys and Tyr stock solution and 100 µL of remaining 18 FAA stock solutions. The concentration of each FAA in this mixture was in the range from 0.28 mmol $L^{-1}$ to 0.93 mmol $L^{-1}$. In order to obtain derivatized amino acid (DAA) solutions in concentration span from 0.028 mmol $L^{-1}$ to 0.930 mmol $L^{-1}$, this mixture was diluted with demineralized water in defined ratios (10: 90; 20: 80; 40: 60; 60: 40; 80: 20; 100: 0). Prepared standard solution was used in sample pretreatment procedure (see subtitle "Supernatant pretreatment"). For preparation of mixtures of 20 FAA in MRS medium without yeast extract, peptone and meat extract and in supernatant of CG medium (10 g $L^{-1}$) the same procedure was applied.

Obtained mixtures of 20 FAA in demineralized water, MRS medium without yeast extract, peptone and meat extract and in supernatant of CG medium, and samples obtained in fermentation experiments were treated as described in subtitle "Su-

**Table 1. Retention times, calibration curve equations and $R^2$ values of amino acids in demineralized water (DW) and in two media, MRS medium without meat extract, yeast extract and peptone (MRS) and supernatant of corn grits medium (CG)**

| AA | $t_R$ (min) | DW | | | MRS | | | CG | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | *Peak height* = | $R^2$ (-) | LR (mmol $L^{-1}$) | *Peak height* = | $R^2$ (-) | LR (mmol $L^{-1}$) | *Peak height* = | $R^2$ (-) | LR (mmol $L^{-1}$) |
| Ala | 10.141±0.004 | 0.7514·*c*+0.0031 | 0.991 | 0.038-0.308 | 1.3885·*c* -0.0542 | 0.993 | 0.077-0.385 | 0.7800·*c* -0.0107 | 0.994 | 0.038-0.385 |
| Asn | 16.195±0.005 | 0.4333·*c* -0.0009 | 0.996 | 0.038-0.308 | 2.3862·*c* -0.1385 | 0.990 | 0.077-0.308 | 0.5538·*c* -0.0094 | 0.998 | 0.038-0.385 |
| Asp | 18.694±0.005 | 0.3553·*c* +0.0034 | 0.995 | 0.038-0.308 | 0.3167·*c* +0.0006 | 0.999 | 0.038-0.385 | 0.2859·*c* -0.0078 | 0.991 | 0.038-0.385 |
| Glu | 20.485±0.002 | 0.3283·*c* +0.0076 | 0.995 | 0.038-0.308 | 0.3821·*c* -0.0027 | 0.990 | 0.038-0.385 | 0.4187·*c* -0.0048 | 0.994 | 0.038-0.308 |
| Gly | 10.265±0.006 | 0.3418·*c* -0.0007 | 0.994 | 0.038-0.308 | 0.6952·*c* -0.0281 | 0.993 | 0.038-0.308 | 0.4645·*c* -0.0007 | 0.995 | 0.038-0.385 |
| His | 21.944±0.002 | 0.3043·*c* -0.0073 | 0.999 | 0.096-0.770 | 0.1472·*c* +0.0159 | 0.993 | 0.096-0.770 | - | - | - |
| Ile | 15.150±0.013 | 1.1912·*c* -0.0026 | 0.993 | 0.038-0.308 | 0.9182·*c* -0.0086 | 0.997 | 0.038-0.385 | 0.4555·*c* -0.0059 | 0.993 | 0.038-0.308 |
| Leu | 14.671±0.011 | 1.4746·*c* +0.0075 | 0.996 | 0.038-0.308 | 2.3784·*c* -0.0368 | 0.991 | 0.038-0.308 | 1.7722·*c* -0.1268 | 0.993 | 0.077-0.308 |
| Lys | 22.552±0.005 | 2.0407·*c* -0.0586 | 0.990 | 0.038-0.308 | 1.9672·*c* -0.0084 | 0.995 | 0.038-0.308 | 1.0593·*c* -0.0385 | 0.998 | 0.038-0.385 |
| Met | 20.037±0.007 | 2.5475·*c* -0.0125 | 0.997 | 0.038-0.308 | 2.8650·*c* -0.0772 | 0.993 | 0.038-0.308 | 2.2246·*c* -0.0448 | 0.997 | 0.038-0.385 |
| Phe | 21.062±0.006 | 5.4279·*c* -0.0114 | 0.991 | 0.038-0.308 | 8.0471·*c* -0.1786 | 0.990 | 0.038-0.308 | 4.4254·*c* -0.0118 | 0.991 | 0.038-0.385 |
| Pro | 15.927±0.011 | 1.1269·*c* -0.0066 | 0.993 | 0.038-0.308 | 2.0206·*c* -0.0439 | 0.990 | 0.038-0.308 | 0.7816·*c* +0.0042 | 0.994 | 0.038-0.385 |
| Trp | 23.344±0.030 | 2.7408·*c* -0.0889 | 0.991 | 0.038-0.308 | 3.6842·*c* -0.1413 | 0.994 | 0.038-0.308 | 1.0376·*c* -0.0311 | 0.991 | 0.038-0.385 |
| Tyr | 23.050±0.008 | 5.5341·*c* -0.0757 | 0.996 | 0.038-0.308 | 6.3829·*c* +0.0020 | 0.990 | 0.038-0.308 | 1.6586·*c* -0.0462 | 0.991 | 0.038-0.385 |
| Val | 12.780±0.011 | 0.7122·*c* -0.0050 | 0.993 | 0.038-0.308 | 0.8427·*c* +0.0024 | 0.997 | 0.038-0.385 | 0.8101·*c* -0.0074 | 0.991 | 0.038-0.308 |

AA, amino acid; $t_R$, retention time; $R^2$, square correlation coefficient; LR, linearity range

pernatant pretreatment". For construction of calibration curves and GC-FID method validation, mixtures of 20 FAA were analyzed at least in triplicates.

**Linearity, precision, recovery and accuracy of GC-FID method**

Method linearity was determined by evaluating the regression curve and it is indicated by the square correlation coefficient ($R^2$). Linearity was achieved with a minimal $R^2$ of 0.990. Intra-day precision was expressed as the coefficient of variation (%CV) of GC-FID method and it was determined by injecting chloroform phase of reaction mixture in five replicates in concentrations pointed out in **Table 1**.

Accuracy (A) was calculated as the percentage relative error of the method:

$$A = \frac{\text{mean calculated concentration - nominal concentration}}{\text{nominal concentration}} \cdot 100 \quad [1]$$

Detection limits were determined by replicate GC-FID analysis of mixture of 20 FAA and IS with the lowest concentration of FAA ($c$ = 0,038 mmol $L^{-1}$ for all 14 quantified FAA, and $c$ = 0,096 mmol $L^{-1}$ for His). The limit of detection (LOD) and quantification (LOQ) were calculated using the following equations:

$$LOD = 3.3\frac{S_{y/x}}{b} \quad [2]$$

**Table 2. Chromatographic conditions for GC-FID analysis**

| | |
|---|---|
| Autosampler | Shimadzu AOC 20s (Shimadzu, Japan) |
| Auto-injector | Shimadzu AOC 20i (Shimadzu, Japan) |
| Injector temperature | 300 °C |
| Inlet liner | deactivated inlet liner suitable for split/splitless injection (cat. no. 220-90784-00; Shimadzu, Japan) |
| Injection mode | split 1:5 |
| Injection volume | 2 µL |
| GC | Shimadzu GC 2010OPlus (Shimadzu, Japan) |
| Analytical column | Rtx®-5ms (30 m L, 0.25 mm ID, 0.25 µm $d_f$; Restek, USA) |
| Carrier gas | Helium 99.999 % (Messer, Croatia) |
| Carrier gas flow rate | 0.7 mL/min |
| Oven program | 70 °C for 3 min,<br>30 °C/min to 120 °C, 2 min hold,<br>5 °C/min to 150 °C, 3 min hold<br>50 °C/min to 330 °C, 5 min hold |
| FID temperature | 300 °C |
| Total analysis time | 26.6 min |

GC, gas chromatograph; FID, flame ionization detector

$$LOQ = 10\frac{S_{y/x}}{b} \quad [3]$$

$S_{y/x}$ is standard deviation of regression line and b is the slope of the calibration curve.

**Chromatographic conditions**

The GC-FID method for detection and quantification of extracellular FAA was carried out with a Shimadzu GC-2010Plus (Shimadzu, Kyoto, Japan) equipped with a flame ionization detector (FID). Chromatographic conditions are listed in **Table 2**. For data acquisition and data processing GCSolutions software ver. 2.3 was used.

## Results and Discussion

**Derivatization of extracellular FAA**

The main problem in analysis of extracellular FAA in the sample by GC-FID method is the need for derivatization. Usually this procedure is laborious and long lasting. The usage of ECF reagent simplifies derivatization procedure (19). To our knowledge there is no data available on derivatization of FAA in MRS medium or supernatant or CG medium with ECF. When multiple sample preparation steps (e.g. derivatization, extraction, evaporation) are necessary to prepare a sample for GC-FID analysis, every one of these steps can increase the error and variability of results. Therefore, in analysis of extracellular FAA esters it is essential to use IS. Quantitation by IS compensates random and systematic errors of the method or detector (20). NorLeu is stable AA and it is readily converted to esters and can be separated from other amino acids naturally occurring in the sample (21).

**Validation of the modified Hušek method**

Calibration curves obtained in demineralized water, MRS medium without yeast extract, meat extract and peptone, and in supernatant of CG medium have unequal slope and intercepts of the calibration curves for corresponding FAA (Table 1). Therefore, using only calibration curves obtained in e.g. demineralized water (DW) for quantification of FAA in the two media would give incorrect results.

Interference from the MRS and CG media were ruled out by injecting chloroform phase of each medium without any addition of 20 AA or IS. The two media were prepared by the same procedure as the standard mixture or supernatants (see subtitle "Supernatant pretreatment"). In obtained chromatograms (data not shown) there were no peaks in the retention times ($t_R$) of FAA.

Separation and detection of 15 AA (Ala, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Asn, Met, His, Pro, Lys, Asp and Glu) out of 20 AA in DW and in MRS medium without yeast extract, meat extract and peptone, and 14 AA (His was not detected) in the supernatant of CG medium was achieved in this work (**Fig. 1 A, B** and **C**).

Amino acids Thr, Ser, Cys, Gln (His) and Arg could not be

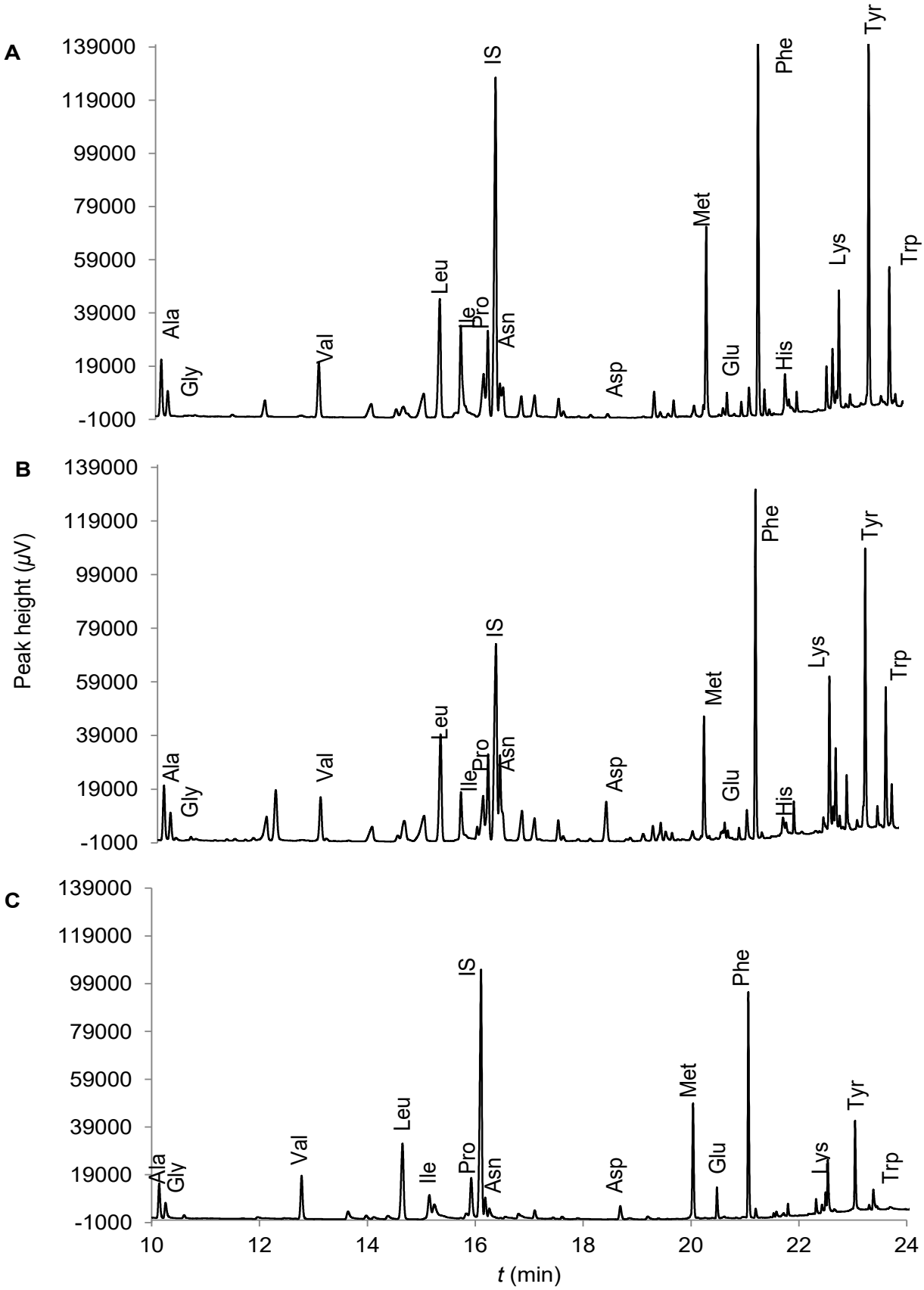


**Figure 1.** Part of the representative chromatogram ($t_R$ = 10-24 min) obtained from mixture of 20 amino acids and internal standard (IS, norLeu) in: (A) demineralized water, (B) MRS medium without yeast extract, meat extract and peptone, and (C) supernatant of corn grits medium with peak identification and retention times ($t_R$).

**Table 3.** LOD and LOQ values in mmol $L^{-1}$ in demineralized water (DW) and in two media, MRS medium without meat extract, yeast extract and peptone (MRS) and supernatant of corn grits medium (CG)

| AA | DW | | MRS | | CG | |
|---|---|---|---|---|---|---|
| | LOD (mmol $L^{-1}$) | LOQ (mmol $L^{-1}$) | LOD (mmol $L^{-1}$) | LOQ (mmol $L^{-1}$) | LOD (mmol $L^{-1}$) | LOQ (mmol $L^{-1}$) |
| Ala | 0.023 | 0.068 | 0.025 | 0.074 | 0.080 | 0.243 |
| Asn | 0.037 | 0.112 | 0.038 | 0.115 | 0.099 | 0.301 |
| Asp | 0.038 | 0.116 | 0.032 | 0.097 | 0.098 | 0.298 |
| Glu | 0.038 | 0.116 | 0.032 | 0.097 | 0.047 | 0.142 |
| Gly | 0.030 | 0.091 | 0.022 | 0.068 | 0.081 | 0.246 |
| His | 0.050 | 0.152 | 0.096 | 0.291 | - | - |
| Ile | 0.021 | 0.063 | 0.096 | 0.291 | 0.094 | 0.286 |
| Leu | 0.027 | 0.082 | 0.032 | 0.097 | 0.036 | 0.111 |
| Lys | 0.035 | 0.106 | 0.033 | 0.100 | 0.063 | 0,191 |
| Met | 0.037 | 0.112 | 0.107 | 0.325 | 0.058 | 0.176 |
| Phe | 0.014 | 0.042 | 0.024 | 0.072 | 0.024 | 0.073 |
| Pro | 0.032 | 0.097 | 0.029 | 0.087 | 0.013 | 0.039 |
| Trp | 0.035 | 0.106 | 0.031 | 0.094 | 0.094 | 0.286 |
| Tyr | 0.035 | 0.106 | 0.108 | 0.326 | 0.036 | 0.109 |
| Val | 0.028 | 0.085 | 0.038 | 0.115 | 0.119 | 0.360 |

AA, amino acid; LOD, limit of detection; LOQ, limit of quantification

quantified with this method. Derivatization of Arg does not result in ester volatile enough to evaporate in injector (22). Solution of only Thr in DW and only Ser in DW were subjected to derivatization and extraction procedure. Afterwards, chloroform phase was analyzed by GC-FID method and $t_R$ for Thr and Ser were obtained ($t_R$ = 15.833±0.008 min and $t_R$ = 16.212±0.008 min). Assigned peaks for Thr and Ser had relatively low intensity. The same procedure was employed for His, Cys and Gln. Retention times for 15 (14) out of 20 FAA esters that were detected are shown in **Table 1**. The $t_R$ for IS - nor-Leu was 16.835±0.005 minutes. As it can be seen in **Fig. 1**, the selected chromatographic conditions resulted in a very good resolution and peak separation. LOD and LOQ values for each amino acid in each matrix are shown in **Table 3**.

Square correlation coefficient for 15 (14) amino acids in DW, MRS medium and supernatant of CG medium is from 0.990 to 0.999 (**Table 1**). Linearity was achieved in the concentration range from 0.038-0.385 mmol $L^{-1}$ (**Table 1**). Therefore, it can be concluded that this method has very good reproducibility and accuracy in DW and the two media. The acceptance criterion for the accuracy (A) was 100 ± 20%, and most of the data was in this range. Reproducibility of the method was in the range from 0.38 to 14.80%, while accuracy was in the range of 0.15 to 27.61% (**Table 4**).

**Quantification of extracellular FAA in samples withdrawn during lactic acid fermentation**

Practical application of the method has been verified by analyzing extracellular FAA concentration in the samples withdrawn during bioprocess of lactic acid fermentation in MRS medium and in CG medium (**Table 5**). It is important to stress that CG medium has a consistency of paste at the beginning of the bioprocess. And even with such heterogeneous medium it is possible to quantify extracellular FAA with this method. Quantification of 15 FAA in MRS medium and 14 FAA in CG medium was achieved, in a single run. Data available on determination of extracellular FAA content during different bioprocesses conducted by LAB is scarce (3-5, 7, 8). Data from Simova (4) clearly show that LABs have different nutritional requirements for AA and, therefore, FAA concentration during kefir production varies significantly depending on which LAB strain is used. Therefore data obtained with this method cannot be compared to any of the data available in the literature.

**Table 4.** Intra day coefficient of variation and accuracy of the method in demineralized water (DW) and in two media, MRS medium without meat extract, yeast extract and peptone (MRS) and supernatant of corn grits medium (CG)

| | | DW | | | MRS | | | CG | | |
|---|---|---|---|---|---|---|---|---|---|---|
| AA | *c* (mmol $L^{-1}$) | mean±SD | CV (%) | A (%) | mean±SD | CV (%) | A (%) | mean±SD | CV (%) | A (%) |
| Ala | 0.038 (0.077*) | 0.235±0.001 | 1.46 | +0.31 | 0.069±0.001* | 0.42 | +15.02 | 0.021±0.001 | 0.82 | +6.34 |
| | 0.308 | 0.032±0.001 | 0.46 | +0.15 | 0.495±0.014 | 2.81 | +2.79 | 0.216±0.006 | 2.57 | -5.81 |
| Asn | 0.038 (0.077*) | 0.018±0.001 | 0.78 | +14.29 | 0.066±0.001* | 1.44 | +11.12 | 0.010±0.001 | 2.67 | -8.25 |
| | 0.308 | 0.131±0.001 | 0.78 | -1.42 | 0.603±0.006 | 0.98 | +0.90 | 0.163±0.003 | 1.56 | +0.97 |
| Asp | 0.038 (0.077*) | 0.016±0.001 | 1.45 | -3.74 | 0.015±0.000 | 1.30 | +17.39 | 0.016±0.001 | 4.61 | +7.60 |
| | 0.308 | 0.116±0.001 | 0.37 | +2.51 | 0.124±0.002 | 1.70 | +1.42 | 0.080±0.001 | 0.50 | +0.27 |
| Glu | 0.038 | 0.019±0.003 | 14.22 | -8.53 | 0.015±0.000 | 1.04 | -12.06 | 0.013±0.001 | 2.96 | +9.41 |
| | 0.308 | 0.111±0.001 | 0.65 | +2.41 | 0.152±0.008 | 5.21 | +1.21 | 0.126±0.001 | 1.11 | +1.68 |
| Gly | 0.038 (0.077*) | 0.014±0.001 | 0.48 | +14.35 | 0.031±0.001* | 0.29 | +9.84 | 0.020±0.001* | 6.70 | -0.07 |
| | 0.308 | 0.107±0.010 | 2.23 | +2.71 | 0.289±0.010 | 3.61 | +18.06 | 0.168±0.003 | 1.52 | +2.69 |
| His | 0.096 | 0,023±0.001 | 3.13 | +2.41 | 0,026±0.004 | 16.59 | -26.24 | - | - | - |
| | 0.192 | 0.049±0.007 | 14.49 | -4.12 | 0,049±0.003 | 6.97 | +17.25 | - | - | - |
| Ile | 0.038 (0.077*) | 0.046±0.001 | 2.46 | +8.00 | 0.041±0.001* | 2.45 | -6.69 | 0.014±0.001 | 1.83 | +16.26 |
| | 0.308 | 0.354±0.010 | 2.07 | -2.70 | 0.356±0.001 | 0.37 | -1.83 | 0.138±0.005 | 3.68 | +2.86 |
| Leu | 0.038 (0.077*) | 0.061±0.001 | 0.96 | -4.53 | 0.143±0.001* | 0.22 | -1.78 | 0.011±0.001 | 7.70 | +0.80 |
| | 0.308 | 0.452±0.006 | 1.28 | -2.22 | 0.993±0.002 | 0.25 | +12.51 | 0.409±0.002 | 0.59 | -1.83 |
| Lys | 0.077 | 0.089±0.007 | 8.00 | -6.00 | 0.150±0.003* | 2.25 | +4.56 | 0.008±0.001 | 3.06 | +16.18 |
| | 0.308 | 0.577±0.011 | 1.89 | +1.10 | 0.589±0.040 | 6.88 | -1.44 | 0.286±0.036* | 12.73 | -0.60 |
| Met | 0.038 (0.077*) | 0.088±0.001 | 1.77 | +4.05 | 0.824±0.048* | 5.84 | -2.11 | 0.052±0.002 | 3.53 | +14.40 |
| | 0.308 | 0.764±0.013 | 1.48 | -1.06 | 0.061±0.001 | 2.09 | +26.50 | 0.659±0.014 | 2.07 | +2.66 |
| Phe | 0.038 (0.077*) | 0.196±0.001 | 0.75 | +0.57 | 0.209±0.004* | 1.72 | -26.71 | 0.245±0.011* | 4.35 | +4.58 |
| | 0.308 | 1.604±0.019 | 1.19 | -3.38 | 2.371±0.127 | 5.35 | +2.86 | 1.308±0.021 | 1.63 | +4.17 |
| Pro | 0.038 (0.077*) | 0.045±0.001 | 1.53 | +3.11 | 0.105±0.001* | 0.42 | -4.02 | 0.067±0.003* | 4.92 | +3.92 |
| | 0.308 | 0.336±0.003 | 0.97 | -0.45 | 0.887±0.010 | 1.14 | +19.66 | 0.258±0.005* | 2.01 | +5.35 |
| Trp | 0.077 | 0.108±0.011 | 10.57 | -6.80 | 0.139±0.006* | 4.01 | -1.33 | 0.031±0.005 | 17.17 | -22.85 |
| | 0.308 | 0.788±0.058 | 7.36 | +3.84 | 1.016±0.303 | 29.86 | +1.95 | 0.357±0.015 | 4.21 | -2.82 |
| Tyr | 0.077 | 0.290±0.017 | 5.97 | -14.08 | 0.182±0.004 | 2.31 | -25.72 | 0.064±0.012 | 18.07 | -13.36 |
| | 0.308 | 1.632±0.052 | 3.20 | +0.19 | 1.989±0.371 | 18.66 | +1.09 | 0.485±0.037 | 7.73 | +4.04 |
| Val | 0.038 | 0.026±0.001 | 0.71 | +13.67 | 0.035±0.001 | 0.77 | +0.51 | 0.029±0.001 | 2.18 | +17.98 |
| | 0.308 | 0.211±0.003 | 1.36 | -1.52 | 0.321±0.005 | 1.45 | +1.93 | 0.251±0.006 | 2.46 | +3.48 |

AA, amino acid; *c*, concentration; SD, standard deviation; CV, coefficient of variation; A, accuracy
*, for amino acids labeled with asterix accuracy of the method was determined at concentration of 0.077 mmol $L^{-1}$

**Table 5. Changes in concentrations of free amino acids in samples withdrawn during lactic acid production in MRS medium by lactic acid bacterium Lactobacillus rhamnosus DSM 20021$^T$ and in corn grits medium (CG) by lactic acid bacterium Lactobacillus amylovorus DSM 20531$^T$**

| LAB / medium | Cultivation time (h) | FAA concentration (mM) | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ala | Gly | Val | Leu | Ile | Pro | Asn | Asp | Met | Glu | Phe | His | Lys | Tyr | Trp |
| *Lactobacillus rhamnosus* DSM 20021$^T$ / MRS medium | 0 | 3.00 | 5.22 | 5.21 | 2.91 | 7.99 | 2.61 | 1.06 | 1.51 | 2.58 | 2.87 | 0.79 | 0.33 | 1.32 | 0.63 | 0.00 |
| | 2 | 3.98 | 6.49 | 6.64 | 3.81 | 10.25 | 3.00 | 1.35 | 2.11 | 2.99 | 5.55 | 0.99 | 1.04 | 1.58 | 0.70 | 0.00 |
| | 4 | 4.41 | 6.99 | 7.66 | 4.39 | 12.43 | 3.43 | 1.56 | 2.53 | 3.43 | 7.93 | 1.22 | 1.42 | 1.81 | 0.74 | 0.00 |
| | 6 | 7.45 | 10.33 | 12.09 | 8.64 | 16.66 | 4.74 | 1.53 | 8.25 | 2.06 | 33.60 | 3.62 | 0.00 | 2.00 | 0.82 | 0.00 |
| | 8 | 7.04 | 10.03 | 12.06 | 8.26 | 16.09 | 5.36 | 2.95 | 9.66 | 2.06 | 33.78 | 3.52 | 0.00 | 2.61 | 0.69 | 0.00 |
| | 10 | 4.33 | 7.39 | 6.42 | 4.75 | 6.25 | 3.51 | 3.03 | 5.43 | 1.09 | 15.30 | 1.84 | 0.00 | 1.62 | 0.59 | 0.00 |
| | 25 | 4.51 | 7.75 | 7.06 | 5.01 | 7.43 | 3.98 | 2.81 | 7 00 | 0.98 | 18.20 | 2.06 | 0.69 | 1.71 | 0.63 | 0.00 |
| *Lactobacillus amylovorus* DSM 20531$^T$ / CG medium | 8 | 0.22 | 0.16 | 0.28 | 0.17 | 0.10 | 0.83 | 0.68 | 0.00 | 0.04 | 0.09 | 0.14 | n.d. | 0.19 | 0,11 | 0.14 |
| | 27 | 0.20 | 0.15 | 0.59 | 0.13 | 0.14 | 2.19 | 2.01 | 0.00 | 0.05 | 0.11 | 0.19 | n.d. | 0.20 | 0.05 | 0.07 |
| | 48 | 0.28 | 0.17 | 0.00 | 0.11 | 0.10 | 2.57 | 2.46 | 0.00 | 0.06 | 0.14 | 0.20 | n.d. | 0.21 | 0.13 | 0.17 |
| | 75 | 0.20 | 0.21 | 0.00 | 0.07 | 0.01 | 1.90 | 1.79 | 0.00 | 0.05 | 0.30 | 0.14 | n.d. | 0.25 | 0.16 | 0.14 |
| | 145 | 0.20 | 0.17 | 0.06 | 0.09 | 0.03 | 3.37 | 3.85 | 0.00 | 0.06 | 0.15 | 0.28 | n.d. | 0.54 | 0.13 | 0.13 |
| | 170 | 0.31 | 0.19 | 0.00 | 0.10 | 0.06 | 3.42 | 3.66 | 0.00 | 0.09 | 0.16 | 0.23 | n.d. | 0.40 | 0.16 | 0.16 |
| | 216 | 0.33 | 0.18 | 0.00 | 0.10 | 0.01 | 4.40 | 5.19 | 0.05 | 0.09 | 0.20 | 0.33 | n.d. | 0.43 | 0.12 | 0.16 |

LAB, lactic acid bacterium; FAA, free amino acid; n.d. not determined

## Conclusions

Derivatization and the extraction procedure of Hušek (18) in combination with GC-FID method applied here is a sensitive and reliable procedure to determine concentrations of extracellular FAA in the samples withdrawn during lactic acid fermentation in MRS medium and CG medium. Using the proposed method, extracellular FAA GC-FID analysis could be completed in a total of 30 min. If sample preparation (centrifugation, derivatization and extraction procedure) is included, time for analysis is prolonged to 50 minutes. The method was validated for the simultaneous determination of 15 and 14 out of 20 FAA in MRS medium and CG medium, respectively. Conditions used during the analysis resulted in very good peak resolution, and therefore the method can be routinely used in laboratories equipped with standard instrumentation like GC with FID. In addition, this method provided good limit of detection and good precision.


### Acknowledgements

This work was financially supported by Croatian Science Foundation under project "Sustainable production of bioethanol and biochemicals from agricultural waste lignocellulosic raw materials" (SPECH-LRM; project No. 9158).


### Conflict of interest statement

The authors declare no commercial or financial conflict of interest.

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