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Liquid chromatographic–mass spectrometric—analyses of anaerobe protein degradation products

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Abstract

A liquid chromatography–electrospray ionisation-mass spectrometry (LC–ESI-MS) method and a liquid chromatography–atmospheric pressure chemical ionisation-mass spectrometry (LC–APCI-MS) method was developed to identify metabolites from the anaerobe protein degradation to biogas. As consequence of a process failure the biogas production breaks down with increasing substrate loading, whereas different metabolites accumulate in the fermentation media. These compounds were identified as metabolites from the anaerobe degradation of the aromatic amino acids phenylalanine, tyrosine and tryptophan and accumulate in concentrations up to 300 mg/L when casein was used as model substrate.

Keywords: Anaerobe protein degradation; HPLC–MS^n; Ion trap; Electrospray ionisation; Atmospheric pressure chemical ionisation

1. Introduction

The anaerobe degradation of protein rich substrates by a complex consortium of microorganisms like liquid manure is not well established. No stable process method for the fermentation of protein rich wastes to biogas is developed until now. Laboratory experiments showed that protein rich wastes from the food industry can be co-fermented (content of ∼25% waste) with cattle manure. With higher substrate loading (∼50%) the process broke down [1].

Therefore, a reliable and rapid method has to be developed in order to identify accumulating metabolites that can serve as marker substances for starting inhibition reactions. This paper describes a high-performance liquid chromatographic–mass spectrometric (HPLC–MS) method for the determination of aromatic compounds resulting from the anaerobe protein degradation using casein as model substrate.

ESI-MS was used for ionisation of phenolic compounds by several authors. Eight phenolic compounds, obtained by in vitro fermentation of quercetin with human faecal flora, were quantified by ESI-MS [2]. Microbial aromatic acid metabolites in human urine were analysed employing a HPLC–ESI-MS–MS method [3]. The application of liquid chromatography using a diode-array detector (DAD) in connection with the analyses of product ions after electrospray ionisation (HPLC–DAD–ESI-MS^n) succeeded identification of phenolic compounds from artichoke heads, juice and pomace [4]. In contrast, ionisation of serotonin and related indoles in human whole blood by APCI was preferred [5].

In the present paper electrospray and atmospheric pressure chemical ionisation were used depending on the chemical properties of the determined compounds.

2. Experimental

2.1. Chemicals

Casein (Merck, Darmstadt, Germany), water (HPLC grade, J.T. Baker, Mallinckrodt B.V., Deventer, The Netherlands), acetonitrile (HPLC grade, Prochem, Weser, Germany), formic acid (50%, HPLC grade, Fluka, Buchs,

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Switzerland), standards for LC–MS (Fluka, Buchs, Switzerland and Merck, Darmstadt, Germany).

2.2. Inhibition experiments

The casein degradation was determined in 1 L fed-batch experiments containing swine manure from a biogas plant (Nordhausen, Germany) as inoculum at 37 °C.

The casein supply was 1 g/L per day. After reaching a stable biogas production, an inhibition of the process was initiated by increasing the casein concentration for 0.3 g/L per day. Samples were taken for LC–MS-analyses before the next substrate addition.

2.3. Sample preparation

Samples taken for analyses were centrifuged (10 min, 5000 × *g*, 4 °C, model 3K12, Sigma Laboratory Centrifuge, Osterode, Germany) and filtered (0.2 μm filter, Schleicher & Schuell, Dassel, Germany).

2.4. LC–MS-analyses

2.4.1. Standard solutions

A standard stock solution of 1000 mg/L of each target compound was prepared by dissolving accurate amounts of pure standard in acetonitrile. Working solutions of the individual standards and of mixture of all of them were achieved by several dilutions in water (HPLC grade). Stock and working standards were stored at 4 °C. Calibration curves were evaluated by the Quant Analysis software (Bruker Daltonics esquire 5.0).

2.4.2. Column liquid chromatography

The HPLC 1100 series consisted of a degasser, binary pump equipped with an autosampler and a ultraviolet (UV) detector (Hewlett Packard Series 1100, Agilent Technologies, Böblingen, Germany). The selected wavelength was 220 nm. Method control and data analysis were performed computer based using the Agilent Chem Station software in connection with the mass spectrometric software (esquire control, Bruker Daltonics Esquire 5.0).

Separation was confirmed with 0.4 mL/min flow rate at 25 °C under gradient elution conditions using water +0.1% HCOOH (v/v) (solvent A) and acetonitrile +0.1% HCOOH (v/v) (solvent B) as mobile phase according to the following solvent programming: from initial conditions of 98:2 (v/v) A–B ratio to a 70:30 (v/v) ratio in 10 min. The final eluent composition was 23:77 after 15 min and then lineary decreased to the initial condition (A–B 98:2, v/v) in 5 min. Samples were separated on a Chromolith Performance RP-18e column (100 mm × 4.6 mm, Merck, Darmstadt, Germany) with 15 μL injection volume.

2.4.3. Mass spectrometry

The MS was operated in negative ion mode electrospray, positive ion mode electrospray or with atmospheric pressure chemical ionisation depending on sample properties (Iontrap Esquire 3000, Bruker Daltonics, Bremen, Germany). Used ionisation parameters are listed in Table 1. Method development was carried out on a syringe pump (Cole-Parmer Instrument Company, London, UK).

Table 1
MS parameters for ionisation with ESI and APCI in ± mode

Parameter	ESI (−)-mode	Ionisation ESI (+)-mode	APCI (+)-mode
Accumulation time (ms)	50.0	50.0	50.0
Averages	10	10	10
Scan range	50–500	50–500	50–500
Ion charge control target	20 000	20 000	20 000
Trap drive	25.0	27.2	27.0
Skim 1 (V)	−15.0	23.4	15.0
Skim 2 (V)	−5.4	7.4	6.6
Octopole radio frequency (Vpp)	50.0	110.0	50.0
Lens 1 (V)	2.05	−4.0	−2.8
Lens 2 (V)	30.0	−46.1	−44.9
Octopole (V)	−1.5	3.61	2.51
Capillary exit (V)	−65.0	73.0	65.0
Capillary exit offset (V)	−50.0	50.0	50.0
High-voltage capillary (V)	3050	−4500	2238
Dry temperature (°C)	300	300	300
Dry gas (L/min)	8.0	8.0	10.0
Nebulizer (psi)	25.0	25.0	25.0
Corona (V)	–	–	4000
APCI temperature (°C)	–	–	450

3. Results and discussion

The LC–MS^n analyses showed accumulation of aromatic hydrocarbons and heterocyclic compounds in the fermentation media with increasing casein addition. The chromatogram of the completely inhibited pro-

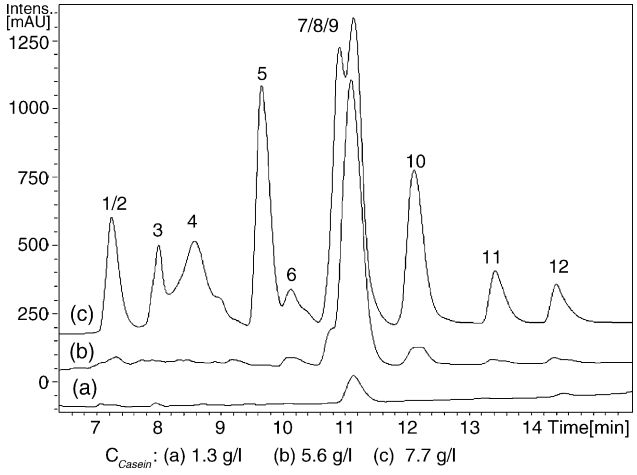

Fig. 1. UV chromatograms at 220 nm of different casein additions. (**1**) Phenylalanine; (**2**) piperidon; (**3**) tryptophan; (**4**) *p*-hydroxyphenylacetate; (**5**) *p*-hydroxyphenylpropionate; (**6**) 5-hydroxyindole; (**7**) benzoate; (**8**) 3-phenylacetate; (**9**) indole-3-acetate; (**10**) 3-phenylpropionate; (**11**) indole; (**12**) skatole.