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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-

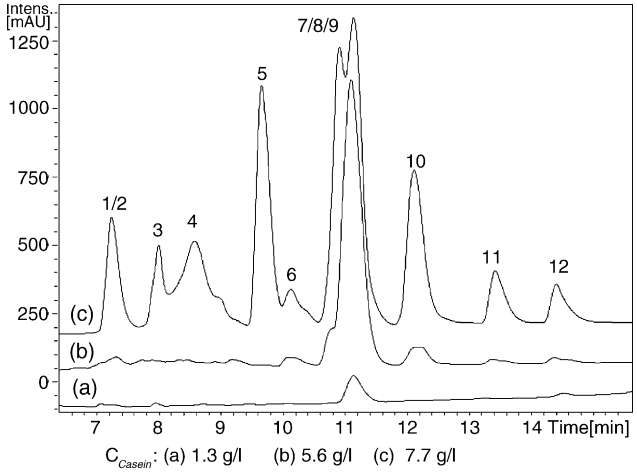


Fig. 1. UV chromatograms at 220 nm of different casein additions. (**1**) Phenylalanine; (**2**) piperidon; (**3**) tryptophan; (**4**) *p*-hydroxyphenylacetate; (**5**) *p*-hydroxyphenylpropionate; (**6**) 5-hydroxyindole; (**7**) benzoate; (**8**) 3-phenylacetate; (**9**) indole-3-acetate; (**10**)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

cess exhibited significant accumulation of at least 12 metabolites (Fig. 1). These intermediates were identified as piperidon (δ-valerolactam), phenylalanine, tryptophan, *p*-hydroxyphenylacetate, *p*-hydroxyphenylpropionate, 5-hydroxyindole, benzoate, 3-phenylacetate, indole-3-acetate, 3-phenylpropionate, indole and skatole (Table 2). Beside piperidon all substances were identified in former experiments as intermediates of the anaerobe metabolic pathway of the amino acids phenylalanine, tryptophan and tyrosine.

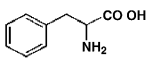
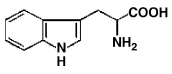
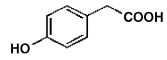
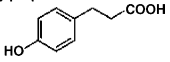
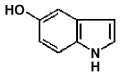
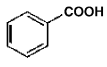
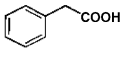
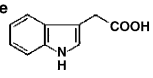
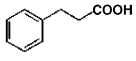
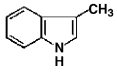
A sufficient HPLC baseline separation of all occurring intermediates was not achieved even after changing HPLC conditions like pH, temperature, gradient, flow and mobile phase. Several substances could only be identified employing the on-line connection with the mass spectrometer. Because of their similar polarity benzoate ($[M + H^+] = 123$ g/mol), phenylacetate ($[M + H^+] = 137$ g/mol) and indole-3-acetate ($[M + H^+] = 176$ g/mol) co-eluted and were only identified by the mass spectrometric analyses (Fig. 2). A separation of phenylalanine and piperidon was also not reached (Fig. 1).

Table 2
Accumulating substances from LC–MS-analyses

Compound	t_R (min)	k'
1 Phenylalanine	7.00	0.823
2 Piperidon	7.20	0.878
3 Tryptophan	7.96	1.079
4 *p*-Hydroxyphenylacetate	9.03	1.356
5 *p*-Hydroxyphenylpropionate	9.70	1.531
6 5-Hydroxyindole	10.10	1.636
7 Benzoate	11.05	1.884
8 3-Phenylacetate	11.14	1.907
9 lndole-3-acetate	11.20	1.923
10 3-Phenylpropionate	12.08	2.152
11 Indole	13.13	2.419
12 Skatole	14.29	2.730

Thus, quantification was achieved by calibrating the mass peaks using their specific parent ions or fragment-ions (MS^2-Mode) to eliminate negative matrix effects (Table 3). Five-level linear calibration curves were generated for all standards. Every calibration level was measured three-fold.

Table 3
Fragment ions used in qualification and quantification of the accumulating compounds

Substance	*M* (g/mol)	Used mass for quantification (*m*/*z*)	Mode ionisation	Intermediate of degradation pathway
Phenylalanine 	165.2	120.1 – $[M + H^+]$ – HCOOH	+ ESI	–
Piperidon 	99.1	100.0 – $[M + H^+]$	+ ESI	Unknown
Tryptophan 	204.2	187.9 – $[M + H^+]$ – OH	+ ESI	Tyrosine, phenylalanine
p-Hydroxyphenylacetate 	152.2	150.9 – $[M - H^+]^-$	– ESI	Tyrosine
p-Hydroxyphenylpropionate 	166.2	164.9 – $[M - H^+]^-$	– ESI	Tyrosine
5-Hydroxyindole 	133.1	106.0 – $[M + H^+]$ – CO	+ ESI	Tryptophan
Benzoate 	122.1	120.9 – $[M - H^+]^-$	– ESI	Tyrosine, phenylalanine
3-Phenylacetate 	136.1	134.9 – $[M - H^+]^-$	– ESI	Phenylalanine
Indole-3-acetate 	175.2	173.8 – $[M - H^+]^-$	– ESI	Tryptophan
3-Phenylpropionate 	150.1	148.9 – $[M - H^+]^-$	– ESI	Phenylalanine
Indole 	117.1	118.0 – $[M + H^+]$	+ APCI	Tryptophan
Skatole 	131.1	132.0 – $[M + H^+]$	+ APCI	Tryptophan

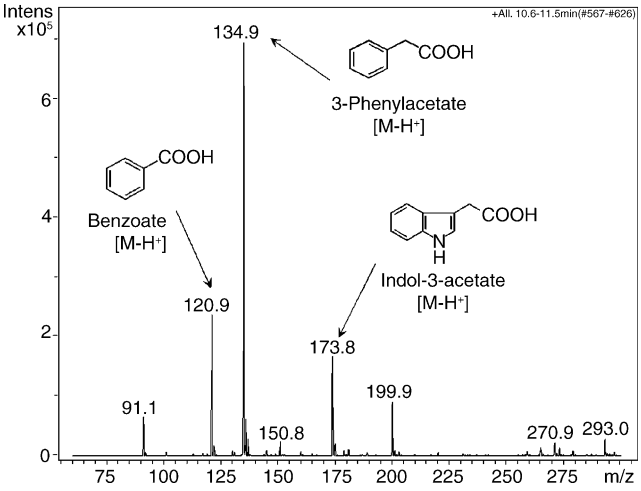


Fig. 2. Mass spectra of benzoate, 3-phenylacetate and indole-3-acetate from 10.6 to 11.5 min (negative ion mode, electrospray ionisation).

A reversed phase-HPLC–ESI-MS–MS method was already successfully applied to analyse eight phenolic compounds, and fragmentation pattern at negative mode was proposed [2]. Typical fragmentation of the phenolic acids was the loss of $m/z = 44$ (CO_2) and $m/z = 18$ (H_2O). Production of m/z 108 by loss of neutral radicals, e.g. COOH, CH_3 or HCO, was also favoured.

In this study it was found that the signal intensity of the aromatic acids at negative mode was significantly increased. The lower background and associated the S/N improvement contributed to better reproducibility. The limit of detection (signal-to-noise ratio = 3) was in the range of 10–200 μg/L according to the nature of the aromatic acids. Fragmentation has found to be similar to Justesen and Arrigoni [2] and to the work of Gonthier et al., who analysed aromatic acid metabolites from the gut [6]. The parent ions $[M - H^+]$ with highest intensity were used for quantification (Table 3).

Correlation of two data sets generated linear regression from 0.08 to 300 mg/L and R^2 values ranging from 0.990 to 0.999.

Ionisation with electrospray of indole and skatole resulted in low intensity of parent and fragment ions with a detection limit of 600 μg/L (S/N = 3). Employing APCI yielded higher intensity. No significant fragment ions could be detected. Thus parent ions $[M + H^+]$ were used for calibration (Table 3) with a detection limit of 100 μg/L. The calculated linearity was for both substances in a range from 3 to 50 mg/L ($R^2 = 0.990$).

Piperidon, phenylalanine, tryptophan, tyrosine and 5-hydroxyindole were detected after electrospray ionisation. Therefore, the positive ion mode was chosen to elucidate their mass spectrometric behaviour because it provided more extensive fragment ions and appeared more sensitive for further LC–MS^n analysis of phenolic compounds in a complex matrix like fermentation media. Beside piperidon, typical fragment ions were used for quantification (Table 3). Similar to the aromatic acids detection limit was 10–300 μg/L with linearity from 0.2 to 300 mg/L ($R^2 = 0.991$–0.996).

Table 4
Unity in % for external standard calibration with correlation of three data sets

Compound	Unity (%)
Phenylalanine	97.8
Piperidon	95.6
Tryptophan	97.3
p-Hydroxyphenylacetate	99.2
p-Hydroxyphenylpropionate	98.9
5-Hydroxyindole	96.3
Benzoate	99.9
3-Phenylacetate	99.8
lndole-3-acetate	99.5
3-Phenylpropionate	98.9
Indole	95.6
Skatole	96.8

Table 5
Relative recoveries in % and RSD in % within parenthesis of aromatic compounds enriched in fermentation media ($n = 4$)

Compound	0.5 mg/L standard	5 mg/L standard	50 mg/L standard
Phenylalanine	94(8)	97(3)	98(10)
Pi peri don	89(6)	95(4)	96(8)
Tryptophan	85(5)	98(2)	94(7)
p-Hydroxyphenyl acetate	98(9)	101(1)	100(2)
p-Hydroxyphenylpropionate	102(2)	99(4)	101(3)
5-Hydroxyindole	100(3)	98(2)	99(2)
Benzoate	100(4)	96(4)	97(3)
3-Phenylacetate	99(6)	99(3)	100(4)
lndole-3-acetate	98(4)	100(5)	104(6)
3-Phenylpropionate	96(3)	101(8)	102(3)
Indole	86(10)	95(7)	91(3)
Skatole	83(7)	96(9)	89(4)

The combination of LC with ESI-MS or APCI-MS provided identification and quantification of different aromatic compounds. Using these procedures with external calibration, good precision and recoveries (95.6–99.9%) have been reached with a fast analyses time of 20 min (Table 4). The overall method reproducibility was determined by supplementing different known standard concentrations in fermentation media. This resulted in unity of 90–98% with relative standard deviations of 5–10% ($n = 4$). Differences especially for the analyses of low concentrations can arise from matrix effects of the fermentation media (Table 5).

The developed LC–MS method is used for identification of metabolites from further protein rich substrates at the present.

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