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J Rousová

K Ondrušová

P Karlová

Alena Kubotava

University of North Dakota, alena.kubatova@und.edu

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Determination of Impurities in Bioproduced Succinic Acid

Rousová J¹, Ondrušová K¹, Karlová P^{1,2} and Kubátová A^{1*}

¹University of North Dakota, Department of Chemistry, 151 Cornell Street Stop 9024, Grand Forks, ND 58202, USA

Abstract

At present, significant research resources are directed towards development of renewable products for replacing petrochemicals such as succinic acid. The critical component of this research is the identification of impurities which have a detrimental impact on further processing of succinic acid. We have adapted derivatization with gas chromatography - mass spectrometry to identify and quantify more than 120 impurities in several succinic acid samples. This study focused on petroleum based succinic acid as well as bio-based samples that use a modified *E. coli* strain for fermentation. To enable an accurate quantification of both the target product and common impurities, we evaluated the acetonitrile extraction efficiency as an alternative to direct derivatization, and then compared several derivatization agents for trimethylsilylation. A prior acetonitrile extraction was shown to be essential to detect impurities in trace concentrations. N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was most efficient for derivatization of saccharides and low molecular weight monocarboxylic acids. However, the presence of pyridine was necessary for derivatization of saccharides and polyalcohols with BSTFA, whereas low molecular weight acids had to be quantified without pyridine.

Fourteen representative bioproduced succinic acid samples differing in production stage, and cultivation method were characterized. The screening of initial process (1st stage of synthesis) samples showed monocarboxylic acids as most abundant and suggested occurrence of saccharides. Thus we have developed method allowing for quantification of carboxylic acids and saccharides with limits of detection between 0.02-0.3 ng. In initial process bacterial samples and also petrochemical sample, formic, acetic, lactic, oxalic, benzoic, citric and malic acids as well as glycerol, butanediol, and glucose were found in a range of 0.02-1160 µg/g. In final processed samples, formic and acetic acid, and glucose were found in concentration lower than 0.001% demonstrating effectiveness of process as well as applicability of the method as quality control of the process.

Keywords: GC-MS; acids; Saccharides; BSTFA; MSTFA; Succinic acid; Bio-based succinic acid

Abbreviations: GC-MS: Gas Chromatography-Mass Spectrometry; BSTFA: N,O-bis(trimethylsilyl)trifluoroacetamide; MSTFA: N-methyl-N-(trimethylsilyl) trifluoroacetamide; HPLC: High Performance Liquid Chromatography; HMDS: hexamethyldisilazane; TMCS: Trimethylchlorosilane; BSA: N,O-Bis(trimethylsilyl)acetamide; ACN: acetonitrile, I.S.: Internal Standard; TIC: Total Ion Chromatogram; LOD: Limit Of Detection; LOQ: Limit Of Quantification; AMDIS: Automated Mass Spectral Deconvolution and Identification System

Introduction

At present, significant research resources are directed towards development of renewable products for replacing petrochemicals [1-3]. Among them, succinic acid, the precursor of a wide range of polyesters, has a market of 270,000 tons per year [2]. Consequently, bio-based succinate is receiving increasing attention, and with rising oil prices it has become a worthy competitor of petrochemical-based succinate [1,2]. The challenge of being cost competitive with petrochemical-based alternatives is being able to obtain high rates of production with little or no by-products, to efficiently use substrates, and to simplify the purification process [1]. The expected by-product of bioproduced succinic acid is acetic acid; however, other impurities, such as organic acids, amino acids, saccharides and polyalcohols might be present in trace amounts [1].

Chromatography is the preferred method of analysis because it adequately addresses the simultaneous identification and quantification of targeted compounds (i.e., carboxylic acids, saccharides, and polyalcohols) [4]. However, not all chromatographic protocols are suitable for the given task. For example, the high performance liquid chromatography (HPLC) of short-chain carboxylic acids (e.g., acetic or formic) is usually performed in the presence of a strong acid,

such as diluted sulfuric acid [4], which is not compatible with mass spectrometry thus preventing the identification of numerous species potentially present in samples. The determination of acetic acid is crucial, because it is considered as the main impurity [1]. The alternative to HPLC is gas chromatography and mass spectrometry (GC-MS). Although the separation using this method generally targets volatile, non-polar species, the use of derivatization for polar low molecular weight species (i.e., the expected impurities) enables detection with a good resolution and sensitivity [4].

Numerous studies addressing acids, saccharides and polyalcohols were performed using GC-MS with trimethylsilylation [5-19] (Supplemental Table S.1 for their overview). Most of these studies characterize food products, focusing on relevant species occurring in fairly high concentration [4-9,16-18]. To our knowledge, no short-chain (i.e., highly volatile) monocarboxylic acids were reported. The shortest-chain acid reported was oxalic acid [11,12,16], which has two carboxylic groups available for derivatization and thus is less volatile than the derivatives of C₁ and C₂ monocarboxylic acids eluting using a non-polar stationary phase after application of a derivatization agent. Similarly, we did not find any study simultaneously addressing

***Corresponding author:** Kubátová A, University of North Dakota, Department of Chemistry, 151 Cornell St. Stop 9024, Grand Forks, ND 58202, USA, Tel: (701) 777-0348; Fax: (701) 777-2331; E-mail: alena.kubatova@UND.edu

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both saccharides and acids. Finally, to our best knowledge, no study has yet addressed the most practical case characteristic for industrial production of pure chemicals when the trace amounts of impurities, such as acids, sugars and polyalcohols, were analyzed in the presence of a high concentration of one major mixture component, e.g., succinic acid.

Several options are available as for selecting the derivatization agents for GC-MS analysis of both acids and saccharides. The most common approach is derivatization with hydroxylamine in pyridine in combination with hexamethyldisilazane (HMDS) with trifluoroacetic acid [5-6,8-10], where hydroxylamine reacts with the saccharide carbonyl group while HMDS functionalizes the moiety containing a reactive hydrogen atom, i.e., carboxyl, hydroxyl and phenyl groups. However, the use of two derivatization agents may lead to uncertainties as the optimal conditions for two different derivatizations may not match. Also, HMDS is not the most efficient derivatization agent, leaving less reactive sources of active hydrogen, e.g., amino groups, unaltered [19]. For a more efficient derivatization of active hydrogen groups, including amino groups, either N-methyl-N-(trimethylsilyl) trifluoroacetamide [13,16] (MSTFA) or N,O-bis(trimethylsilyl) trifluoroacetamide [11,12,14,15] (BSTFA) is typically employed. The derivatization with BSTFA is often catalyzed with trimethylchlorosilane [11] (TMCS) or, in specific cases, trimethylsilylimidazole [7] (TMSI). Because trimethylsilylation is water-sensitive, the most common pretreatment of samples is either evaporation [5,-9,15,16] or lyophilization [13]. However, the short-chain monocarboxylic acids are volatile and thus may be lost together with the solvent, which might lead to underestimation of their content.

Thus, in order to provide a comprehensive characterization of impurities in bioproduced succinic acid samples, we developed a method for simultaneous saccharide and carboxylic acid determination using a GC-MS analysis and ensuring efficient derivatization. The efficiency of prior acetonitrile extraction compared to direct derivatization, and effectiveness of several derivatization agents/conditions for trimethylsilylation was evaluated. Finally, the effectiveness of the manufacturing processing and purification were assessed based on the concentrations of target species found in the samples.

Materials

Studied samples

Fifteen samples of succinic acid were used (labeled A–P; the complete list including detailed sample descriptions is provided in Supplement Table S.2). Samples C–P were produced on a large scale with *E. coli* bacteria using adapted protocol [20]. Briefly, the fermentation took place for 36 hours at 35°C using glucose based media enriched with ammonia as nitrogen source. The purification was accomplished via anion and cation exchange followed by electrodialysis to remove ammonium. Crystallization was used to further improve quality (samples G and L). Samples M–O were produced using a corn steep liquor, which is a by-product of corn wet milling. An analytical standard of succinic acid (99% purity; Sigma-Aldrich, St. Louis, MO, USA) and sample A were used as references, where sample A was petroleum based succinic acid.

Chemicals

Acetonitrile (ACN), methanol (both LCMS Optima grade), and dichloromethane (DCM, GC quality) were purchased from Fisher Scientific (Waltham, MA, USA). Water was purified using a Direct-Q3 water purification system with incorporated dual wavelength UV

lamp (Millipore, Billerica, MA, USA) for low total carbon content (the manufacturers claimed impurity is less than 5 ng/g). Derivatization agents N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA, 99%) with 1% of trimethylchlorosilane (TMCS), BSTFA with 10% of TMCS, N-methyl-N-trimethylsilyl-trifluoroacetamide (MSTFA), were obtained from Sigma-Aldrich. Pyridine (99%) was obtained from Alfa Aesar (Ward Hill, MA, USA). The compounds quantified are listed in Table 2 along with their suppliers.

Sample preparation

Direct BSTFA derivatization: Samples (1.0 mg) were directly mixed with 50 μ L BSTFA and derivatized overnight at 60°C. The amount of BSTFA was calculated to be in a 20-fold molar excess, considering the amounts of succinic acid in the samples. Samples were diluted to 200 μ L using DCM together with 5.0 μ L of an internal standard (*o*-terphenyl) to control the volume changes, and analyzed in vials with 400 μ L inserts.

Extraction: Bioproduced succinic acid samples (1.00 ± 0.05 g) were sonicated overnight with 1 mL of acetonitrile. After sonication, the samples were filtered through some purified glass wool inserted into a Pasteur pipette.

BSTFA derivatization: Filtered ACN extracts (100 μ L aliquot) were mixed with 50 μ L BSTFA (99% + 1% TMCS), then derivatized for 1 h at 60°C. Alternatively, samples were derivatized for 18 h at 70°C in order to achieve a complete derivatization of saccharides and polyalcohols.

BSTFA derivatization with ACN: Acid and saccharides standards (100 μ L) were dried and subsequently mixed with 50 μ L BSTFA and 100 μ L ACN and derivatized for 18 h at 70°C.

BSTFA derivatization with pyridine: Filtered ACN extracts (100 μ L aliquot) were mixed with 60 μ L BSTFA (99% + 1% TMCS) and 60 μ L of pyridine and derivatized for 18 h at 70°C.

MSTFA derivatization: Acid and saccharides standards (100 μ L) were mixed with 50 μ L MSTFA and derivatized for 18 h at 70°C.

Calibration: Stock solutions of individual compounds were prepared and combined into two mixtures, i.e., acids (the final concentration ~ 0.5 mg/mL per analyte) and saccharides (the final concentration ~ 0.2 mg/mL per analyte). The calibration range was between 0.001–50 μ g/mL, where the highest calibration point corresponded to ~ 30 μ moles of carboxylic or hydroxy groups. The list of compounds with their retention times, target and confirmation ions used for data processing is provided in Table 1.

Prior to the analysis an internal standard, *o*-terphenyl (10 μ L, ~ 1 mg/mL), was added to all samples, and the solution was diluted to 1.0 mL using DCM unless stated otherwise.

Instrumentation

GC analyses were performed using a 5890 GC with 5972 MS equipped with an autosampler (6890 series, Agilent Technologies, Santa Clara, CA, USA). Injections were performed in the splitless mode for 0.50 min at 250°C and the injection volume was 1 μ L. The separation was performed using a 52-m long DB-5MS capillary column, with 0.25 mm internal diameter (I.D.) and 0.25 μ L film thickness (J&W Scientific, Folsom, CA, USA). A constant carrier gas (helium) at a flow rate of 1.0 mL/min was maintained during the analysis. The temperature program used was adapted from our previous work [21,22], and started at 35°C held for 5 min, followed by a gradient of 15°C/min to 300°C and

	Supplier	t_r^a [min]	r_{12}^b	MW ion	Target ion	Confirmation ions	LOD [ng]
formic acid	Fluka ^c	2.8	0.1	118	103	73, 45	0.2
acetic acid	Fisher ^d	3.9	0.2	132	117	75, 45	0.3
lactic acid	Sigma-Aldrich ^e	12.3	0.5	230	191	147, 117	0.2
oxalic acid	Sigma-Aldrich	13.6	0.6	230	190	219, 147	0.2
3-hydroxybutyric acid	Sigma-Aldrich	13.8	0.6	244	191	233, 117	0.04
butanediol	Sigma-Aldrich	13.9	0.6	234	177	147, 116	0.02
benzoic acid	Sigma-Aldrich	15.3	0.7	192	179	135, 105	0.4
glycerol	Fisher	15.7	0.7	308	205	218, 117	0.2
proline	Sigma-Aldrich	15.9	0.7	259	142	216, 73	0.1
malic acid	Sigma-Aldrich	18.5	0.8	344	233	245, 147	0.04
phthalic acid	Sigma-Aldrich	20.8	0.9	310	295	147, 73	0.1
xylitol	Supelco ^f	21.0	0.9	502	307	319, 217	0.04
arabitol	Supelco	21.1	0.9	502	307	319, 217	0.02
ribitol	Supelco	21.2	0.9	502	319	307, 217	0.06
citric acid	Sigma-Aldrich	22.2	1.0	480	273	465, 73	0.03
glucose	Supelco	23.1	1.0	530	204	191, 147	0.02
sucrose	Supelco	29.8	1.3	902	361	217, 73	19

^a Retention time

^b Relative retention time (retention time/IS retention time)

^c Fluka – St. Louis, MO, USA

^d Fisher – Waltham, MA, USA

^e Sigma-Aldrich – St. Louis, MO, USA

^f Supelco – St. Louis, MO, USA

Table 1: List of acids, saccharides and polyalcohols studied, their suppliers, the GC–MS retention times, target and confirmation ions (used for quantification) of their trimethylsilyl derivatives used for data processing, and limits of detection (LODs).

held for 1 min. The MS data in total ion chromatograms (TIC) were acquired in the mass range of m/z of 35–1000 at a scan rate 2.66 scan/s using the EI of 70 eV. The MS was turned off to eliminate signal from the derivatization agents and their by-products in periods determined by observing the increase of pressure in MS. Namely, for BSTFA with pyridine, the MS was off for the first 2.5 min, 2.90–3.60 min, 4.40–7.00 min, 8.00–8.70 min; for MSTFA, the MS was off for the first 4 min.

Data processing

GC-MS data were processed using ChemStation (version E.02.02.1431) and AMDIS software (Automated Mass Spectral Deconvolution and Identification System, version 2.71) [23]. Compounds' identification was based on confirmation with the corresponding analytical standard, or as isomers of standards with similar mass spectra and/or using NIST 05 Mass Spectra library.

AMDIS software was used for the deconvolution of MS ion spectra and tentative identification of impurities for which the analytical standards are not available. The tentative identification was based primarily on the reversed match of >80% and compared to the weighted match requiring at least 80% for both matching methods. Peaks found in the pure succinic acid standard and in the BSTFA blank were not considered. Based on TIC, the AMDIS program provided a percent response, which allowed for semi-quantification of impurities (Table 3) and their comparison between samples, by normalizing to the response of the internal standard.

The limits of detection and quantification (LODs and LOQs) were determined using the target ions m/z , which were selected based on the highest signal-to-noise ratio (ions listed in Table 2). The instrumental LODs were calculated from calibration curves (within one order of magnitude of LOD) using the formula $LOD=3.3 \cdot s_y/k$, where k is a slope of the calibration curve and s_y is the standard error of the predicted y -value for each x -value; s_y was obtained by a least square linear regression. In order to report the low amounts of impurities we have

used for quantification, lower limits of quantification were defined as $LOQ=5 \cdot s_y/k$.

The repeatability of the quantification method was evaluated using a representative sample of bioproduced succinic acid (C), which was chosen on the basis of preliminary testing. The sample was prepared in triplicate and analyzed in the following ways: 1) the same sample was analyzed three times in a row to assess the intraday GC repeatability; 2) the same sample was analyzed throughout the sequence on two consecutive days, to evaluate the interday GC repeatability; and 3) the extraction triplicate was analyzed to assess the extraction repeatability.

Results and Discussion

Extraction v/s direct analysis

The selection of a sample preparation method strongly affects the impurities detected. Thus we first compared the extraction using ACN followed by derivatization with BSTFA with direct BSTFA derivatization (no extraction). Figure 1 shows that the ACN extraction was essential for characterization of impurities. A range of peaks representing impurities was observed in the majority of ACN extracted and BSTFA derivatized samples (Figure 1b and Table 3). We expected enhanced derivatization when eliminating the extraction step and using BSTFA in molar excess; however no additional impurities were found when the direct analysis was applied (Figure 1a). The higher responses observed after extraction could be explained by a higher solubility of impurities in acetonitrile than in the derivatization agent alone, combined with a lower solubility of succinic acid in ACN.

Initial identification of impurities

The initial method of analysis was adapted from our previous work [21] allowing for quantification of a wide range of mono- and di-carboxylic acids. Over 120 peaks were observed in the initial process bacterial succinic acid samples upon derivatization with BSTFA. Table 3 shows the normalized data for the most abundant species (the detailed

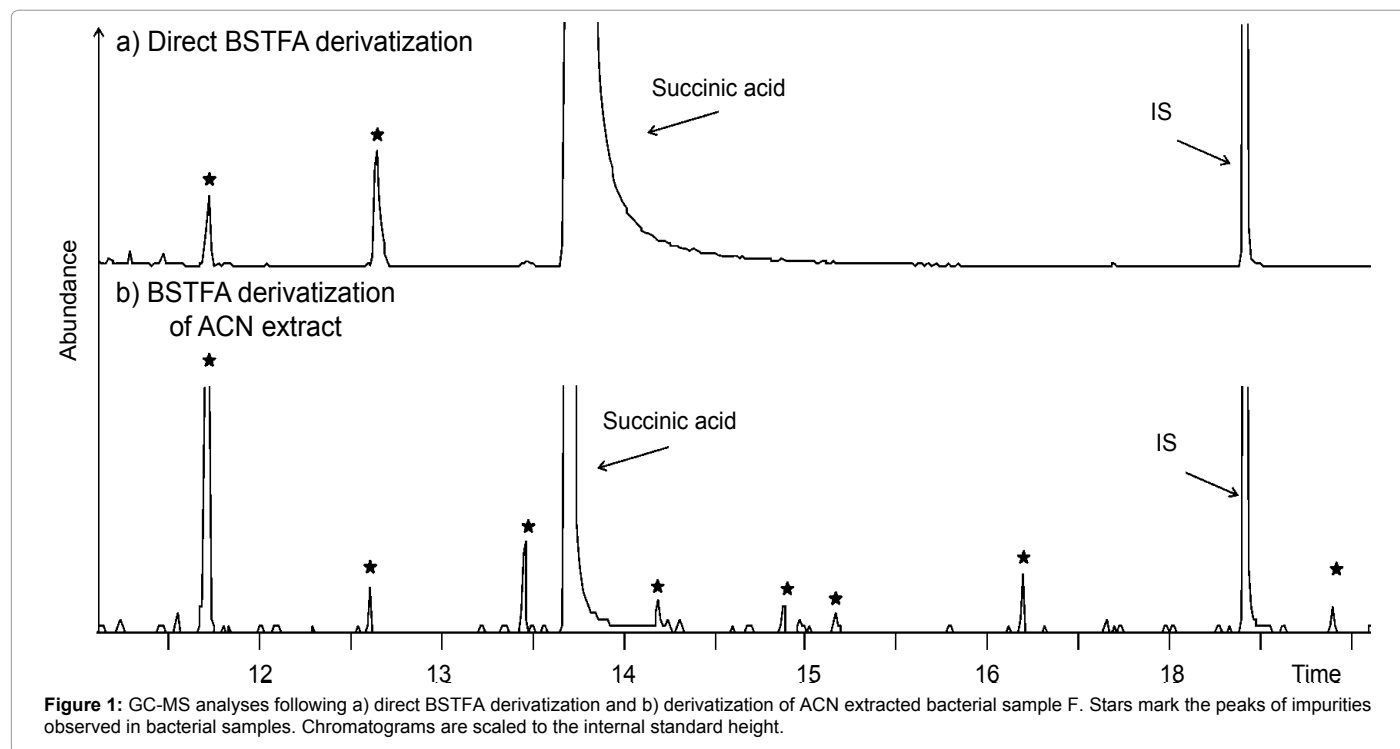


Figure 1: GC-MS analyses following a) direct BSTFA derivatization and b) derivatization of ACN extracted bacterial sample F. Stars mark the peaks of impurities observed in bacterial samples. Chromatograms are scaled to the internal standard height.

r_{12}^a	Identified compounds	A (petroleum)	F (bacteria)	K (bacteria)	Confirmed ^b
0.319	formic acid	0.01	0.03	0.03	*
0.406	acetic acid	0.02	0.12	0.17	*
0.570	methyl-propanoic acid		0.03		
0.604	alanine			0.03	
0.608	dimethylsulfone			0.01	*
0.631	ethanediol	0.04			*
0.663	butanediol		0.02	0.03	*
0.672	lactic acid		0.74	0.30	*
0.694	alanine			0.01	
0.715	methyl butanol	0.01			
0.720	3-hydroxybutyric acid			0.02	*
0.722	oxypentanoic acid			0.02	
0.724	hydroxymethylbutyric acid		0.05		
0.736	pentenoic acid			0.03	
0.747	L-valine (bisTMS)			0.08	*
0.759	ethyl succinate			0.04	*
0.770	glycerol			0.04	*
0.773	phosphoric acid		0.10		
0.792	methyl succinic acid	0.03			
0.798	pyrimidine			0.02	
0.815	malic acid		0.03	0.08	
0.821	pentanedioic acid			0.02	*
0.854	malic acid	5.40	0.03	0.02	
0.860	hexanedioic acid		0.01		*
0.930	phthalic acid	0.03	0.05		
0.967	citric acid			0.07	*
0.992	heptanol derivative			0.04	
1.000	o-terphenyl (IS)	1.00	1.00	1.00	IS
1.012	glucose			0.02	

^a Relative retention time (retention time/IS retention time)

^b Confirmed using the analysis of standard.

Table 2: Contaminants and their percent responses, with respect to an internal standard, observed upon BSTFA derivatization of an ACN extract of petroleum produced succinic acid and initial process bio-based succinic acid samples

list is in Supplemental Table S.3). The common impurities of higher abundance in the bacterial samples were formic, acetic, lactic and malic acids, butanediol and L-valine (Figure 2). Using this screening method, we also observed incompletely derivatized saccharides. Other compounds found in a lower abundance were oxalic, benzoic, phthalic, hexadecanoic, and octadecanoic acids (Table 3). These acids might be from the sample preparation contamination; however their abundance in controls (experiment performed without analytes) seemed to be lower.

The screening results showed primarily acids, saccharides and polyalcohols, which are essential for production control on large scale [1,3], and thus, the further quantification efforts targeted these species.

Development of quantification method for analysis of acids and saccharides as the most abundant impurities

Based on our previous work [23] and reported data, several trimethylsilylation methods were compared to determine the most efficient approach for a simultaneous derivatization of saccharides and acids. These methods included the derivatization with MSTFA in the presence of ACN, and BSTFA (1% TMCS) with/without ACN or pyridine. The application of these derivatization agents to saccharides resulted in only an incomplete derivatization in MSTFA with or without ACN and in BSTFA without either pyridine or ACN (Figures 3a and b). Xue et al. [24] reported multiple peaks for glucose derivatized with MSTFA, however, the problem was not addressed. By contrast, BSTFA in the presence of either ACN or pyridine resulted in a complete derivatization of saccharides and polyalcohols (Figures 3c and d). Nevertheless further tests of derivatization evaluation of BSTFA with ACN and pyridine resulted in higher peaks of glucose in presence of pyridine (Figure 4). The comparison of extracted ion chromatograms of acetic acid (ion 117, [M-15]⁺) demonstrates that the MSTFA (Figure 5a) and BSTFA derivatization with ACN (Figure 5c) resulted in higher peaks compared to the derivatization using BSTFA with pyridine.

Perhaps pyridine had a negative effect on the transfer of volatile analytes from the GC injection port to the column due to its relatively high boiling point and tendency to bind acids due to the formation of pyridinium salts. Therefore, the derivatization using BSTFA with ACN seemed to be optimal for acids, while BSTFA with pyridine was more effective for saccharides (Figures 3-5). We also tested the separation of succinic acid and its isomer, methylmalonic acid. Those compounds were completely separated as shown in Supplemental Figure S.1.

Limits of detection and repeatability

Table 2 lists the obtained instrumental LODs, which were in a range of 0.03-0.6 ng for acids and 0.03-0.2 ng for saccharides and polyalcohols. The values obtained for acids are comparable to those reported in our previous study [23], while we achieved ten-fold lower values for sugars than in the study of Adams et al. [10], where HMDS was used as derivatization agent, possibly due to a more effective derivatization or greater calibration range. LOD's in other studies [11,13,15] were not comparable because they have been reported in different units, e.g. Pietrogrande and Bacco [11] reported as air volume concentrations.

The repeatability of the developed quantification method on representative sample C is demonstrated in (Table 4). The GC intra- and interday repeatability as well as sample preparation were similar, with relative standard deviation (RSD) <10%, with exception of glycerol, where intraday reproducibility was 12%

Characterization of succinic acid samples

The developed quantification method was applied to bioproduced succinic acid samples, as an application for monitoring the product quality. The targeted compounds were the most abundant acids, as well as saccharides, and polyalcohols, i.e., formic, acetic, lactic, oxalic, 3-hydroxybutyric, benzoic, malic, phthalic and citric acids, butanediol, glycerol, xylitol, arabinol, glucose, and sucrose (Table 2).

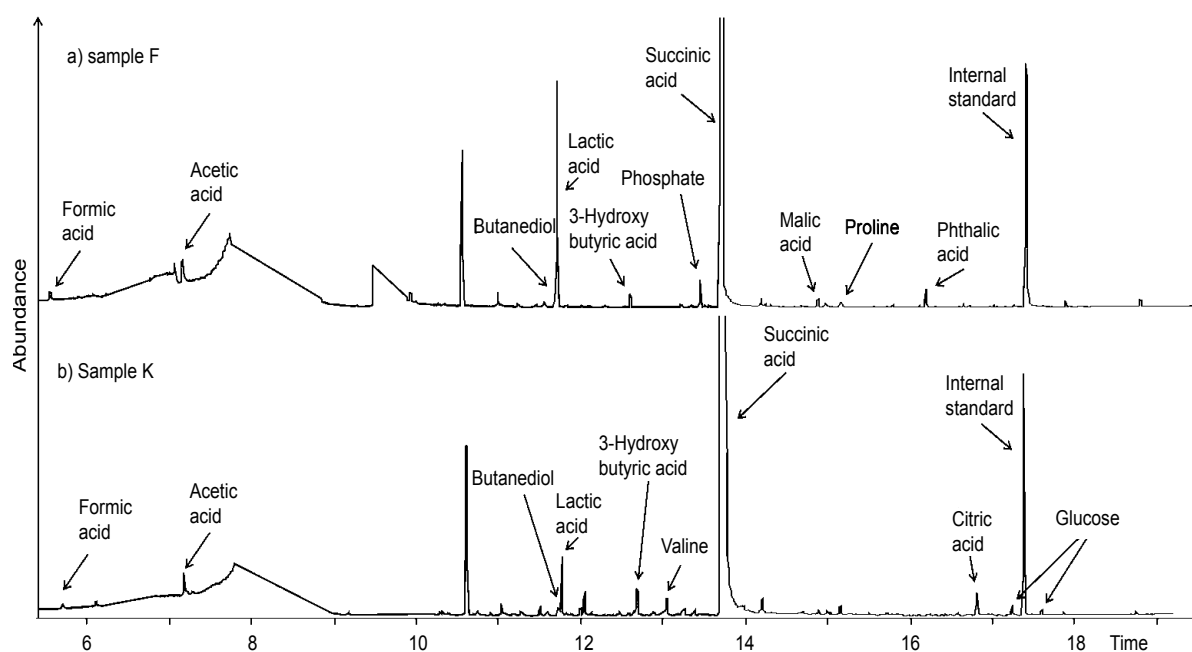


Figure 2: Comparison of GC-MS chromatograms of analysis BSTFA derivatization of ACN extracted bioproduced succinic acid samples, normalized to the same percent response of internal standard. Samples F(a) and K(b) were initial process samples.

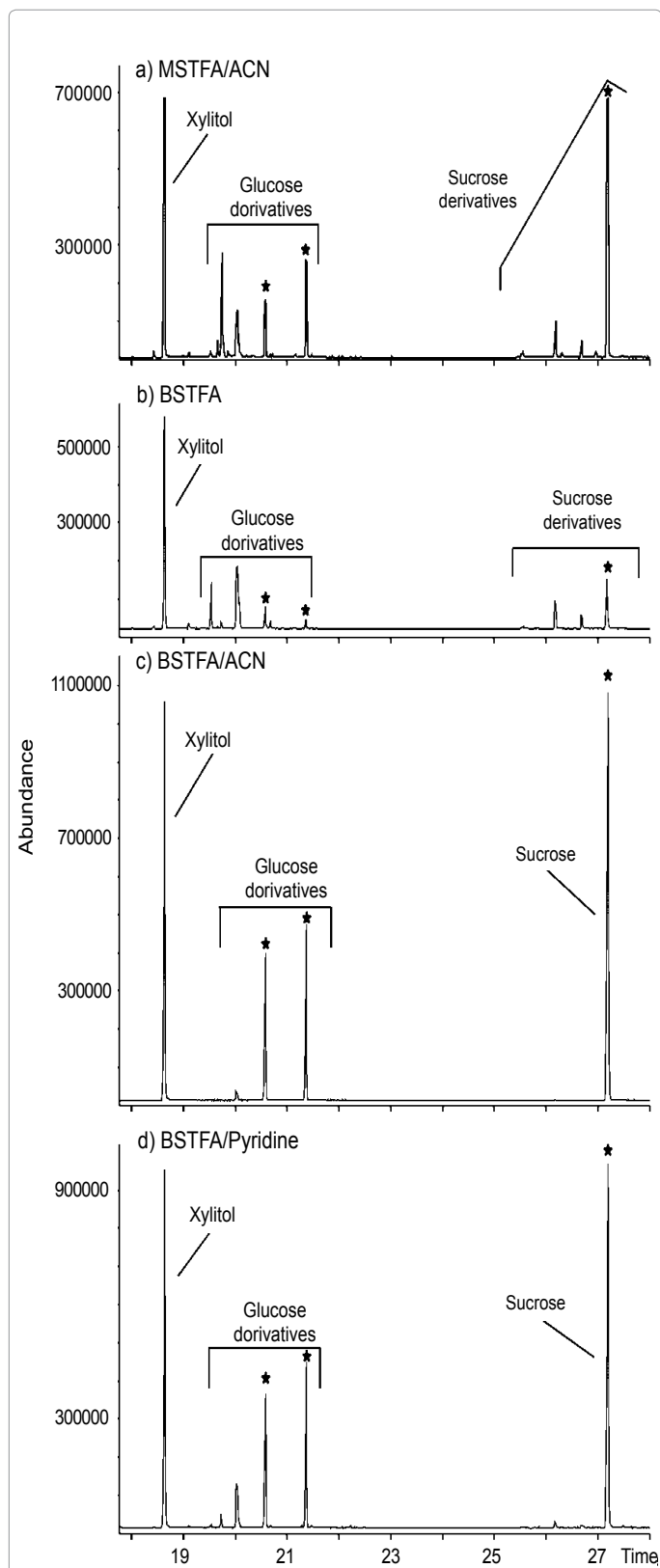


Figure 3: GC-MS extracted ion chromatograms ($m/z = 217$) of a mixture of standard saccharides and polyalcohols upon derivatization (18 h at 70°C) with a) MSFTA with ACN, b) BSTFA (1% TMCS), c) BSTFA 1% TMCS with ACN, d) BSTFA (1% TMCS) with pyridine. The stars mark peaks of the completely derivatized sucrose and glucose.

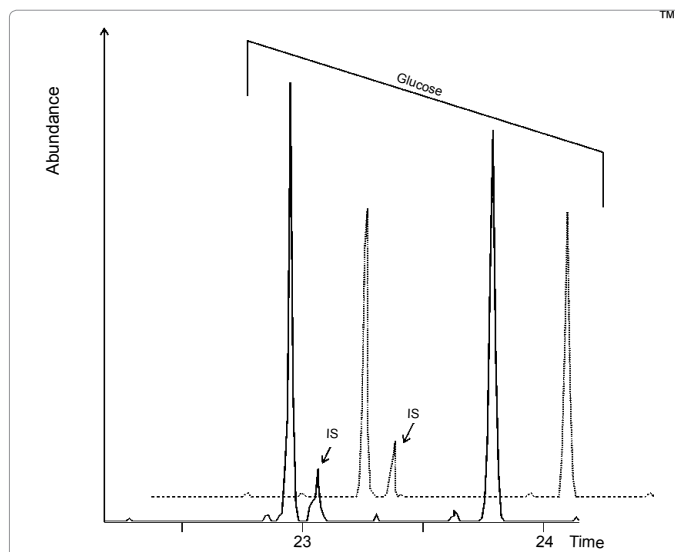


Figure 4: GC-MS extracted ion 204 chromatograms of bio-produced succinic acid (sample F) upon derivatization with various derivatization agents for 18 hours at 70°C. The derivatization with pyridine (solid line) provided a higher response than that with ACN (dashed line). IS denotes internal standard. The IS co-elutes with other derivatized hexose, which is believed to be an impurity in the glucose standard.

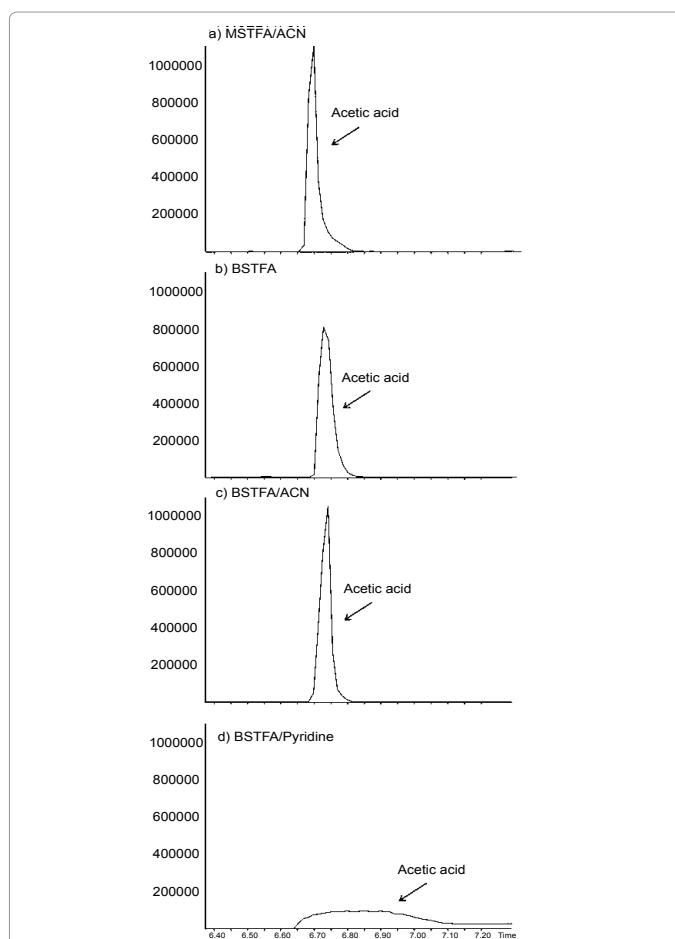


Figure 5: GC-MS extracted ion chromatograms ($m/z = 117$) of an acetic acid standard upon derivatization (18 h at 70°C) with a) MSFTA with ACN, b) BSTFA (1% TMCS), c) BSTFA 1% TMCS with ACN, d) BSTFA 1% TMCS with pyridine.

Due to the low concentrations of some of these compounds in the samples, quantification is reported only for a narrower range of these compounds featuring the concentrations above the corresponding LODs (Table 4).

Abundance of acids, saccharides, and polyalcohols: Quantification confirmed occurrence of all tested acids and glucose (Tables 3 and 4). The polyalcohols in samples were found as well but xylitol, arabitol and ribitol were below their LOD.

As mentioned above, acids were the prevailing impurities in the bacterial samples. Acetic acid is a common contaminant of biologically produced succinic acid [1], and for its unpleasant smell was an undesirable impurity. It has been abundant in samples F and K (13 µg/g and 20 µg/g, respectively), but its concentration decreased in purified sample G (3 µg/g). Formic acid, which has also undesirable odor, had been determined in all samples between 1 µg/g in samples K, L, and M (Table 4) and 16 µg/g in samples A (petroleum-based sample). Similarly to acetic acid, formic acid concentration decreased after purification from 5 µg/g (sample F) to 1 µg/g (sample G). Malic acid, also used in industry for polymer production [3], was the major impurity in sample A (1.2 mg/g) and lactic acid was found in samples F and K (0.2 mg/g and 27 µg/g, respectively).

Polyalcohols found in the samples were glycerol, butanediol (Table 4). Glycerol was found in samples F and L (0.5 and 0.3 µg/g, respectively). Butanediol was also found in sample F (5 µg/g) and sample K (4 µg/g). Ethanediol was observed in petroleum based sample but it was not quantified in other samples. Sugar polyalcohols were not detected, with exception of arabitol, which was detected in sample N, but it was below its limit of quantification. Glucose was only representative of saccharides with concentration up to 8 µg/g in sample K (Table 4).

The effect of production media on the purity of succinic acid

was evaluated for samples K–O comparing the product produced by bacteria in a defined medium (sample K) and in corn steep liquor (samples M, N, O). Corn steep liquor is less expensive as it is a by-product of corn wet milling and so it is preferred in industry; however, the product obtained using this complex organic mixture was expected to contain more impurities. In contrast to this expectation, samples M, N, and O and other initial process samples contained similar impurities (formic acid, acetic acid and glucose), suggesting that the production medium had a lower impact on generation of the observed impurities than the production microorganism. Only oxalic acid was observed in a 4-fold higher abundance in sample M with corn steep, compared to sample K produced using a defined medium.

Final bacterial process samples: The effectiveness of the product purification was evaluated by comparison of samples F and K (initial process), and G and L (final product) where G was purified F. While most of the targeted compounds were detected in initial process samples, only formic and acetic acids were quantified in purified sample G, showing a decrease from 0.13 µg/g to 0.06 µg/g for formic acid and from 0.3 µg/g to 0.1 µg/g for acetic acid. Sample L showed also some glycerol present. Lactic and malic acids were both detected in initial process samples, but were not found in refined samples (Table 4). Thus the developed method was demonstrated to be suitable for the quality control of the process as well as demonstrated purity of the final products.

Conclusions

We have developed a protocol for characterization and quality control of bioproduced succinic acid. A prior ACN extraction was found to be essential to detect impurities. The optimization of derivatization was critical for low molecular weight polar acids as well as saccharides; a procedure using BSTFA with pyridine as a catalyst was determined to be suitable for both polyalcohols and saccharides whereas the BSTFA with ACN treatment was found to be the suitable for quantification

Analyte	GC intraday			GC interday			Extraction		
	Mean	±	SD	Mean	±	SD	Mean	±	SD
lactic acid	6.3	±	0.5	6.2	±	0.4	6.0	±	0.1
benzoic acid	0.63	±	0.02	0.67	±	0.06	0.61	±	0.03
glycerol	0.12	±	0.01	0.11	±	0.01	0.12	±	0.01
glucose	0.08	±	0.01	0.08	±	0.01	0.071	±	0.004

Table 3: GC intra, interday, and extraction method repeatability for a bioprocessed sample of succinic acid (sample C) reported as a mean value (in µg/g) ± one standard deviation (n=3).

Analyte	A (petroleum)			F (initial process)			K (initial process)			G (final process)			L (final process)		
	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD	Mean	±	SD
formic acid	15	±	5	5	±	2	1.1	±	0.03	1	±	0.06	1.5	±	0.8
acetic acid	Below LOQ			13	±	3	20	±	6	3.2	±	0.6	3.9	±	0.8
oxalic acid	8	±	5	Below LOQ ^a			Below LOQ			ND ^b			ND		
lactic acid	ND			186	±	19	27	±	4	ND			ND		
3-hydroxybutyric acid	Below LOQ			Below LOQ			1.1	±	0.1	ND			ND		
butanediol	Below LOQ			5.1	±	0.3	3.6	±	0.1	ND			ND		
benzoic acid	2.00	±	0.03	Below LOQ			ND			ND			ND		
glycerol	ND			0.49	±	0.06	ND			ND			0.23	±	0.03
malic acid	1159	±	24	10	±	2	Below LOQ			Below LOQ			Below LOQ		
phthalic acid	7	±	2	ND			ND			ND			ND		
citric acid	ND			Below LOQ			8	±	1	ND			ND		
glucose	Below LOQ			3.1	±	0.2	0.07	±	0.01	0.02	±	0.001	Below LOQ		

^a Below LOQ – below quantification limit

^b ND – not detected

Table 4: Concentrations of acids and saccharides in bioprocessed succinic acid samples reported as a mean value (in µg/g) ± one standard deviation (n=3).

of low molecular weight carboxylic acids. The presence of short chain monocarboxylic acids, i.e. formic and acetic acid, has an effect on odor of final product, which is undesirable in the industrial process. Presence of saccharides might lead to caramelization or Maillard reactions, resulting in coloring the final product. We achieved LODs as low as 0.02 ng for saccharides and 0.03 ng for acids, which makes the quantification method advantageous for detection of trace-level impurities even in the presence of one major compound at a high concentration, e.g., succinic acid. The final process samples showed removal or decrease of all quantified compounds.

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