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Limits of detection for the determination of mono- and dicarboxylic acids using gas and liquid chromatographic methods coupled with mass spectrometry☆

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ABSTRACT

The chromatographic separation and instrumental limits of detection (LODs) were obtained for a broad range of C_1–C_{18} monocarboxylic (MCAs) and C_2–C_{14} dicarboxylic acids (DCAs) employing either chemical derivatization followed by gas chromatography–mass spectrometry and flame ionization detection (GC–MS/FID) or direct analysis with liquid chromatography high resolution MS and tandem MS (LC–MS). Suitability, efficiency and stability of reaction products for several derivatization agents used for esterification (BF_3/butanol), and trimethysilylation, including trimethylsilyl-*N*-*N*-dimethylcarbamate (TMSDMC) and *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were evaluated. The lowest limits of detection for the majority of compounds below 10 pg (with the exception of acetic acid) were obtained for derivatization with BF_3/butanol followed by GC–MS in the total ion current (TIC) mode. Further improvements were achieved when applying either selected ion monitoring (SIM), which decreased the LODs to 1–4 pg or a combination of SIM and TIC (SITI) (2–5 pg). GC-FID provided LODs comparable to those obtained by GC–MS TIC. Both trimethylsilylation (followed by GC–MS) and direct LC–MS/MS analysis yielded LODs of 5–40 pg for most of the acids. For volatile acids the LODs were higher, e.g., 25 and 590 ng for TMSDMC and BSTFA derivatized formic acid, respectively, whereas the LC–MS methods did not allow for the analysis of formic acid at all.

1. Introduction

A number of chromatographic methods have been employed for the determination of low and high molecular weight mono- and dicarboxylic acids (MCAs and DCAs) in various matrices including food products, biological materials, and atmospheric samples [1–9]. Although a wide range of acids C_1–C_{18} can be found in those matrices, the methods applied usually targeted only a limited range of low and/or high molecular weight carboxylic acids (CAs). Gas and liquid chromatography (GC and LC) are the two methods of choice for analysis of a broad spectrum of CAs. Ion chromatography (IC) offers high sensitivity for smaller molecular weight acids (up to C_5) but is not applicable for determination of hydrophobic (i.e., long carbon chain) CAs [5,6,9–11]. Recent developments in reverse phase LC columns, which are specifically modified for analysis of CAs, may help to overcome difficulties in separating a wide range of water-soluble and insoluble acids. Yet, the full capabilities of these relatively new stationary phases for CA separations were not fully explored. The earlier applications focused either on the low molecular weight ($<C_6$) [1,2,12,13] or some high molecular weight CAs ($>C_6$) [3,4,14–16].

The majority of applications using a direct liquid injection to GC focused only on heavier acids ($>C_5$) [17–22]. To our knowledge, only one study, by Sun et al., reported a GC analysis of MCAs from C_1 to C_{18} [23].

The polar nature, low thermal stability, and low volatility of CAs require their derivatization prior to their analysis on a non-polar GC column. The esterification including alkylation and trimethylsilylation is the most commonly utilized CA derivatization method [24]. Methylation of the carboxylic group is typically performed by using diazomethane or methanol/BF_3 derivatization agents [24]. Reactions with diazomethane produce almost no side-products; however, due to the diazomethane's toxicity and explosive nature, many labs refrain from its use [24]. The drawback of methylation, in general, is the increased volatility of acid methyl esters, which may result in analyte losses during the subsequent sample preparation and/or lead to a co-elution of derivatized volatile MCAs with a solvent [24]. Significant improvements in the analysis of volatile MCAs were achieved by performing the CA esterification with propyl and, particularly, butyl groups. For example, the increased alkyl ester chain length from C_3 to C_4 (i.e., going from propylation to butylation) enabled the analysis of more volatiles

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acids starting with C_4 and C_2 MCAs, respectively [24,25]. Similarly to butylation, trimethylsilylation significantly increased the resulting derivative's molecular weight thus enabling the analysis of both low and high molecular weight CAs [18,26–29].

The limits of detection (LODs) achieved using the two most common derivatization techniques (BF_3/butanol and trimethylsilylation with BSTFA) were compared for the analysis of C_3–C_9 DCAs in atmospheric aerosols by Pietrogrande et al. [29]. In this study, BSTFA was suggested for practical use as being more suitable due to lower detection limits of its DCA derivatives, below 2 ng [29]. Another BSTFA derivatization study demonstrated that volatile MCAs (particularly C_1 and C_2) exhibit LODs that are one order of magnitude higher than those of the other MCAs [26]. This issue may be related to high interferences from solvents, derivatization agents, and matrix, which are common in the analysis of low molecular weight MCAs and DCAs. The LODs for butylated C_6–C_{34} MCAs were in a range of 0.01–2 ng [22].

LC with UV detection also tends to require derivatization to ensure high sensitivity [12,24,30]. Limits of detection for direct (i.e., without derivatization) LC analyses of MCAs and DCAs can be significantly improved by the use of mass spectrometric detectors (MS) [14,15,28]. The LODs reported for ultra high performance LC with high resolution MS were as low as 0.001–0.3 ng for C_{12}–C_{28} MCAs. The most suitable mobile phase modifiers for LC separation of CAs, however, include non-volatile inorganic salts, which are not compatible with the atmospheric pressure ionization MS. Alternative modifiers suited for the electropray ionization (ESI) MS are organic acids (e.g., formic, acetic acid) [14,15,28] and their salts (e.g., ammonium formate, ammonium acetate). These electrolytes may, however, impair the detectability of low molecular weight CAs (e.g., formic, acetic acid) due to the high background of quantitation ions.

The goal of our study was to provide a comprehensive evaluation of common derivatization techniques including the esterification with BF_3/butanol, as well as trimethylsilylation using frequently used derivatization agents, such as *N*,*O*-bis(trimethysilyl)trifluoroacetamide (BSTFA), *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and also a novel trimethylsilylation agent, trimethylsilyl-*N*-*N*-dimethylcarbamate (TMSDMC). Chemical derivatization coupled with a GC analysis was also compared to two alternate methods, LC with high resolution time-of-flight MS, and tandem MS. Chromatographic limitations and instrumental LODs reported in this paper for a broad range of both hydrophilic and hydrophobic MCAs (C_1–C_{18}) and DCAs (C_2–C_{14}) may be used for the analysis of CAs in food products, biofuels, biological matrices and atmospheric aerosols.

2. Experimental

2.1. Chemicals

Acetonitrile (ACN), methanol (both LCMS Optima grade) and dichloromethane (DCM) of GC quality were purchased from Fisher Scientific (Waltham, MA, USA); *n*-hexane (GC quality) was from Sigma–Aldrich (St. Louis, MO, USA). Water was purified using a Direct-Q3 water purification system with an incorporated dual wavelength UV lamp (Millipore, Billerica, MA, USA) for low total organic carbon content (the manufacturer's claimed purity is less than 5 ng/g).

Derivatization agents *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane, *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and BF_3/butanol solution (10%, w/w) were purchased from Sigma–Aldrich. The pertinent information on MCAs and DCAs considered in this study is provided in Table 1, including the supplier names, retention times and ions (*m*/*z*) used in the GC–MS analysis. To control for volume changes, *o*-terphenyl (Sigma and Aldrich) was employed as an internal standard (~5 mg/mL) for GC analysis. Two internal standards, ^{13}C-octanoic acid (MCA ^{13}C-C_8) and propanedioic acid-d_2 (DCA C_3-d_2), were used for LC analysis in concentrations of 9.0 μg/L.

2.2. Sample preparation

For GC, four mixtures of stock solutions were prepared including DCAs (C_2–C_7 ~7 mg/mL and C_8–C_{14} ~1.5 mg/mL, both in ACN) and MCAs (C_1–C_{10} ~100 mg/mL and C_{11}–C_{18} ~25 mg/mL in DCM), respectively. The solutions were stored at −18 °C. The highest final concentration of acids tested in the derivatized solution was ~100 μg/mL per analyte, corresponding to ~140 μmoles of carboxylic groups. For LC, the stock solutions of individual acids were prepared in water or methanol in concentrations of ~5 mg/mL and stored at −18 °C. Desired concentrations for method optimization were obtained by appropriate dilutions into water or acetonitrile.

MSTFA derivatization. Samples (100 μL) were mixed with an equal volume of MSTFA (534 μmoles), derivatized at 60 °C for 1 h and the solution was diluted to 1.0 mL using DCM; 5.0 μL of an internal standard was added to control the volume changes.

BSTFA derivatization. Similarly to MSTFA derivatization, acid samples (100 μL) were mixed with an equal volume of BSTFA (370 μmoles), derivatized at 60 °C for 1 h, and then diluted to 1.0 mL using DCM. An internal standard (5.0 μL) was added prior to the GC analysis.

TMSDMC derivatization. Acid samples (100 μL) were mixed with 50 μL of the derivatization agent (258 μmoles). After the derivatization, the samples were diluted to 1.0 mL using DCM and 5.0 μL of an internal standard was added. The effectiveness of derivatization was evaluated at different temperatures (8, 20, and 60 °C) and times (15, 30, 60 min). The final conditions of 8 °C for 15 min were used for the determination of LODs.

BF_3/butanol derivatization. Butyl esters of organic acids were formed upon the CA reaction with BF_3/butanol using the adopted protocol [31–33]. An appropriate amount (100 μL) of the acid mixture was spiked into 50 μL of BF_3/butanol solution. The reaction took place at 60 °C for 60 min. When the samples cooled down, 0.5 mL of water saturated with NaCl was added into the reaction mixture. Butyl esters were 3 times extracted with *n*-hexane (2 mL total volume). The fractions were combined and the internal standard (5.0 μL) was added. The resulting *n*-hexane solution was filtered through anhydrous Na_2SO_4 to remove residual water.

2.3. Instrumentation

GC analyses were performed using a GC-FID/MS (7890N GC, 5975C MS) equipped with an autosampler (7386B series) and a split/splitless injector (Agilent Technologies, Santa Clara, CA, USA). Separations were accomplished using a 30-m long DB-5 capillary column, 0.25 mm internal diameter (I.D.) and 0.25 μm film thickness (J&W Scientific, Rancho Cordova, CA, USA) at a constant helium flow rate of 1.2 mL/min. Samples (1.0 μL) were injected with a splitless time of 0.4 min at 250 °C using a single gooseneck splitless liner with glasswool. The temperature programs were evaluated to allow for an efficient separation of all analytes, solvents, and derivatization agents. The final column temperature program started at 35 °C with a hold of 5 min, followed by the gradient of 20 °C/min to 300 °C and hold for 5 min.

The MS and FID data were simultaneously acquired employing a two-way splitter with a makeup gas (helium at a constant pressure of 28 kPa); the split flow ratio was 1:2 (MS:FID). The length of the connecting capillaries (0.15 mm I.D.) to the detectors (MS and FID) was 1.71 m and 0.31 m, respectively. The FID temperature was

Table 1
List of mono and dicarboxylic acids (MCAs and DCAs) studied as well as the corresponding GC–MS retention times, target and confirmation ions of their derivatives used for data processing.

Acid	Supplier	Trimethylsilyl esters of acids					Butyl esters of acids				
		t_R (min)	Target ion	Conformation ions		MW ion	t_R (min)	Target ion	Conformation ions		MW ion
MCA C1	Fluka	3.65	103(75)[a]	75(80)	45(15)	118[b]	6.08	56(100)	41(50)	73(10)	102
MCA C2	Fisher	5.41	117(70)	75(100)	73(15)	132	7.60	43(100)	56(50)	73(20)	116
MCA C3	Aldrich	7.36	131(70)	75(100)	73(40)	146(1)	9.00	57(100)	56(30)	87(10)	130
MCA C4	Aldrich	8.70	145(60)	117(10)	75(100)	160(1)	10.00	71(100)	89(65)	101(10)	144
MCA C5	Sigma	9.81	159(80)	129(10)	117(30)	174(1)	10.92	85(100)	103(70)	56(60)	158
MCA C6	Acros	10.72	173(100)	129(15)	117(40)	188(1)	11.72	99(100)	117(90)	56(70)	172(1)
MCA C7	Acros	11.52	187(90)	129(20)	117(60)	202(1)	12.45	113(100)	131(90)	56(100)	186(1)
MCA C8	Acros	12.25	201(100)	129(20)	117(55)	216(1)	13.13	145(100)	56(100)	127(90)	200(1)
MCA C9	MP Biomedicals	12.93	215(100)	129(30)	117(75)	230(1)	13.78	159(90)	56(100)	141(75)	214(1)
MCA C10	Acros	13.57	229(100)	129(20)	117(40)	244	14.39	173(90)	56(100)	155(70)	228(1)
MCA C11	Acros	14.17	243(100)	129(30)	117(70)	258(2)	14.96	187(80)	56(100)	169(60)	242(3)
MCA C12	Aldrich	14.75	257(100)	129(35)	117(75)	272(3)	15.52	201(80)	56(100)	183(50)	256(5)
MCA C13	MP Biomedicals	15.30	271(100)	129(40)	117(80)	286(3)	16.04	215(80)	56(100)	197(50)	270(10)
MCA C14	Alfa Aesar	15.83	285(100)	129(25)	117(45)	300(4)	16.54	229(70)	56(100)	211(40)	284(10)
MCA C15	Acros	16.33	299(90)	129(50)	117(100)	314(7)	17.02	243(70)	56(100)	225(40)	298(10)
MCA C16	Acros	16.81	313(100)	129(35)	117(70)	328(4)	17.49	257(70)	56(100)	239(40)	312(10)
MCA C17	Alfa Aesar	17.28	327(100)	129(25)	117(50)	342(5)	17.86	271(60)	56(100)	253(40)	326(20)
MCA C18	Acros	17.72	341(100)	129(35)	117(50)	356(6)	18.35	285(65)	56(100)	267(35)	340(20)
DCA C2	Aldrich	11.18	219(5)	147(100)	117(3)	234	12.91	57(100)	41(50)	56(25)	202
DCA C3	Aldrich	11.76	233(15)	147(100)	117(3)	248(1)	13.44	105(100)	57(50)	143(30)	216
DCA C4	Aldrich	12.58	247(17)	147(100)	117(2)	262(1)	14.22	101(100)	157(20)	57(17)	230
DCA C5	Aldrich	13.20	261(25)	147(100)	117(5)	276	14.81	115(100)	171(30)	87(20)	244
DCA C6	Unknown	13.85	275(30)	147(60)	117(15)	290	15.40	185(100)	129(100)	111(70)	258
DCA C7	Aldrich	14.44	289(50)	147(75)	117(25)	304	15.93	199(100)	125(100)	143(60)	272
DCA C8	Unknown	14.99	303(50)	147(45)	117(30)	318	16.45	213(100)	157(60)	138(30)	286
DCA C9	Aldrich	15.53	317(100)	147(40)	117(35)	332	16.94	227(100)	171(70)	152(40)	300
DCA C10	Aldrich	16.04	331(90)	147(30)	117(35)	346(1)	17.41	241(100)	185(80)	199(20)	314
DCA C11	Unknown	16.53	345(100)	147(20)	117(25)	360(1)	17.86	255(100)	199(85)	213(20)	328
DCA C12	Unknown	17.00	359(100)	147(20)	117(35)	374(1)	18.29	269(100)	213(95)	227(20)	342
DCA C13	Acros	17.44	373(75)	147(20)	117(40)	388(1)	18.72	283(85)	227(100)	241(20)	356
DCA C14	Aldrich	17.87	387(70)	147(17)	117(35)	402	19.19	297(90)	241(100)	255(25)	370

[a] The number in parentheses denotes relative abundance of particular ion vs. the base peak.
[b] If no number in parentheses is provided the molecular ion was not observed.

set to 350 °C and that of the transfer line to 300 °C. The gas supplies for the FID were hydrogen, air, and helium at flow rates of 30, 400, and 25 mL/min, respectively. The performance of the two-way splitter was regularly checked using a custom-made test mix. The MS data (total ion chromatogram, TIC) were acquired in the full scan mode (m/z of 35–550) at a scan rate of 2.82 scan/s using the electron ionization (EI) with an electron energy of 70 eV. Analyses using the selected ion monitoring (SIM) mode were acquired using two ions at each retention time window (100 ms each). The 5975C MS offers an additional mode which allows one to couple both the SIM and TIC scans (SITI). The scan time using SITI was 2.1 scan/s. The solvent delay was set as appropriate, depending on the derivatization agents, their by-products, and solvent retention times. Namely, for BF_3/butanol it was set to the first 5.5 min; for BSTFA, the MS was off for the first 3.4 min, 4.5–5.2 min, 6.1–7.0 min, and 8.1–8.2 min; for TMSDMC, the MS was off at 0–3.4 min, 4.15–5.20, and 9.35–9.78 min. The accurate determination of solvent delays was based on simultaneously acquired GC-FID data.

The LC–MS analyses were performed using an Agilent 1100 LC system coupled to a 6200 (model G1969A) high resolution (HR) time-of-flight MS from Agilent Technologies equipped with an ESI source set in the negative mode. LC/MS/MS analyses were carried out using an Agilent 1100 LC system coupled to an API3000 triple-quadrupole (3Q) MS equipped with a turbo-ion spray in negative mode from Applied Biosystems (Foster City, CA, USA).

Optimizations of HRMS parameters were performed using the flow injection analysis (FIA). Aliquots (20 μL) of individual acid solutions were injected into 50% ACN in water. The flow rate of 200 μL/min was directed to the MS without a prior separation on a LC column. To achieve a maximum response in MS, four mobile phase modifiers (i.e., ammonium formate, ammonium acetate, ammonium hydroxide, and acetic acid), ESI potentials (between −2000 and −4000 V), and collision-induced dissociation (CID) potentials between 110 and 200 V were evaluated. The nebulizer pressure, drying gas flow rates, and drying gas temperatures were optimized in ranges of 20–60 psig, 7–13 L/min, and 300–350 °C, respectively. Data were acquired in the m/z range of 30–1000. LODs were computed for deprotonated ions $(M-H)^-$. Mass accuracy of the instrument was ±5 ppm.

The ESI parameters (ionization potentials, temperatures, and gas flow rates) for 3Q MS/MS were optimized using the FIA with the same conditions as for TOF-MS. Direct infusion using a syringe pump with a flow rate of 12 μL/min was employed for optimization of selected reaction monitoring (SRM) parameters. Each standard was introduced separately at a concentration of ∼1 mg/L. Precursors/products' ion pairs, declustering and focusing potentials, and collision energies are provided in Table 2.

The LC conditions employed were the same for both LC–HRMS and LC–MS/MS systems. The LC reverse phase column suitable for 100% aqueous conditions was Prevail Organic Acid 3 μm, 100 mm long, 2.1 mm internal diameter and packed with 3 μm particles from Alltech Associates, Inc. (Deerfield, IL, USA). The flow rate of 200 μL/min was kept constant. The optimal separation was achieved using a gradient elution of water modified with 1.0 mM formic acid (eluent A) and acetonitrile (eluent B). The elution gradient was programmed as follows: 100% A for 20 min, followed by a linear gradient to 90% B from 20 to 40 min, then held at 90% B from 40 to 70 min, followed by a linear gradient to 100% A from 70 to

Table 2
MS/MS parameters used for the analysis of organic acids.

Acid	LC t_R (min)	Mass m/z	Precur. ion m/z	DP (V)	FP (V)	Target ion m/z	CE (V)	CEP (V)
MCA C1	2.0	46	45	−26	−300	45	−118	−1
MCA C2	n.d.	60	59	−16	−290	59	−74	−23
MCA C3	9.2	74	73	−16	−230	73	−6	−11
						55	−26	−9
MCA C4	25.9	88	87	−16	−240	87	−6	−5
						69	−25	−10
MCA C6	35.7	116	115	−21	−310	115	−8	−19
						71	−16	−11
MCA C7	38.4	130	129	−21	−290	129	−10	−9
						111	−16	−9
MCA C8	40.6	144	143	−21	−320	143	−10	−11
						125	−18	−5
MCA C9	42.7	158	157	−21	−60	157	−12	−9
						139	−18	−25
MCA C10	44.6	172	171	−26	−350	171	−10	−15
						153	−18	−13
MCA C12	48.6	200	199	−21	−300	199	−10	−13
						181	−26	−33
MCA C14	53.0	228	227	−26	−350	227	−7	−1
						209	−18	−27
MCA C16	61.0	256	255	−21	−280	255	−10	−7
						237	−28	−13
MCA C18	76.6	284	283	−16	−270	283	−12	−13
						265	−34	−23
DCA C2	1.9	90	89	−11	−160	45	−14	−5
						61	−12	−9
DCA C3	2.4	104	103	−11	−210	59	−18	−9
						41	−38	−5
DCA C4	5.8	118	117	−16	−220	73	−18	−11
						55	−24	−7
DCA C5	13.6	132	131	−16	−250	87	−18	−15
						69	−22	−11
DCA C6	26.5	146	145	−21	−270	83	−20	−13
						101	−18	−7
DCA C8	31.5	174	173	−16	−260	111	−20	−9
						129	−20	−11
DCA C9	33.1	188	187	−11	−210	125	−22	−21
						143	−24	−25

t_R, retention time. Precur. ion, precursor ion. DP, declustering potential. FP, focusing potential. Prod. ion, product ion. CE, collision potential. CEP, collision exit potential.

72 min and held for 15 min at 100% (equilibration time). The total analysis time was 87 min. The injection volume was 20.0 μL.

2.4. Data processing

The LODs for derivatization with GC–MS were determined using the target ions of m/z, which were selected based on the highest signal-to-noise ratio (the ions are listed in Table 1).

Instrumental LODs were calculated from the calibration curves using the formula LOD = 3.3 * s_y/k [34], 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. From the acquired calibration profiles, only the points within one order of magnitude of the LOD, were used for the LOD calculations. The LODs were expressed per injected volume.

Since for the GC/MS analyses the column flow was split between the FID and MS detectors in a 2:1 ratio, the LODs were adjusted by this factor as well.

3. Results and discussion

In this work, we first considered several derivatization methods for GC analysis and compared them to LC/MS analyses of a wide range of mono- and dicarboxylic acids. The critical parameters, i.e., derivatization for GC and MS ionization for LC, are discussed below. Upon completion of the method development, we evaluated and compared these techniques based on the instrumental LODs.

3.1. Optimization of CAs' TMSDMC derivatization protocol

The derivatization methods with BSTFA, MSTFA, and BF_3/butanol were adopted from previously published studies [29,31–33,35,36] and are not discussed here in detail. By contrast, the derivatization with TMSDMC was optimized only for phenolic compounds [37]; only the qualitative applicability to acids was reported for acetic and benzoic acid [38]. To our knowledge, the protocol for TMSDMC derivatization within the full range of acids was not previously evaluated. The previously published protocols involved the CA derivatization both at room temperature and at 50 °C, thus suggesting that temperature had no effect on the derivatization efficiency [37,38]. However, the low recoveries of derivatized formic acid were notable. This may be explained by the formation of dimethyl amine as a product of derivatization of acids [38], which, presumably, may further react with reactive formic acid to form dimethyl formamide, thus preventing the correct derivatization.

Contrary to the previously made assumptions, evaluation of several derivatization temperatures in this study showed that the reaction performed at 8 °C for 15 min resulted in a significant derivatization efficiency of formic acid, similar in response (area) to that of the BSTFA trimethylsilylated formic acid. Increase of the reaction temperature to 20 °C resulted in a 2-fold decrease in the formic acid derivatization efficiency (Fig. 1). Perhaps, the above-mentioned competing side reaction was less pronounced at lower temperatures. All other acids were not affected by changes in the reaction temperature. Similar detector responses were obtained for

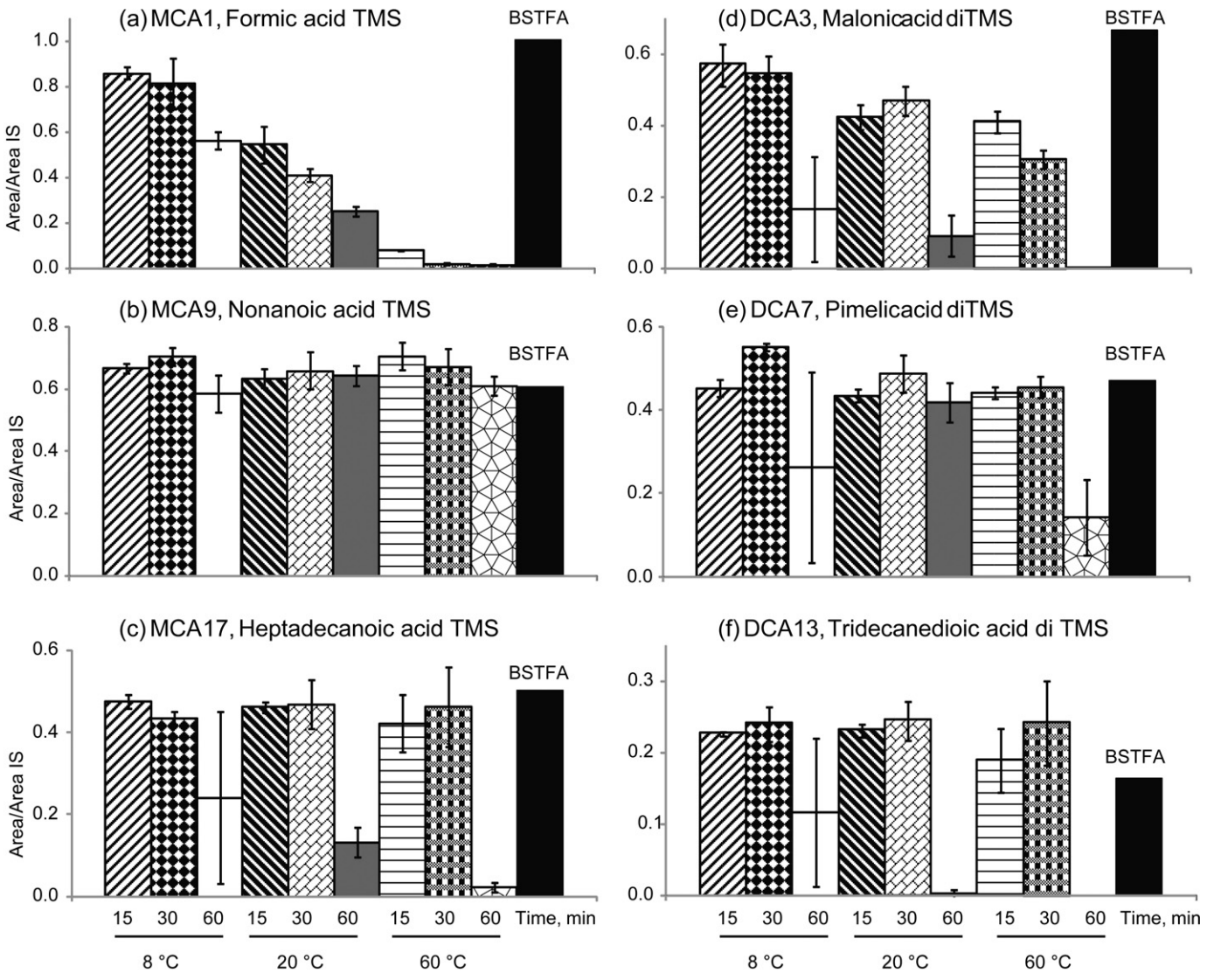

Fig. 1. The optimization of TMSDMC derivatization conditions (temperature and time) compared to BSTFA derivatization (at 60 °C for 1 h) for selected acids: (a) formic acid, (b) nonanoic acid, (c) heptadecanoic acid, (d) malonic acid, (e) pimelic acid, and (f) tridecanedioic acid.

the entire temperature range from 15 to 60 °C and reaction times of 15–60 min for MCAs 3–10. However, the extension of derivatization time seemed to have a negative impact on derivatization recoveries of selected species, DCAs and heavier MCAs in particular. It is of note that acetone, when used as a solvent, was found to have a detrimental impact on the CA derivatization with TMSDMC.

3.2. GC separation and aging of TMS and butylester derivatives

The separation of short chain MCAs and DCAs may be complicated due to a coelution with derivatization reagents and solvents used for the sample dilution. Thus, we evaluated the temperature program affecting the separation. Obviously, the column selection would also affect the analyte separation. In this study, we used a DB-5MS column of common specifications (30 m × 0.25 mm I.D. and 0.25 μm film thickness) typically applied in most laboratories.

Separation and aging of the TMS esters obtained using MSFTA and BSTFA. We found that the MSTFA derivatives of MCA C_1 and MCA C_3 (formic and propionic acids) could not be resolved using our instrumental setup. By contrast, the TMS esters were separated when BSTFA was used as a derivatization agent. Nevertheless, the analysis of BSTFA derivatives was complicated due to the occurrence of the remaining derivatization agent as well as derivatization by-products. As shown in Fig. 2, apart of the solvent peak, the analysis of volatile MCAs (C_1–C_4) was affected by the original BSTFA (peak no. 4), trifluoroacetamide (peak no. 1), and two by-products with major ions 77(100), 120(50), 170(50), 143(10) (peak nos. 2 and 3). The formation of one of these by-products could be explained by the occurrence of 2,2,2-trifluoro-*N*-(trimethylsilyl)acetamide [39], the other one was tentatively identified by us as 2,2,2-trifluoro-*O*-(trimethylsilyl)acetamide. The peak shapes of volatile MCAs were affected by these interfering species, thus negatively influencing their LODs as shown in the section on LODs. The separation of derivatization agent from analytes may be affected by the amount of the derivatization agent introduced onto the column. In this work, we used a 10-fold (moles) excess of BSTFA compared to the highest concentration of acids.

When monitoring the stability of derivatized products over time (i.e., aging effect), we have found that all samples were stable for at least 12 h at ambient temperature. However, after 1 week of aging oxalic acid (DCA C_2) was lost. Further aging for period of two weeks lead to decrease of the BSTFA peak (no. 4) while the peaks of by-products (peak nos. 2 and 3) increased, perhaps due to the derivatization of water penetrating into the samples as ubiq-

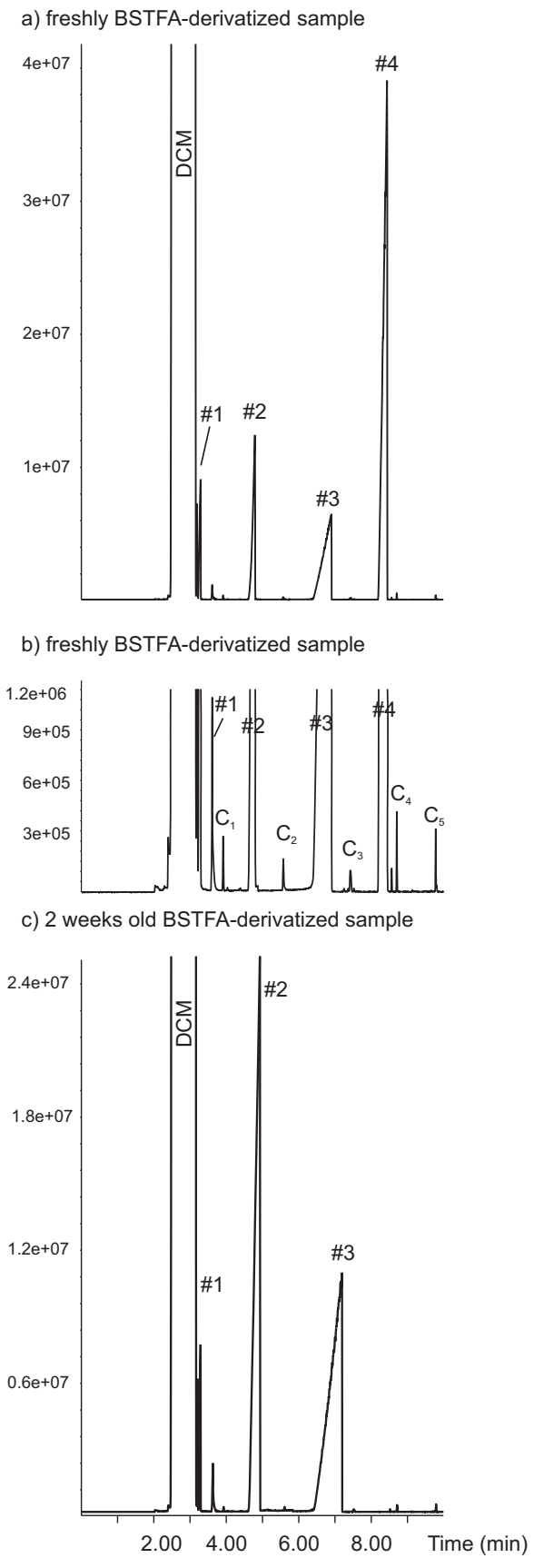

Fig. 2. Effect of aging on the BSTFA-derivatized samples: (a) freshly derivatized sample, (b) freshly derivatized shown in enlarged scale vs. (c) 2-week old sample. The numbers 1–4 refer to BSTFA and its by-products described in Section 3.3, C_1–C_5 symbols refer to monocarboxylic acids. For derivatization, the molar ratio of BSTFA to acids was 10:1.

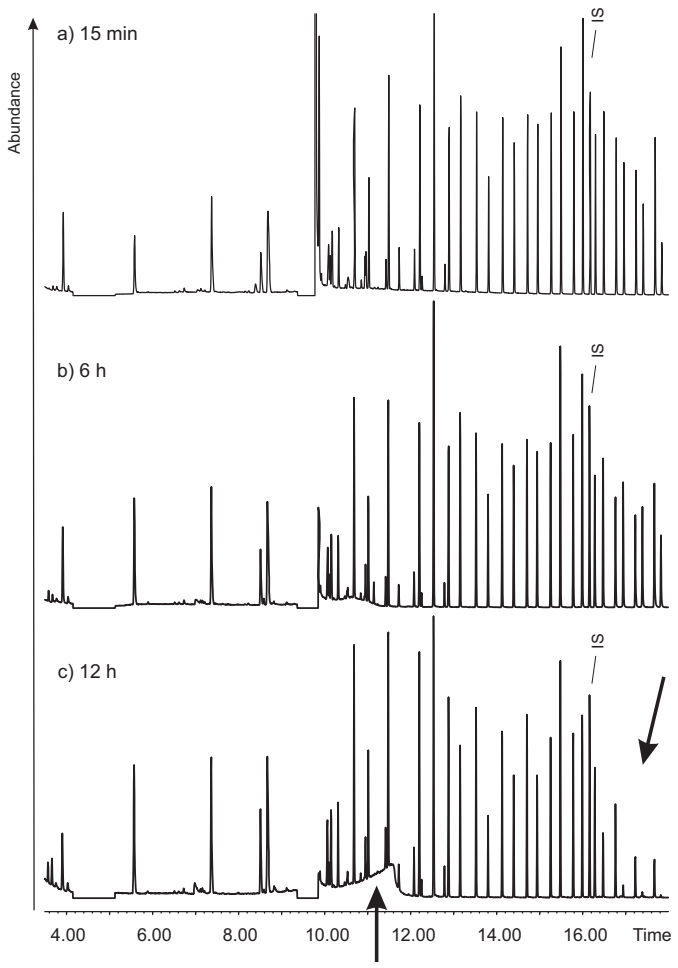

Fig. 3. Aging of a sample derivatized with trimethylsilyl dimethylcarbamate analyzed (a) 15 min, (b) 6 h, and (c) 12 h after the derivatization. The arrows highlight the affected response, which can be compared to the response of the internal standard.

uitous moisture (Fig. 2c). As a result, upon 2 week storage the C_1 and C_3 MCAs could not be determined as they coeluted with these by-products.

Trimethylsilylation with TMSDMC. Initially, we considered the derivatization with TMSDMC as the most promising protocol due to a fairly late elution of the derivatization agent, and thus effective separation of the volatile MCAs. However, when evaluating the sample stability over time, the higher molecular weight species, particularly DCAs, significantly deteriorated after >6 h (Fig. 3). This artifact was accompanied by the formation of a large unresolved "hump" below the signals of C_5–C_7 MCA.

Separation and aging of butyl esters. A substantial separation of all butylated MCAs and DCAs was readily achieved. The only compound observed after the solvent delay was the residual butanol. We did not observe any aging of derivatized acids even after 1 week of storage.

3.3. Optimization of a ESI-time of flight mass spectrometry protocol for HRMS

In order to maximize the detectability of CAs in HRMS, several electrolytes enhancing the ionization efficiency as well as some key ESI parameters including ionization potential, CID potential, drying gas flow rate/temperature and nebulizer pressure were optimized. The effects of ESI parameters were evaluated based on the response changes of the deprotonated molecule ($[M-H]^-$), which provided the greatest signal-to-noise ratio for all acids.

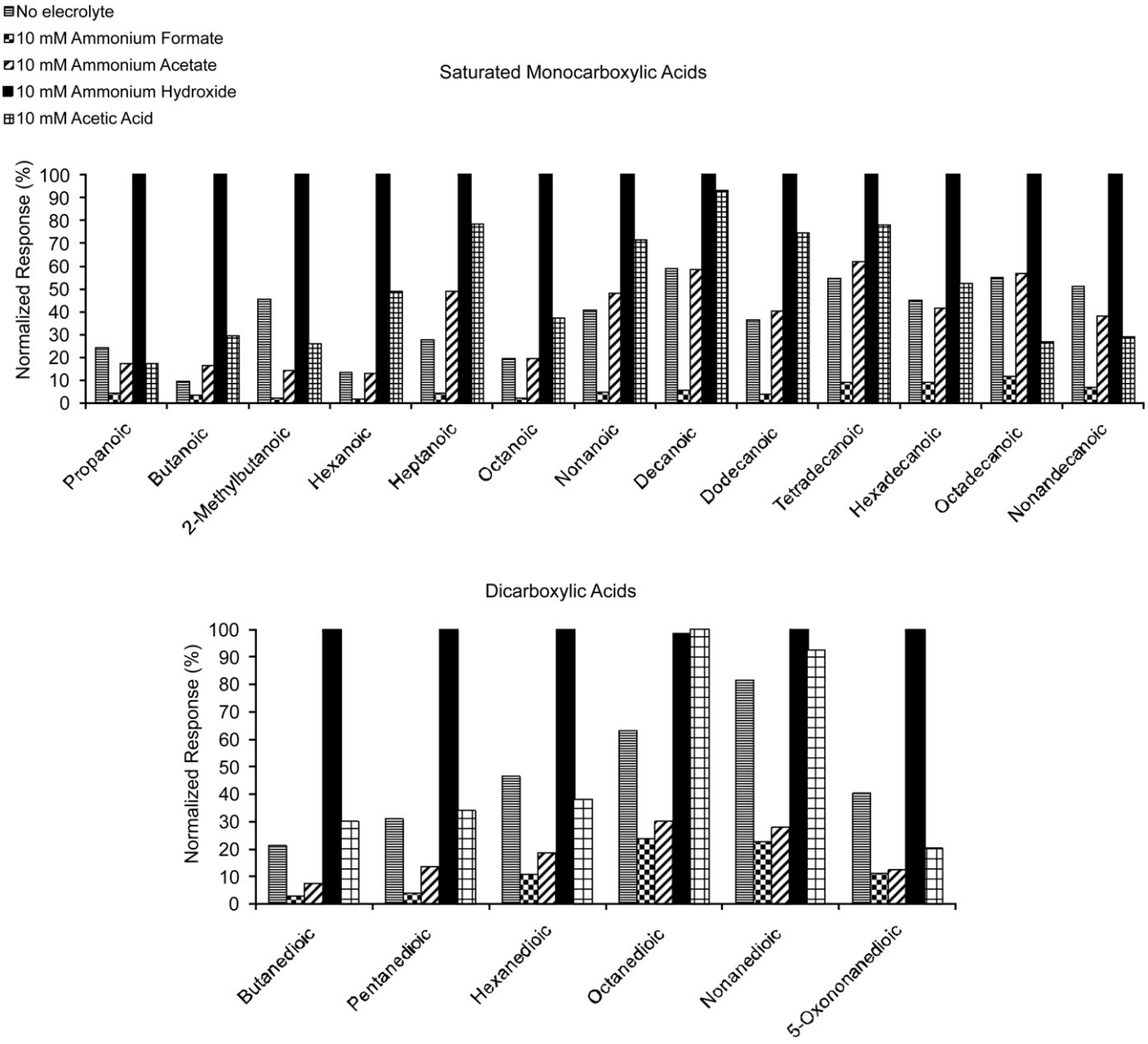

Fig. 4. Effect of electrolyte addition on the electrospray ionization efficiency in TOF MS for mono- and dicarboxylic acids.

The addition of electrolytes appeared to have the most significant impact on the responses of individual CAs among all parameters tested (Fig. 4). Ammonium formate suppressed the analyte ionization and thus lowered the signal intensity when compared to the analysis with no ionization agent added. In agreement with published results, acetic acid added as an electrolyte increased the response for some longer chain MCAs and DCAs [14]. The addition of ammonium hydroxide had the most pronounced positive effect on the ionization efficiency for all analytes. Adjusting the concentration of this electrolyte to 5.0 mM contributed to slight improvements in the responses of some analytes. Responses of acids declined with a further decrease of the electrolyte concentration to 2 mM.

Another important parameter was the CID potential. The increased potential from 110 to 200 V caused an extensive fragmentation of deprotonated molecules. The signal of small dicarboxylic acids, in particular, significantly deteriorated at a CID of 150 V; therefore, 110 V was selected as optimal even though the manufacturer's recommended range for CID is 150–300 V. Variation of other ESI parameters did not have as pronounced impact on the response of the tested analytes as the selection of electrolyte and CID potential. The following conditions, ionization potential = −4500 V, drying gas flow rate = 13 L/min, drying gas temperature = 350 °C, and nebulizer pressure = 60 psig were optimal for the majority of analytes and were selected for the determination of LODs (Table 3).

3.4. Optimization of triple quadrupole mass spectrometry for LC–MS/MS

Similarly to HRMS, addition of ammonium hydroxide in a concentration of 5.0 mM to the FIA mobile phase assured a great response for the majority of analytes. The ESI conditions for 3Q

Table 3
Comparison of instrumental limits of detection expressed in picograms injected obtained by GC–MS/FID and LC–MS analyses. The MS quantification was based on the target ions listed in Tables 1 and 2 with further details provided in Section 2.

	GC–MS/FID analysis (pg)							LC–MS (pg)	
	MS-TIC analysis			MS-SIM	MS-SITI	FID	FID	LC–HRMS	LC–MS/MS
Acid	TMSDMC	BSTFA	BuOH–BF_3	BuOH–BF_3	BuOH–BF_3	BuOH–BF_3	BSTFA		
MCAs									
MCA C1	25	591	61	2.1	NA	9.4	927	ND[a]	ND
MCA C2	16	36	62	20	20	66	153	234,032	ND
MCA C3	11	228	6.0	1.8	0.6	2.8	127	25,607	55
MCA C4	18	25	3.9	2.2	1.5	11	55	16,844	115
MCA C5	39	15	5.2	2.4	1.6	38	66	NA[b]	NA
MCA C6	20	34	5.2	1.5	2.4	50	13	51	46
MCA C7	13	32	3.9	1.6	1.9	30	4.7	52	4
MCA C8	5.0	3.0	2.8	2.5	2.0	2.6	6.9	36	13
MCA C9	5.1	15	3.7	1.6	3.5	7.1	4.4	25	9
MCA C10	5.7	17	3.7	2.3	2.8	3.7	4.7	19	5
MCA C11	7.0	10	5.5	1.4	2.4	7.0	5.9	NA	NA
MCA C12	13	20	7.1	1.8	2.8	5.6	8.1	26	8
MCA C13	8.0	18	7.6	2.2	3.1	7.2	8.0	NA	NA
MCA C14	4.7	7.1	8.3	2.3	3.4	5.7	8.7	18	4
MCA C15	13	15	3.8	2.2	1.6	3.5	8.1	NA	NA
MCA C16	6.1	7.2	6.9	5.1	5.5	19	16	93	12
MCA C17	21	9.4	21	3.3	2.4	4.3	7.5	NA	NA
MCA C18	15	23	12	1.0	0.8	15	41	64	54
DCAs									
DCA C2	220	216	4.8	1.1	0.2	6.2	256	ND	ND
DCA C3	50	77.5	3.8	2.2	4.0	7.8	70	2926	69
DCA C4	2.4	2.0	2.4	1.6	2.7	6.0	2.3	6735	12
DCA C5	8.3	18	5.1	4.7	5.0	8.3	13	3070	10
DCA C6	20	19	4.1	2.1	4.9	9.3	13	322	82
DCA C7	9.4	18	2.8	2.0	3.3	10	13	NA	NA
DCA C8	36	18	5.0	1.5	3.3	5.4	8.5	47	25
DCA C9	7.1	15	8.8	3.2	4.1	9.5	8.3	65	8
DCA C10	5.3	10	6.5	2.1	3.7	11	10	NA	NA
DCA C11	18	15	13	1.9	2.8	6.3	7.9	NA	NA
DCA C12	17	34	19	1.8	2.6	4.4	9.3	NA	NA
DCA C13	111	39	38	2.4	2.3	8.2	12	NA	NA
DCA C14	104	41	27	1.6	3.0	9.0	5.9	NA	NA

[a] ND denotes not detected.
[b] NA denotes not available.

MS/MS were evaluated similarly as for TOF MS. The ionization potential had the most significant impact among the evaluated parameters. The conditions which were optimal for the majority of analytes were as follows: The ionization potential (i.e., ion spray voltage) was set to −4200 V, the ionization source temperature was 550 °C, the nebulizer pressure was 15 psig and the drying gas (air) flow rate (i.e., curtain gas) was 14 L/min. These conditions were selected for the subsequent LC–MS/MS analysis.

MS/MS fragmentation parameters were optimized using a direct infusion of individual standards. It was difficult to achieve an efficient MS/MS fragmentation of MCAs. The only product ion observed in spectra was $[M-19]^-$, which is $[M-H_3O]^-$. However, this fragment provided a rather low response for each of the MCAs used despite the optimization. Ultimately, the precursor ion, i.e., $[M-H]^-$, was also scanned in the second MS and employed for quantitation (i.e., data acquisition using the SIM). The product ion $[M-19]^-$ was used as a confirmation ion in the subsequent quantitative analysis of acids. DCAs were mainly losing their carboxylic group upon fragmentation thus yielding the $[M-45]^-$ (i.e., $[M-COOH]^-$) product ion. The second product ion was $[M-63]^-$, perhaps $[M-CO_3H_4]^-$. For the majority of DCAs, this specie was used as the confirmation ion in quantitative analysis. To our knowledge, this is the first systematic study of fragmentation patterns in MS/MS for a wide range of CAs.

3.5. Comparison of the chromatographic methods used based on the values of instrumental LODs

In this work, we report instrumental LODs addressing sample derivatization and analysis process *per se*. However, when implementing this method, a special attention should be paid to sample preparation steps (i.e., extraction and preconcentration). Particularly, evaporation may lead to the losses of low molecular weight acids and therefore their increased LODs. In this study, we minimized the acid losses by using small extraction volumes for butylation, thus avoiding their evaporation.

The instrumental LODs obtained using different GC and LC methods are summarized in Table 3. When comparing all results obtained, butylation in the presence of BF_3 provided the lowest LODs in a range of 2–60 pg (analyzed using GC–MS TIC). Moreover, the butylation derivatives' LODs were at least one order of magnitude lower than those previously reported [40]. Significantly higher LODs ~3000 pg in SIM mode were observed for this derivatization agent for acetic acid in the presence of ACN (not shown). This observation can be explained by the Pinner reaction, in which nitriles reacting with the excess of alcohols under acidic catalysis in the presence of water can form esters [41]. Thus, a special attention needs to be paid when selecting a solvent for sample preparation. Much lower LODs, 20 pg in the SIM mode, were obtained when DCM was employed for preparation of the acetic acid stock solution.

GC–MS acquisition experiments were initially performed in the total ion current mode and quantified using the selected extracted ions. As expected, further improvements in detection limits were achieved by the use of either selected ion monitoring or recently introduced parallel scanning in TIC and SIM (i.e., SITI). Similarly as observed in previous method development, SITI scan provided comparable data to those obtained by SIM [42]. The SITI mode thus showed an advantage of high sensitivity while maintaining the benefit of providing the TIC mass spectra, thus allowing for the identification of unknowns. It is of note that GC with FID, which is considered, due to its nearly universal character, to be less sensitive than GC–MS, provided comparable LODs to those obtained by GC–MS TIC.

As far as the derivatization technique is concerned, butylation provided the lowest LODs and all analytes were resolved. However, this protocol involved the most labor intensive sample preparation as it required the removal of the unreacted derivatization agent. Trimethylsilylation methods showed, at most, 2-fold higher LODs (thus still being in a picogram range) than those obtained for butylation. Moreover, the same final sample dilution was employed for both processes which was a mandatory step for butylation but not required for BSTFA derivatization. Even though lower LODs may be observed when omitting the dilution step, the resulting higher BSTFA concentration may also lead to an incomplete separation of low molecular weight acids from the derivatization by-products and possible co-elution. Consequently, this technique may be preferred due to an easier sample preparation, although the setup of solvent delays may be considered a setback for the GC–MS analysis, due to a near elution of volatile MCAs and the derivatization agents (discussed in Section 3.2). The main disadvantage for the TMSDMS derivation was the above mentioned limited stability of samples.

In general, the LODs obtained with LC–MS and tandem MS provided comparable results to those obtained using GC with trimethylsilylation. The clear advantage of LC methods is a fairly easy sample preparation. However when adopting this method, one should be aware of possible common matrix effects causing ion suppression and requiring additional purification within the sample preparation procedure [43].

The LODs obtained by HRMS were comparable to those previously reported [15]. Due to a merely minor fragmentation of MCAs in MS/MS, data had to be recorded in selective monitoring through two quadrupoles (i.e., selection of the same precursor/product ions). Fragmentation for all DCAs exhibited the same pattern (as described in Section 3.4) improving LODs when compared to HRMS and providing an important means of qualitative analysis for these acids. To our knowledge, no LC protocol with tandem MS capable of achieving the CAs' LODs in a 5–100 pg range was previously reported.

Both LC–HRMS and LC–MS/MS showed some limitations in the determination of low molecular weight acids. As mentioned in the previous section (Section 3.4), the LOD for formic acid was compromised due to its use as a mobile phase modifier. In addition, acetic and oxalic acids were not detected due to the presence of common background ions. The excessive thermal decomposition of oxalic acid may also contribute to the observed low detectability of this analyte. LODs of other acids were not affected as they were deprotonated due to the post-column addition of 5 mM ammonium hydroxide (optimized for the maximum ESI efficiency).

4. Conclusions

In this work, we have compared several derivatization GC–MS approaches and direct LC–MS methods for determination of wide range of aliphatic MCAs and DCAs. The most critical factor for all techniques was the separation and quantification of volatile acids. The lowest LODs were obtained by using butylation followed by GC–MS TIC and further improved when using SIM or SITI. Although GC-FID is expected to provide a nearly universal detection and thus being of limited sensitivity, the LODs obtained while using this method were comparable to those achieved by GC–MS TIC.

Although the use of BF_3/butanol for CA derivatization seems to be ideal from the perspective of achieving the lowest LODs, this technique turned out to be the most laborious compared to the others. For the majority of CAs, approximately one order of magnitude higher LODs, still in a range of 5–60 pg, were achieved with trimethylsilylation followed by GC. In this work, the LODs are strictly limited to the derivatization and GC analysis method. The sample preparation steps such as evaporation may need further evaluation or addition of appropriate recovery standards. Furthermore, the CA derivatization with BSTFA provided a significant stability of the derivatized analytes for at least 24 h. After week lost of oxalic acids was observed, but all other analytes seemed to be stable. This was in contrast to the use of TMSDMC, which provided an easy method for the CA derivatization, but the stability of analytes limited to 6 h.

As an alternative to GC, LC–MS/MS analyses (using the sample precursor/product ion) yielded LODs comparable to GC–MS with trimethylsilylation, yet providing an advantage of a direct analysis. However, when applying this method to real-world samples, one would have to validate the possible contribution from a matrix, which can cause the ionization suppression.

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