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Research Article

Application of Standard HSQC-ME Pulse Sequence on Benchtop NMR Devices for Quantitative Applications

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The heteronuclear single quantum correlation (HSQC) NMR method is widely used for the structural characterization of complex mixtures. In this study, the application range of multiplicity-enhanced HSQC (HSQC-ME) spectroscopy was broadened to quantitative analysis on low-field NMR devices with standard measurement possibilities. Acquisition parameters such as number of scans and t1 increments as well as repetition time were optimized to achieve the best signal-to-ratio and resolution requiring minimum measurement time. Standardization with internal standard using correction factor and external calibration approaches for active pharmaceutical ingredients/stimulants in pharmaceutical products and dietary supplements showed average absolute bias of 5.0% and 7.7%, respectively. The HSQC-ME NMR spectroscopic method was characterized by measurement uncertainty below 4% and limits of detection below 3 mg/mL for 2-h measurement time. Semiquantitative HSQC-ME analysis of organic acids in e-cigarettes can be performed within the accuracy of 25% at low-field NMR instruments in case of overlap in 1D NMR dimension and in the presence of huge solvent signals.

Keywords: active pharmaceutical ingredient; benchtop NMR; electronic cigarettes; HSQC-ME NMR; qNMR

1. Introduction

Nowadays, quantitative NMR (qNMR), especially ^{1}H qNMR, is a well-established procedure to estimate the purity of chemical compounds and concentrations of multiple compounds in complex mixtures. The main applications of qNMR include pharmaceutical and natural product analysis [1–3], food and beverage control [4], and clinical diagnostics [5]. Quantitative approaches usually deal with the usage of an internal standard (IS), as the peak area of a ^{1}H-signal is proportional to the number of nuclei responsible for the signal. This, along with a rather simple sample preparation, makes NMR spectroscopy an attractive method for obtaining both structural and quantitative information from a single experiment.

As the size of molecules and the number of metabolites in mixtures increase, it is no longer possible to find an undisturbed resonance of analyte or a "place" in spectrum, where a signal of a suitable reference compound can be placed. This is especially the case for metabolomic mixtures and biopolymer samples such as proteins or nucleic acids, where spectral overlap in ^{1}H NMR impedes data evaluation even using deconvolution techniques [6, 7].

To resolve this issue, two-dimensional (2D) NMR techniques can be applied, which display recorded spectrum onto a second dimension, thereby spreading signals and reducing overlap. One of the most popular 2D NMR techniques, the heteronuclear single quantum coherence (HSQC) NMR, reveals cross-signals at the position of the

corresponding ^{1}H-^{13}C bonds. Recently, it has become possible to acquire 2D ^{1}H–^{13}C NMR spectra within a second by a single scan 2D NMR technique also known as ultrafast NMR [8, 9]. HSQC NMR at high-field NMR (HF NMR) devices strongly improved structural analyses and was used, for example, for the investigation of human biofluids, complex carbohydrates, and plant metabolome [10–12].

However, quantitative considerations provide a challenge for 2D NMR, since peak volumes are not only dependent on concentration and number of nuclei but also on a variety of factors including relaxation time, signal offsets, coupling constants, and parameters of the pulse sequence [13]. Different generic approaches have been recently described to determine absolute concentrations by 2D NMR including sensitivity-enhanced pulse sequences, theoretical calculations, or analytical approaches such as external calibration and standard addition [13]. Achieving quantitative results with HSQC using HF NMR devices has been the focus of several research groups over the past 20 years, and several pulse sequences (such as QHSQC and HSQC0) were developed and implemented [14, 15].

For benchtop devices, a few studies on HSQC spectroscopy have performed mostly for qualitative purposes [16–18]. However, the investigation of the use of a 2D HSQC experiment for the quantification of small molecules in mixtures on benchtop devices is urgent due lower resolution in ^{1}H dimension and more prominent signal overlap than at HF NMR spectrometers. The aim in this study therefore was to perform quantitative analysis using standard HSQC pulse sequences implemented on benchtop devices. This study is aimed at standard benchtop NMR instrumentation, where only standard HSQC and multiplicity enhanced HSQC (HSQC-ME) pulse sequences are implemented. An external calibration approach was systematically compared to the IS quantitation using a correction factor. Several validation parameters such as robustness, inter- and intraday variations, and precision of manual integration were evaluated. The developed method was applied for several analytical tasks, such as analysis of low-chain fatty acids in e-cigarettes, stimulants in dietary supplements, and active pharmaceutical ingredients in medicinal products.

2. Materials and Methods

2.1. Samples and Chemicals. In total, 10 pharmaceutical samples and one dietary supplement with different active ingredients (AIs) were purchased in local drug stores in Germany in 2023-2024 (Table 1). Refill fluids for e-cigarettes were purchased in local e-cigarette shops in Germany.

Ibuprofen (> 98%) was obtained from Glentham Life Sciences (Planegg, Germany). Maleic acid (> 99%), paracetamol (> 98%), caffeine, succinic acid (> 99%), levulinic acid (98%), and 1,2-propanediol (PG, > 99.5%) were bought from Sigma-Aldrich (Taufkirchen, Germany). Benzyl benzoate (BB) (> 99%), L-nicotine (> 99%), and taurine (99%) were purchased from Thermo Scientific Chemicals (Waltham, Massachusetts, USA). L-ascorbic acid (> 99%), malic acid (> 99%), citric acid (> 99.5%), propionic acid (> 99.5%), valeric acid (> 98%), and butyric acid (> 99%) were obtained from Carl Roth (Karlsruhe, Germany). Vegetable glycerol (VG, > 99%) was provided by Janssen Chimica (Neuss, Germany). Dimethyl sulfoxide DMSO-d_6 (D 99.8%), deuterated water D_2O (D 99.9%), and chloroform $CDCl_3$ (D 99.8%) were bought from Deutero (Kastellaun, Germany).

2.2. Sample Preparation. A dosage of pharmaceutical sample/dietary supplement corresponding to approximately 50 mg of AI and 50 mg of IS were dissolved in 1 mL of appropriate solvent (see Table 2). BB was used as an IS. The mixtures were treated with an ultrasonic bath at room temperature for 30 min. Subsequently, the samples were transferred into a centrifuge (Universal 320 by Hettich, Kirchlengern, Germany) for 15 min at 9000 rpm. Six hundred μL of the clear supernatant were transferred to an NMR tube for measurement. If necessary, the solutions were filtered through a membrane filter.

Synthetic mixtures of organic acids were prepared in D_2O or in a mixture of PG/VG/H_2O (25/25/50 v/v%) containing 5 mg/mL nicotine to simulate the e-cigarette solvent composition. Moreover, 0.5 mL of authentic e-cigarettes were diluted with 0.5 mL H_2O and were spiked with different organic acids. Maleic acid 30 mg/mL was used as an IS for quantification.

2.3. LF- and HF-NMR Measurements. Benchtop NMR measurements at 80 MHz were performed on a Spinsolve 80 Carbon spectrometer equipped with automatic sample changer for 20 samples (Magritek GmbH, Aachen, Germany). The data were recorded automatically under the control of Spinsolve software 14.2.1 (Magritek GmbH, Aachen, Germany). ^{1}H NMR spectra were acquired using 16 number of scans (NS), 3.2-s acquisition time (AQ), 10-s repetition time (RT), and a 90° pulse angle (PA) with 64K data points (TD). Solutions in mixed PG/VG/H_2O solvents were measured using ^{13}C-satellite-suppressed ^{1}H-NMR experiment with the simultaneous suppression of three regions (5.5–4.0 ppm, 3.9–3.0 ppm, and 2.0–1.0 ppm) and AQ of 3.2 s, RT of 30 s, NS 512, and a PA of 90°. T1 was determined for target signals of pharmaceuticals and ISs using inversion recovery experiment. All T1 values were found to be below 1.0 s (Table 3).

HSQC-ME spectra were measured using 64 NS, 1 s RT, and 125 ppm with the center at 60 ppm (f1) and 25 ppm (f2) SWs with 4K TD in f2 and 64 t1 increments. Other not adjustable parameters were dwell time of 500 μs, 1024 points along t2, coupling delay 1/4J(C, H) = 1.8 ms (for J(C, H) = 140 Hz), and multiplicity editing delay 1/2J(C, H) = 3.6 ms (for J(C, H) = 140 Hz). These parameters were set for all measurements. Measurement time is 2 h and 18 min.

High-field ^{1}H qNMR (HF ^{1}H qNMR) measurements were performed on a Bruker Avance III 500-MHz spectrometer with BBO cryo probe equipped with Bruker Automatic Sample Changer for 60 samples (Bruker Biospin, Rheinstetten, Germany). ^{1}H NMR spectra for the samples were recorded with AQ of 5.45 s, relaxation delay (RD) of 40.0 s, NS of 128, TD of 65 K, and a PA of 90° at 298 K. The measurement time was about 60 min.

Table 1: List of the investigated pharmaceutical products and dietary supplements.

Sample[a]	Tablet weight (mg)	AI		Main matrix components (selection)
		Compound	AI pro tablet (mg)	
S1	830	Ibuprofen	400	Lactose, microcrystalline cellulose
S2	550	Paracetamol	500	Potato starch, povidone
S3	520	Paracetamol Caffeine	350 50	Sodium carboxymethyl starch, corn starch, silica
S4	465	Paracetamol	325	Corn starch, dimethicone 350
S5	375	Paracetamol Ascorbic acid Caffeine	200 150 25	Lactose
S6	600	Ibuprofen	400	Lactose, microcrystalline cellulose, corn starch, croscarmellose sodium, silica
S7	3225	Paracetamol	500	Maltodextrin, lactose, sorbitol
S8	840	Ibuprofen Paracetamol	400 200	Calcium phosphate, microcrystalline cellulose, croscarmellose sodium hydroxypropyl methylcellulose, talcum
S9	210	Caffeine	100	Microcrystalline cellulose
S10	490	Ibuprofen	400	Microcrystalline cellulose, croscarmellose sodium, hypromellose, macrogol 400
S11	525	Caffeine Taurine	100 150	Calcium carbonate, hydroxypropyl methylcellulose

[a]Medicines except S11 (dietary supplements).

Table 2: Quantitative results for AI in pharmaceutical and dietary supplement samples based on one-point calibration using internal standard (IS) and external calibration (EC); information of the integrated signals are listed in Table 3.

Sample	Solvent	AI		IS		AI content (mg/mg tablet)			Bias (%)	
		Compound	SNR[a]	Compound	SNR	^{1}H qNMR 500 MHz	80 MHz			
							IS	EC	IS	EC
S1	$CDCl_3$	Ibuprofen	132	BB[b]	68	0.51	0.54	0.46	6.4	−10.2
S6	$CDCl_3$	Ibuprofen	138	BB	79	0.80	0.71	0.70	−12.0	−12.4
S8	$CDCl_3$	Ibuprofen	78	BB	60	0.32	0.34	0.35	−6.3	9.4
S10	$CDCl_3$	Ibuprofen	143	BB	93	0.77	0.81	0.82	5.7	6.4
S2	DMSO-d_6	Paracetamol	228	BB	145	1.04	0.93	0.90	−9.9	−13.2
S3	DMSO-d_6	Paracetamol	182	BB	116	0.67	0.69	0.63	2.4	−6.2
S4	DMSO-d_6	Paracetamol	212	BB	124	0.92	0.97	0.86	5.1	−9.7
S5	DMSO-d_6	Paracetamol	156	MA[c]	70	0.50	0.47	0.50	−6.9	−0.3
S7	DMSO-d_6	Paracetamol	157	BB	114	0.14	0.14	0.11	1.9	−3.0
S8	$CDCl_3$	Paracetamol	73	BB	60	0.26	0.26	0.24	2.0	−9.4
S3	D_2O	Caffeine	18	BB	116	0.10	0.10	0.07	−5.3	−8.3
S9	D_2O	Caffeine	26	MA	24	0.49	0.47	0.46	−3.1	−6.1
S5	DMSO-d_6	Ascorbic acid	45	MA	70	0.41	0.37	—[d]	−8.1	—[d]
S5	D_2O	Caffeine	12	MA	70	0.06	0.06	0.04	−2.2	−9.9
S11	D_2O	Caffeine	29	MA	22	0.18	0.19	0.19	1.3	3.4
S11	D_2O	Taurine	22	MA	22	0.34	0.33	—[d]	1.2	—[d]

[a]SNR: signal-to-noise ratio.
[b]BB: benzyl benzoate.
[c]MA: maleic acid.
[d]was not calculated.

Table 3: HSQC integration range and multiplicities of integrated peaks; the exact positions may slightly vary in different solvents.

Substance	Group[a]	Multiplicity[b]	f2 (ppm)	f1 (ppm)	T1 time (s)
Ibuprofen	CH_3	d	1.3–1.8	16–20.5	0.7
Paracetamol	CH_3	s	1.7–2.6	22–26	0.7
Ascorbic acid	CH_2	d	3.2–4.0	60–65	0.5
Caffeine	CH_3	s	3.7–4.0	32–36	1.0
Taurine	CH_2	t	3.0–3.5	45–49	0.8
Benzyl benzoate	CH_2	s	5.1–5.6	64–69	0.9
Maleic acid	CH	d	5.9–6.6	4–12	—[c]
Citric acid	CH_2	s	2.6–3.2	40–46	—
Malic acid	CH_2	s	2.5–3.2	34–39	—
Succinic acid	CH_2	t	2.6–3.2	36–40	—
Levulinic acid	CH_2	t	2.5–3.0	25–31	—
Propionic acid	CH_2	q	2.3–2.8	25–29	—
Butyric acid	CH_2	q	2.1–2.6	33–38	—
Valeric acid	CH_2	q	2.1–2.6	31–35	—
Nicotine	CH_3	s	2.6–3.1	37–41	—

[a]the absolute value of CH_2 group signal volume was used for quantification.
[b]s: singlet, d: doublet, t: triplet, and q: quadruplet.
[c]Was not measured as semiquantitative analysis was performed.

NMR spectra were manually processed using Mestrenova 14.2.3 (Mestrelab Research S.L., Santiago de Compostela, Spain). All 1D and 2D NMR spectra were first phase- and baseline-corrected. For the processing of HSQC-ME spectra in MNova, a 90° shifted sine square function was applied in both dimensions to the free induction decay (FID), as well as exponential and Gauss apodization functions (5 Hz each) in f2. Zero filling was applied to f2 from 1K to 2K.

The signal-to-noise ratio (SNR) was calculated using an interactive tool implemented in MNova software, which considers the height of the 3D peak versus noise from an empty area of the 2D map. To obtain reproducible results, the noise level was calculated in the region 0-1 ppm and 10–20 ppm for ^{1}H and 13C dimensions, respectively. The calculation precision was below 5% for SNR when different noise regions were used. Integration of HSQC-ME signals was performed using ellipsoidal regions in MNova by summation of all TD. Absolute volume values were used for a quantitative analysis.

2.4. HSQC-ME Quantification and Validation. For the quantification of AIs (ibuprofen, paracetamol, caffeine, ascorbic acid, and taurine), two approaches were applied and

compared. For external standardization, five calibration solutions were prepared for ibuprofen (20–200 mg/mL), paracetamol (10–100 mg/mL), and caffeine (5–20 mg/mL) in appropriate solvents along working ranges (Table 2, Figure 1). To prepare calibration solutions, we used dilution series to avoid weighing small amount of analytes. Absolute volume values were plotted against the concentration of an analyte.

As mentioned in the introduction, using a single IS is not compatible with the intrinsic characteristics of 2D NMR. Therefore, the response factor has to be calculated for peak volumes of the IS–analyte mixture. For this, a standard solution with a known concentration of analyte and IS was prepared to calculate a proportionality factor F according to the following equation:

$$F = \frac{V_{\mathrm{IS,cal}}}{V_{A,\mathrm{cal}}} * \frac{m_{A,\mathrm{cal}}}{m_{\mathrm{IS,cal}}}, \quad (1)$$

where V is the peak volume (integral), m is the amount (mg), IS is the internal standard, and A is the analyte.

The same amount of IS was then added to an unknown mixture/pharmaceutical product, and the amount of analyte can be calculated as

$$m_{A,x} = \frac{V_{A,x}}{V_{\mathrm{IS}}} * F * m_{\mathrm{IS},x}. \quad (2)$$

This approach can be used to simultaneously calibrate several compounds in one matrix. However, reference standards are necessary to calculate response factors in comparison with inherently quantitative 1D qNMR methodology. The integration ranges for all compounds are listed in Table 3.

Reference concentrations were obtained from ^{1}H HF qNMR spectra by conventional calculation using the IS [1].

To evaluate accuracy, the relative deviation (Bias) for different standardization methods on LF NMR compared to the reference HF NMR concentrations was used (as shown in the following equation):

$$\mathrm{Bias}[\%] = \frac{c_{\mathrm{HF}} - c_{\mathrm{LF}}}{c_{\mathrm{HF}}} * 100, \quad (3)$$

where c_{HF} and c_{LF} concentrations are found by HF qNMR and LF qNMR, respectively.

For validation measurements, Samples S5, S6, and S11 underwent sample preparation five times. Additionally, three samples were measured on five different days. The following analytes were evaluated: ibuprofen, paracetamol, ascorbic acid, caffeine, and taurine (see Table 3 for selected signals). Limit of detection (LOD) and limit of quantification (LOQ) were determined by interpolating SNR for standard solutions to three and 10, respectively. For one selected Sample S11, the integration on MNova for three different compounds (two analytes and one IS) was executed by three different operators. For an evaluation of the method's robustness, the NS and the number of t1 increments varied between 32 and 128 each. To evaluate accuracy, the calculated concentrations were referenced to the concentration obtained from HF NMR measurements.

3. Results and Discussion

3.1. HSQC-ME Method Development at 80 MHz. Only HSQC and HSQC-ME pulse sequences are implemented in the standard automation software of the LF NMR device used in this study. The HSQC-ME technique, in comparison with HSQC experiment, differentiates negative amplitude of CH_2 signals and positive signals to CH and CH_3 groups. Phase-sensitive detection yields an HSQC-ME spectrum with higher resolution (narrower peaks) than the standard HSQC (which is an absolute-value experiment) but takes twice as long to run for a given NS and RT. HSQC-ME is widely employed for the qualitative and semiquantitative analysis of complex mixtures on HF NMR spectrometers [19, 20]. However, no studies aimed at optimization of acquisition parameters for quantitative applications of LF NMR devices are known.

Different adjustable acquisition parameters (RT, NS, and t1 increments) systematically varied for different compounds/functional groups. It was found that the SNR of HSQC signals does not depend on RT in our case as justified by Grubbs's outlier test. Therefore, the HSQC-ME measurements at RT = 1 s were performed at full longitudinal relaxation (see Table 3 for T1 values). It is also in accordance with the study, where the maximal SNR values per time unit is reached for RT delay nearby $1.25 \times$ T1 for a 90° excitation [21].

A logarithmic correlation was obtained for NS and the number of t1 increments (see Figure 2 for ibuprofen as an example). As the increase of these parameters leads to proportional rise in measurement time, it was decided to keep RT at a minimum of 1 s and NS and the number of t1 increments at 64. To increase the digital resolution of HSQC signals for more accurate integration, F1 bandwidth was set at 125 ppm with the center at 60 ppm (the lowest possible values for F1 bandwidth and F1 center, respectively). These settings led to the folding of aromatic signals, therefore, in this study, preliminary aliphatic signals were investigated. The total measurement time was 2 h 18 min, which was a compromise between expenditure of time and accuracy of the HSQC-ME experiment at low-field NMR spectrometer.

Using these experimental conditions, different quantitative applications of HSQC-ME were developed. This included analysis of pharmaceutical and dietary supplement samples with different degrees of overlap in ^{1}H dimension. Secondly, several synthetic mixtures of organic acids in D_2O with great overlap in ^{1}H dimensions were explored. Lastly, organic acid mixtures were quantitatively determined in a viscous solvent of PG/VG/H2O, where ^{1}H-NMR spectroscopy was found hardly feasible even for quantitative purposes.

3.2. Active Pharmaceutical Ingredients and Stimulants. The first group of samples was over-the-counter medicines containing ibuprofen, paracetamol, and caffeine for which ^{1}H qNMR analysis has been already well-established [22]. As an example, Figure 3 shows the HSQC-ME spectrum with the integrated regions of Sample S4, where, similarly to the

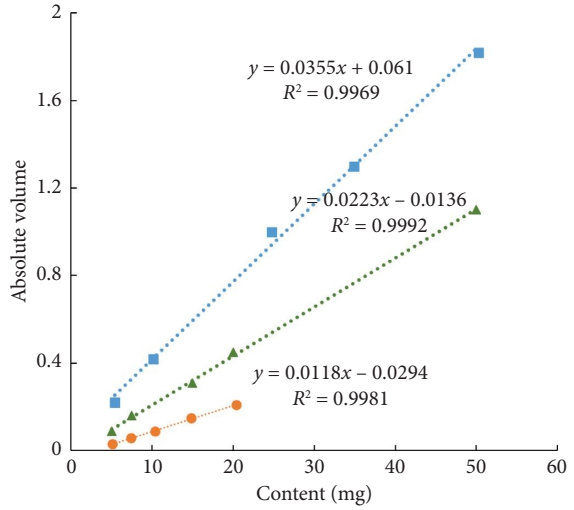

FIGURE 1: External calibration curves for caffeine in D_2O, ibuprofen in $CDCl_3$, and paracetamol in DMSO-d_6. HSQC integration ranges and multiplicities of integrated peaks are summarized in Table 3.

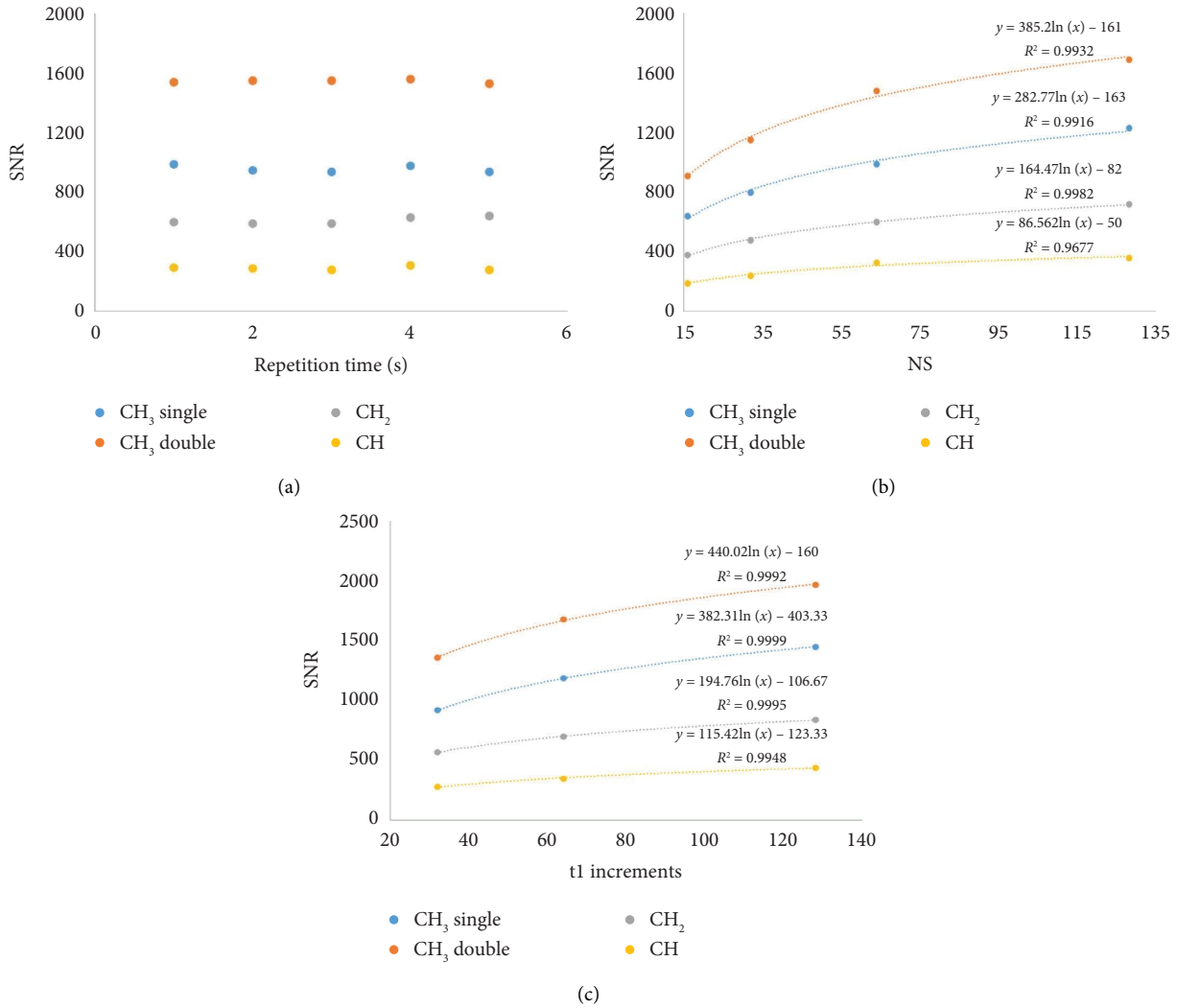

FIGURE 2: Influence of RT (a), NS (b), and t1 increments (c) on SNR of HSQC-ME signals for different groups of ibuprofen (500 mg/mL in $CDCl_3$). Other acquisition parameters were NS = 64, t1 = 64 (A); t1 = 64, RT = 1 s (B); NS = 64, RT = 1 s. (C) The averaged SNR values for three different noise regions are shown.

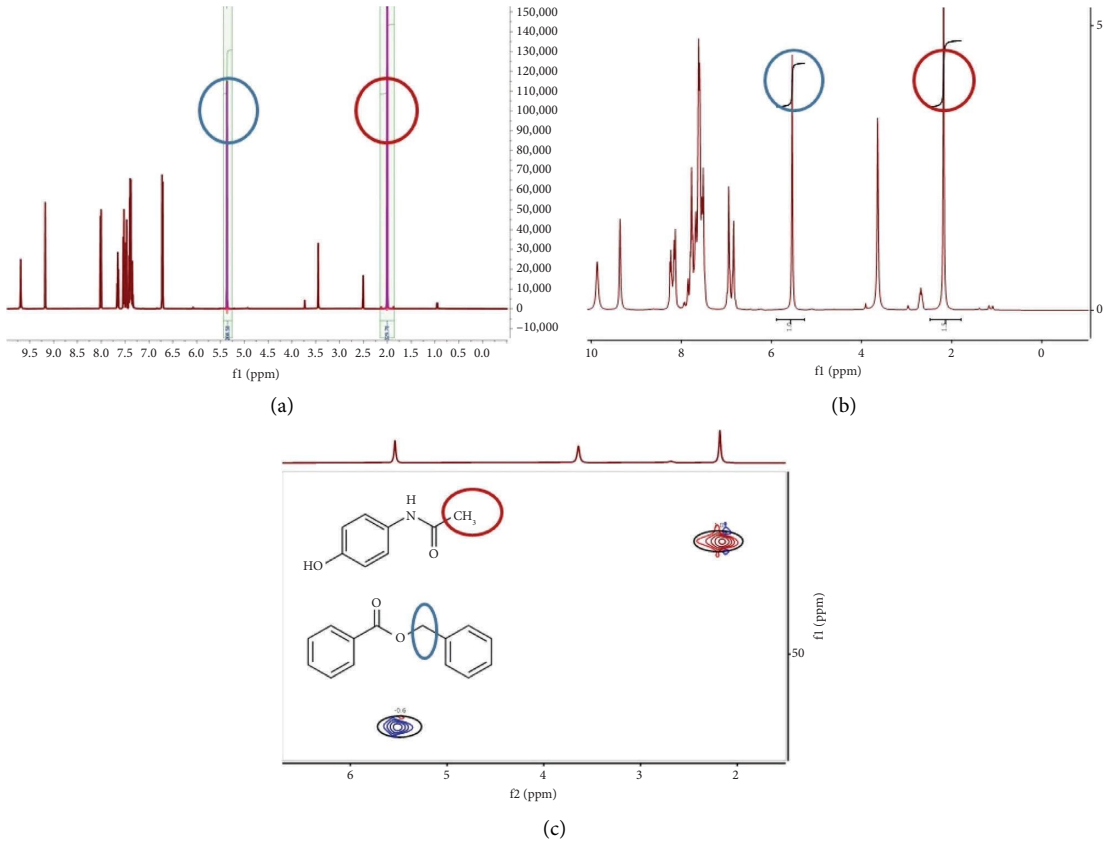

FIGURE 3: ^{1}H NMR spectra at 500 MHz (a) and 80 MHz (b) as well as HSQC-ME spectrum at 80 MHz (c) of Sample S4 with BB as internal standard.

^{1}H NMR spectrum at 80 MHz and 500 MHz, 2D cross-peaks of paracetamol and BB were separated from matrix compounds.

The results of IS quantification with a response factor and external calibration were referenced to the results obtained from HF qNMR measurements (Table 2). External calibration curves for caffeine in D_2O, ibuprofen in $CDCl_3$, and paracetamol in DMSO-d_6 are shown in Figure 1. Calibration curves were linear in the investigated concentration ranges ($R^2 > 0.99$). This also shows that there was no variation in T1 over the working range. HSQC integration ranges for both quantification approaches, and multiplicities of integrated peaks are summarized in Table 3. These quantification cases included different solvents, multiplicities, functional groups, and SNRs between 22 (caffeine and taurine in S11) and 228 (paracetamol in S2) to evaluate the applicability of the standard HSQC-ME experiment in quantification settings. It can be stated that both approaches provided reliable results of the quantitative analysis with the average absolute value of bias of 5.0% and 7.7% for IS with a response factor and EC methodology, respectively. The maximum bias based on IS and EC were 12.0% and −13.2% for S6 and S2, respectively, which is appropriate for 2D LF NMR measurements. Keeping in mind the increased workload from preparing and measuring an external calibration for each analyte, the application of IS calibration for HSQC-ME spectroscopy, where only one reference mixture was sued, is more sustainable.

Except medicines, more complicated sample (dietary Supplement S11) was investigated. Contrary to medicines, where targeted analyte signals were already well-resolved in the 1D NMR spectrum, taurine signals, and the CH_3, signals of caffeine overlapped with each other and with matrix components (Figure 4). Conventional ^{1}H NMR spectroscopy at benchtop devices therefore did not allow simultaneous quantification of taurine and caffeine, a rather common combination in dietary supplements. The HSQC-ME spectrum showed clear separation of the analytes' signals, and the quantitative results obtained supported the suitability of the HSQC method for this complex application (Figure 5, Table 2).

3.3. Validation of HSQC-ME Method for Pharmaceuticals. Detailed method validation was performed for the investigated AIs. The LOD for ibuprofen, paracetamol in DMSO-d_6, and caffeine in D_2O have been determined to be 3.3 mg/mL, 0.8 mg/mL, and 0.6 mg/mL, respectively. The signals used for determination of analytical limits are listed in Table 3. The uncertainties for sample preparation ($n = 5$) varied between 2.5% and 4.5% for taurine and ascorbic acid, respectively. Measurement precision (intraday, $n = 5$) varied between 1.3% and 4.1%.

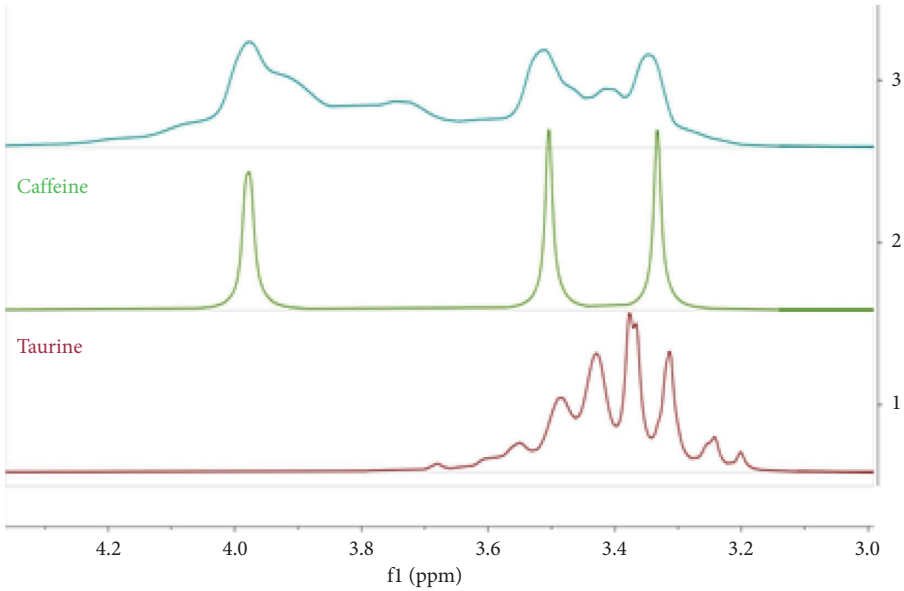

FIGURE 4: ^{1}H NMR spectra at 80 MHz (excerpts) in D_2O of Sample S11 as well as standard of caffeine and taurine.

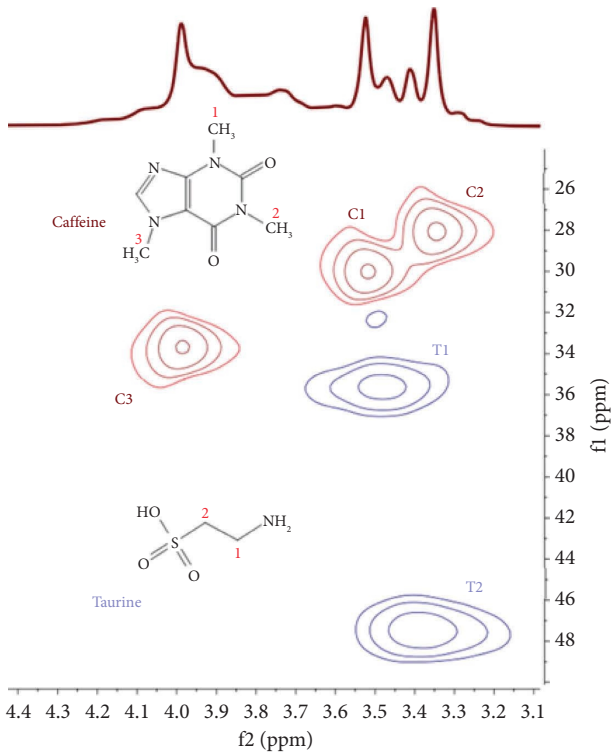

FIGURE 5: HSQC-ME spectrum (excerpt) of Sample S11 showing the separation of taurine and caffeine signals.

Integration precision was evaluated for Sample S11 by integrating 12 times across three different operators. For the two analytes (caffeine at [4.15; 33.4] ppm and taurine at [3.55; 47.3] ppm), there was no significant deviation ($p = 0.95$) among operators and the variation coefficient for the integration being below 3%.

For the evaluation of the method's robustness, the NS and the number of t1 increments varied between 32 and 128 for Samples S5, S6, and S11. It was found that the increase of NS and the number of t1 increments to 128 had no significant impact on the accuracy compared to the standard parameters. The deviation when decreasing the parameters to 32 scans and t1 increments was noticeably higher. The effect observed for a reduced NS could be traced back to the lower SNR. A lower number of t1 increments also had a negative effect on the quantification, possibly tracing back to the lower resolution in f1. For the optimal accuracy of the method with the lowest measurement time, NS and the

Table 4: Quantification results for organic acid in synthetic mixtures in D_2O.

Mixture	Substance 1			Substance 2			Substance 3			IS (mg)
	Acid	Amount (mg)		Acid	Amount (mg)		Acid	Amount (mg)		
1a	Malic	20.0		Citric	30.0		—			30.0
2a	Malic	30.0		Levulinic	22.8		—			32.5
3a	Malic	25.3		Levulinic	34.2		—			36.4
4a	Butyric	19.2		Propionic	29.7		Citric	19.9		29.7
5a	Butyric	28.8		Valeric	18.6		—			30.0
6a	Butyric	24.0		Valeric	27.9		Propionic	19.8		30.2
7a	Succinic	20.4		Levulinic	34.2		—			30.9
		Actual amount (mg)	Recovery (%)		Actual amount (mg)	Recovery (%)		Actual amount (mg)	Recovery (%)	
1b	Malic	29.5	78	Citric	20.9	76	—			30.6
2b	Malic	20.5	112	Levulinic	22.8	91	—			30.0
3b	Malic	30.5	94	Levulinic	22.8	111	—			29.8
4b	Butyric	28.8	110	Propionic	19.8	112	Citric	24.6	91	30.3
5b	Butyric	19.2	111	Valeric	27.9	96	—			30.0
6b	Butyric	28.8	113	Valeric	18.6	106	Propionic	24.8	96	30.2
7b	Succinic	20.3	109	Levulinic	45.6	90	—			32.5

Note: Mixtures 1a–7a were used for calibration for Mixtures 1b–7b, respectively. Information about integrated signals is listed in Table 3. Maleic acid (MA) was used as an internal standard (IS).

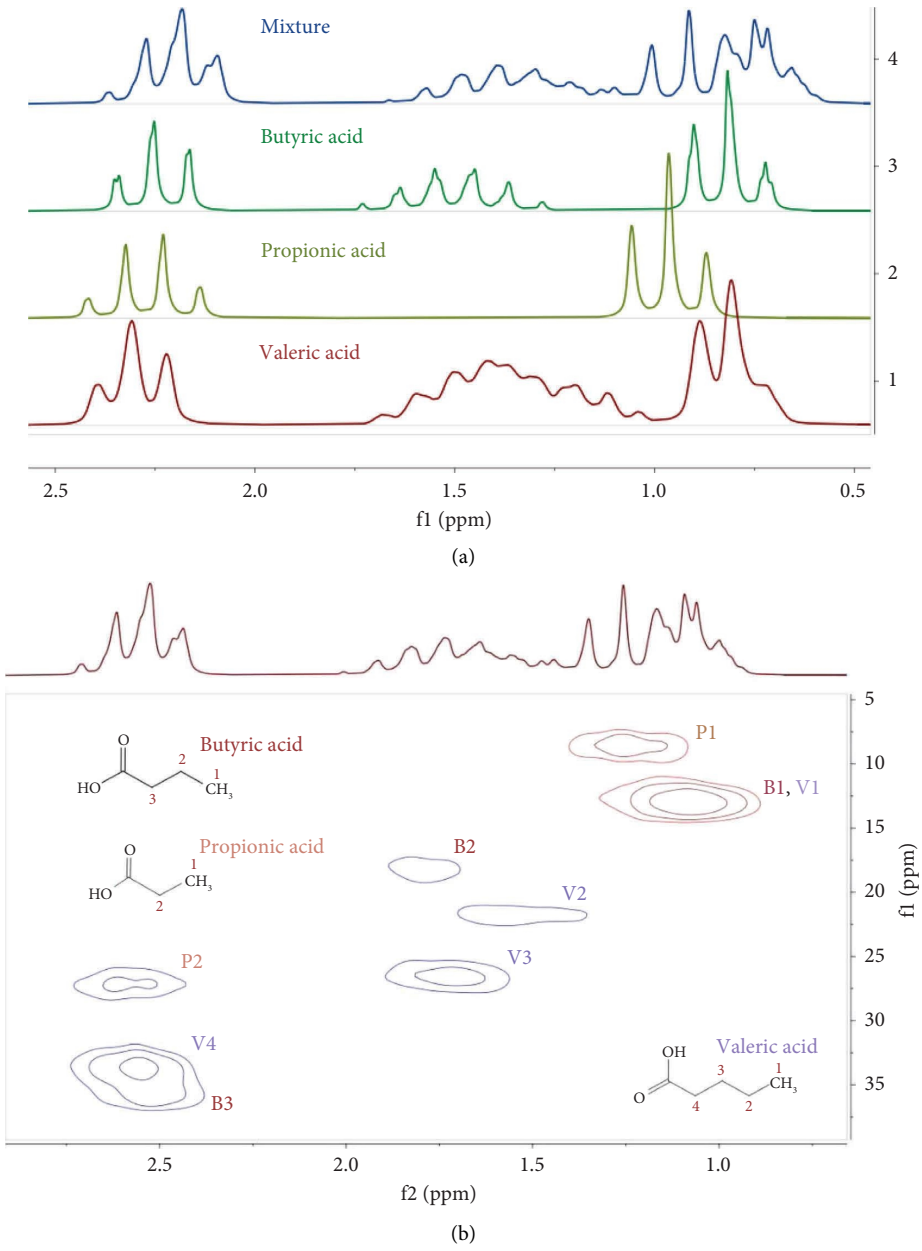

Figure 6: (a) ^{1}H NMR spectra at 80 MHz of butyric, propionic, and valeric acids in D_2O compared with their mixture. For the mixture composition, please refer to Table 4, Mixture 6a. (b) HSQC-ME spectrum of this mixture with signal assignments.

number of t1 increments should be set to 64. In general, the HSQC-ME method at 80 MHz has proved to be fit-for-purpose to determine the concentration of analytes with SNR > 20 with the accuracy below 10%.

It should be mentioned that the standard HSQC-ME pulse sequence on benchtop NMR devices provides acceptable results in case of small molecules (such as pharmaceuticals investigated in this study) that have similar JCH, JHH, and T2 values, leading to comparable peak response factors.

3.4. Simultaneous Determination of Organic Acids. Another group of compounds that showed severe spectral overlap in ^{1}H NMR due to their structural similarity is organic acids. Since they tend to simultaneously appear in different products such as food and beverages, their determination on low-field NMR can be a valuable application of this technique. Internal standardization with a response factor was used for quantification, as this method showed appropriate results for pharmaceutical samples.

Table 5: Quantification results for organic acid in synthetic mixtures in PG/VG/H_2O (25/25/50 v/v%) and e-cigarettes.

Mixture	Substance 1			Substance 2			IS (mg)
	Acid	Amount (mg)		Acid	Amount (mg)		
1a	Propionic	20.0		Butyric	20.0		20.0
2a	Malic	14.3		Citric	14.7		21.0
		Actual amount (mg)	Recovery (%)		Actual amount (mg)	Recovery (%)	
1b.1	Propionic	15.0	114	Butyric	25.0	94	20.6
1b.2	Propionic	25.0	89	Butyric	15.0	95	20.9
2b.1	Malic	11.2	120	Citric	10.1	81	21.2
2b.2	Malic	12.7	112	Citric	8.2	85	20.6
C1.1	Propionic	18.7	88	Butyric	18.3	114	26.8
C1.2	Malic	10.9	130	Citric	10.3	71	19.7

Note: Mixtures 1a and 2a were used for calibration for Mixtures 1b–2b. Samples C1.1 and C1.2 refer to the spiked authentic e-cigarette sample. Maleic acid (MA) was used as internal standard (IS).

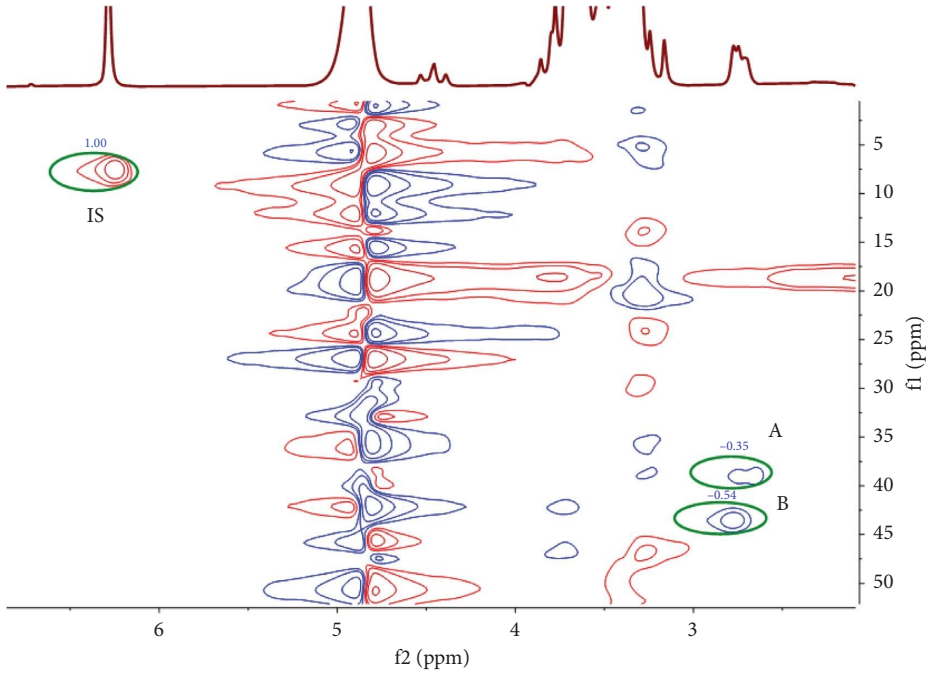

Figure 7: HSQC-ME NMR spectrum of the e-cigarette sample spiked with 10.9 mg malic and 10.3 mg citric acid. Signals A and B refer to CH_2 groups of malic and citric acids, respectively. Maleic acid was used as an internal standard (IS).

First, different binary and ternary mixtures were prepared in D_2O and analyzed using HSQC-ME spectroscopy (Tables 3, 4). For example, Figure 6 shows the benefit of using HSQC spectroscopy in case of the signal overlap in ^1H NMR spectra for ternary mixtures of butyric, propionic, and valeric acids. The recoveries for synthetic mixtures varied between 91% for citric acid in Mixture 4b and 113% for butyric acid in Mixture 6b. The accuracy of organic acid quantification in synthetic mixtures is compared with that for pharmaceuticals and dietary supplements (Tables 2, 4).

Organic acids are currently frequently added to e-cigarettes to increase flavor and sensory effects associated with the domination of monoprotonated nicotine form of these products [23]. Previously, the possibility of ^1H NMR at 80 MHz for identification and semiquantitative analysis of organic acids added to e-cigarettes was investigated by the authors in [24]. However, to obtain precise quantitative information liquid–liquid extraction was necessary due to intensive signals of PG and VG, which obscure the signals of minor compounds. An additional analytical challenge for HSQC-ME measurements was high viscosity of neat e-cigarettes.

The quantitative results based on internal standardization with a response factor for the synthetic mixtures are summarized in Table 5. The recoveries ranged from 81% for citric acid to 120% for malic acid. Figure 7 shows the ^1H and HSQC-ME NMR spectra of an e-cigarette sample spiked with malic and citric acids. Spiked concentrations were selected based on common literature values and, therefore, correspond to real conditions [24]. The identification was unambiguously performed using the HSQC-ME experiment at [2.65 ppm, 39.01 ppm] and [2.78 ppm, 43.65 ppm] for malic and citric acids, respectively. The observed recoveries were higher than for synthetic mixtures and ranged between

71% and 130% for malic and citric acids, respectively (Table 5). The reasons for this were huge solvent signals, viscous samples, and small SNR values (nine and 18 for malic and citric acids, respectively). The results suggested that even in this complicated case, quantitative HSQC-ME at 80 MHz is applicable to a semiquantitative analysis of absolute concentration of minor compounds in complex mixtures. The implementation of the solvent suppression block (presaturation, WET, and Watergate) in the standard HSQC-ME experiment on benchtop devices could decrease high dynamic range for the receiver and increase the accuracy of quantification in nondeuterated solvents.

4. Conclusions

This study proved the applicability of HSQC-ME spectroscopy for quantitative purposes on benchtop devices. The recording of spectra in two dimensions enabled substances with high overlap in ^{1}H NMR to be quantitatively analyzed. This approach was especially useful for complicated matrices or those containing multiple substances with a similar structure. This approach provided satisfactory results relatively small molecules with similar JCH, JHH, and T2 values.

Previously, only Steimers et al. applied a partial least squares (PLS) regression for the quantification of HSQC data on benchtop devices, which require substantial amounts of data for model training. In this study, the technique was used to monitor the multistep reaction of glycerol and acetic acid [25]. Our study is the first where acquisition parameters for HSQC-ME were systematically optimized to achieve the best accuracy/measurement time ratio. Additionally, our method was able to quantify pharmaceuticals, stimulants, and organic acids with satisfying accuracy, demonstrated on a representative set of samples.

More research is needed to resolve overlapping 2D HSQC signals on LF and HF NMR devices in the future. To increase the accuracy of LF QHSQC quantification, it is necessary to introduce advanced HSQC pulse sequences, such as QHSQC and HSQC0 and solvent suppression blocks (presaturation, WET, and Watergate) in the HSQC experiment on benchtop devices.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Conflicts of Interest

The authors declare no conflicts of interest.

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