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Comprehensive determination of flavouring additives and nicotine in e-cigarette refill solutions. Part II: Gas-chromatography–mass spectrometry analysis

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a b s t r a c t

Flavouring compounds are an essential part of e-liquid products for cigarettes. In general, they are regarded as safe for ingestion, but they may have unrecognized risks when they are inhaled. In some cases, manufactures do not currently abide by the Tobacco Products Directive (2014/40/EU) and do not declare the detailed contents of e-liquids on their labels. To help evaluate the health impact of flavouring substances, there is a need for comprehensive approaches to determine their concentrations in e-liquids. For this purpose, a GC-EI-MS method was developed and validated for the simultaneous determina-tion of 46 commonly used flavour additives in e-liquids. The proposed method performed well in terms of the key validation parameters: accuracy (84–113%), inter-/intra-day precision: 0.1–10% and 1–11%, respectively, and sensitivity (limit of detection: 3–87 ng/mL). The sample preparation step was based on a simple "dilute & shoot" approach. This study is a complementary method to the LC–MS/MS procedure described in Part I. Both approaches are suitable for the comprehensive determination of 88 flavouring compounds and nicotine and can be used as tools for the rapid evaluation of the quality and safety of e-cigarette products.

Keywords: Electronic cigarettes, Gas chromatography, Mass spectrometry, Flavouring compounds

1. Introduction

The use and sale of electronic cigarettes have increased extremely quickly worldwide. Business Intelligence and Strategy Research expects that the global e-cigarette industry will grow to $50 billion by 2025 at an estimated CAGR (compound annual growth rate) of 22.36% from 2015 to 2025 [1]. Despite the increasing popularity and availability of e-cigarettes, there is controversy surrounding the unknown, long-term health effects and inadequate data about the compounds in e-liquids and their emission ratios. Little attention has been paid to the presence of flavouring compounds in e-liquids. Most of the compounds are generally recognized as safe when ingested, but their influence on human health through inhalation into the respiratory tract is unknown [2–4].

Some studies on the determination of the hazardous compounds in e-liquids have been published. Most of them used hyphenated techniques, such as gas chromatography (GC) and liquid chromatography (LC) coupled with mass spectrometry (MS) [5–8]. Based on the literature research, headspace (HS) and thermal desorption (TD) techniques coupled with GC–MS have been widely applied to the analysis of volatile organic compounds (VOCs) [9,10], carbonyls [11] and polycyclic aromatic hydrocarbons (PAHs) [12] or glycols [13,14]. In addition, HPLC is a plausible technique with tandem mass spectrometry (MS/MS) or diode array detection (DAD) for the analysis of tobacco specific nitrosamines (TSNAs) [15,16], nicotine and related impurities [7,17], carbonyls [6] and simple saccharides [18]. According to the literature data and to the best of the authors' knowledge, there is limited information on the determination of flavour compounds in e-liquids. *Farsalinos* et al. presented a GC–MS procedure for the determination of diacetyl and acetyl propionyl in 159 e-liquids [19], while in another study, a LC–MS method was developed for the determination of the 5 main flavour ingredients: ethyl maltol, ethyl vanillin, 2,5-dimethylpyrazine, methyl cyclopentenolone and 3,4-dimethoxybenzaldehyde [13]. However, these studies are limited to a relatively short target flavour chemicals list. *Hutzler* et al. published a GC–MS method for the analysis of 141 flavour chemicals in 28 e-liquids, but the method tended to be only qualitative in nature. In another study, 90 flavouring compounds in e-liquids were determined with the aid of a GC–MS technique [20], but there is a lack

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of information about the method validation. This may cause problems in reproducibility and raises uncertainties about whether the method fulfils the acceptance criteria for analytical method validation. There is not a "one size fits all" approach for the determination of different compounds in e-liquids and flavouring compounds.

Taking into account all the issues described, the present study focused on developing and validating a GC–MS with electron-impact (EI) method to determine 46 popular volatile flavour compounds in e-liquids. The proposed method is supplementary to the LC–MS/MS approach described in Part I. Both procedures are versatile and allow for comprehensive determination of 88 frequent flavouring compounds and nicotine in e-liquids in terms of quality and safety. The target analytes were chosen from compounds previously detected in other studies [13,20–22] and other commonly used flavouring additives for e-liquids. The sample preparation step involved only the dilution of the e-liquid with acetonitrile (ACN). The applicability of the method was verified by analysing 25 previously investigated e-liquid samples to fully characterize the e-liquid flavour composition.

2. Material and methods

2.1. Chemicals

All standards were supplied by Sigma Aldrich (St. Louis, USA): Δ-tetradecalactone, 1-amyl alcohol, 2-isopropyl-5-methyl-2-hexenal, 3,4-dihydrocoumarin, anisyl acetate, benzaldehyde, α,α-dimethylphenethyl butyrate, benzyl acetate, benzyl alcohol, citral *(*mixture of *cis and trans;* neral and geranial), *cis*-3-hexenyl acetate, *cis*-3-hexenyl-valerate, Hedione (mixture of *cis* and *trans*), citronellol, decanal, ethyl heptanoate, ethyl caproate, eugenol, furfural, furfuryl alcohol, geranyl propionate, hexyl acetate, hexyl hexanoate, isoamyl butyrate, isoamyl isovalerate, isopentyl acetate, leaf aldehyde, leaf alcohol (*cis*-3-hexen-1-ol), limonene, linalyl acetate, L-menthyl acetate, melonal, n-hexanol, phenethyl alcohol, phenethyl isovalerate, raspberry ketone, styralyl acetate, tetrahydrolinalool, theaspirane, *trans*-2-hexenol, α-terpineol, γ-nonanolactone, γ-butyrolactone, γ-decalactone, γ-dodecalactone, and δ-decalactone. Naphthalene-d_8 was used as the internal standard and was purchased from Isotec/Sigma-Aldrich (St. Louis, USA). Vegetable glycerine (VG) and propylene glycol (PG) were purchased from Anwit (Warsaw, Poland). HPLC grade acetonitrile (ACN) was obtained from Merck (Darmstadt, Germany).

2.2. Samples

Twenty-five e-liquid samples were evaluated for their contents of the compounds investigated in this study. The e-liquids were divided into the following groups by flavour: fruit, tobacco, menthol and other. The labels and bottle descriptions did not indicate the flavour compounds. Only nicotine and the main ingredients (PG, VG) were mentioned.

2.3. Standards and calibration solutions

Standard stock solutions of the analytes were prepared in ACN at a concentration of approximately 5 mg/mL. Naphthalene-d_8 was used as the internal standard (IS). The standard working mixture were prepared by diluting the individual stock solutions with ACN to obtain a concentration of 50 μg/mL for each analyte. A stock solution of the IS was prepared in the same solvent at a concentration equal to 100 μg/mL.

Matrix-matched calibration solutions were prepared in a 10-mL volumetric flask by adding approximately 100 mg of a laboratory-made e-liquid (65% propylene glycol, 30% glycerine, 5% water) spiked with appropriate amounts of the standards to obtain seven concentration levels. Subsequently, the IS was added to each calibration solution to maintain a concentration of 500 ng/mL, and the mixture was diluted with ACN. The calibration curve range was set for each compound by considering the linearity and LOD. All stock solutions and the working solution were stored in a refrigerator at +4 °C until GC–MS analysis.

2.4. Sample preparation and fortification procedures

The e-liquid sample (approximately 100 mg) was placed in a 10-mL volumetric flask. Subsequently, 50 μL of the IS solution (100 μg/mL) was added, the flask was filled to the mark with ACN, and the sample was injected into the GC–MS system. In cases when the concentrations of the compounds in the samples were outside of the calibration curve range, the samples were appropriately diluted and reanalysed.

To evaluate the key validation parameters, fortified samples were prepared using the laboratory-made e-liquid as the matrix. The laboratory-made e-liquid was spiked with an appropriate amount of the working standard at three concentration levels. The fortified samples were prepared in the same manner as the real samples.

2.5. GC–MS conditions

The analyses were performed using a gas chromatograph 7890A equipped with an autosampler 7683b, a split/splitless injector and a MS detector 5975C (Agilent Technologies). The analytes were separated on a capillary column ZB-5, 30 m x 0.25 mm i.d., 0.25 μm film thickness (Phenomenex), with helium (purity ≥ 99.999%) as a carrier gas in a constant flow mode (1.0 mL/min). The splitless injection mode was applied for 1 min to achieve low LOD values. The oven temperature was programmed as follows: 4 min at 50 °C, then 10 °C/min up to 130 °C, and finally a 25 °C/min ramp to 300 °C. The post-run conditioning was set at 300 °C for 5 min (to avoid carry-over effects), and 3 min was applied for equilibration. The temperatures of the injector, interface, ion source, and quadrupole were set at 250, 285, 230 and 150 °C, respectively. The MS was operated in a positive electron-impact mode (electron energy 70 eV). Quantification was performed in a selected ion monitoring mode (SIM). Two ions (one quantifier, one qualifier) were chosen based on their selectivity and abundance to obtain the best signal-to-noise ratio in the matrix extracts. For identification, a deviation of ± 0.1 min of the expected retention time was allowed, and a quantifier/qualifier ratio within 20% was required. The detection parameters for all the analytes are listed in Table 1. The bold ions were used for quantification purposes. All data were collected and processed using MSD ChemStation E.02.02.1431 version (Agilent Technologies).

2.6. Method validation

The GC–MS method was validated in terms of selectivity, linearity, LOD and LOQ values, matrix effects, accuracy, as well as intra- and inter-day precision according to the guidelines for analytical method validation [23–25].

Selectivity experiments were carried out to verify the presence of interfering substances in the retention times of the investigated analytes. For this purpose, six blank matrix samples were injected into the GC–MS system.

An assessment of the matrix effect (ME) was performed according to the strategy described in previous studies [26–29] and was arranged as follow: seven calibration solutions (n = 3) were prepared in the solvent and in the homemade e-liquid as the matrix. The matrix effect [%] was evaluated by comparing the slopes of the calibration curves for each compound and was calculated as:

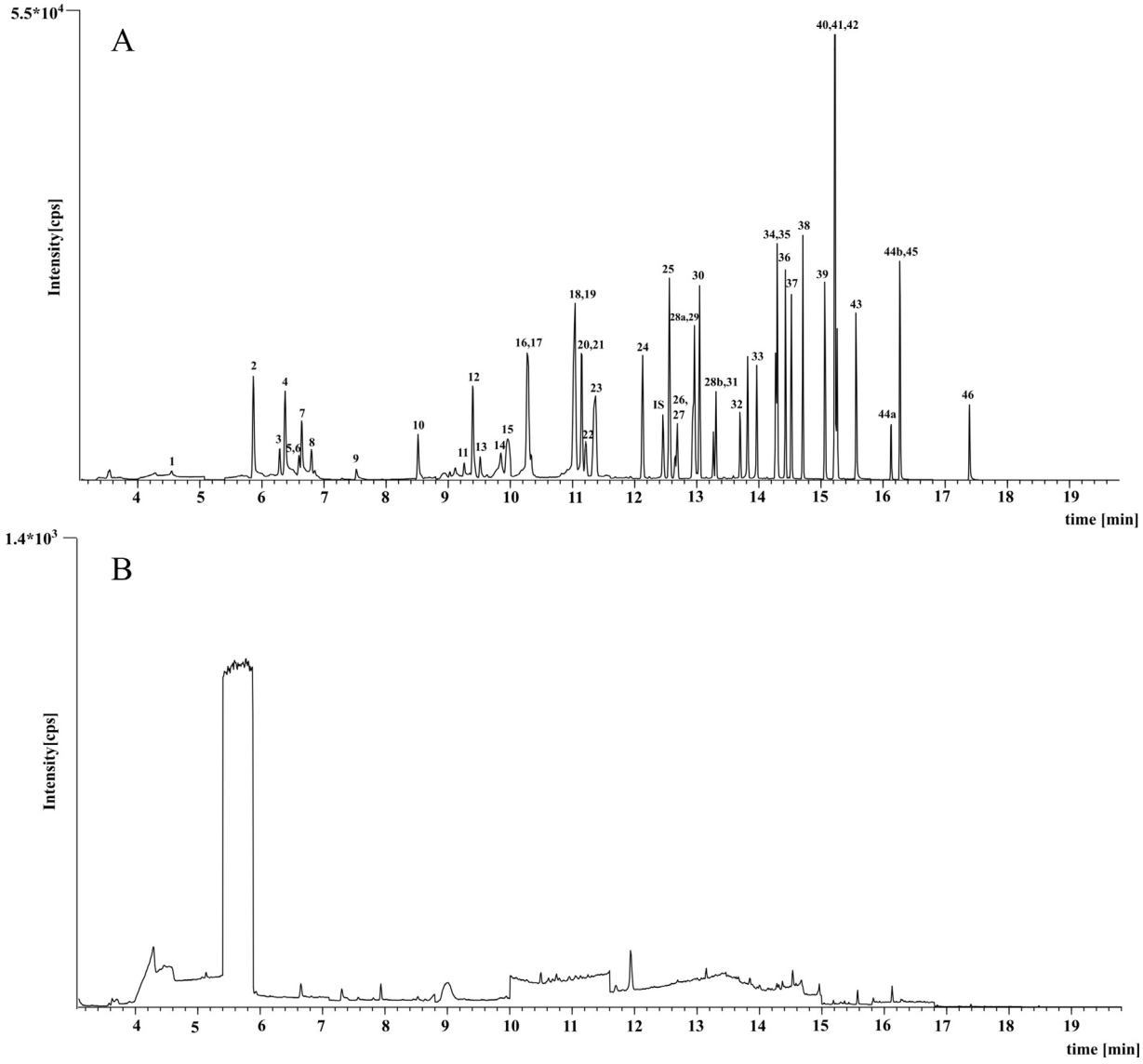

Fig. 1. Total ion chromatograms (TIC) from the GC–MS-SIM analysis (the peak numbers correspond to the numbers of compounds given in Table 1 and Table 2): A) chromatogram of mixture of all analytes and IS in matrix-matched calibration solution at a concentration of 500 ng/ mL; B) chromatogram of blank reference e-liquid sample.

*ME[%] = (am/as – 1)*100%*, where a_m is the slope of the calibration curve in the matrix, and a_s is the slope of the calibration in the solvent.

To evaluate the linearity, seven matrix-matched calibration solutions were prepared in triplicate using the laboratory-made e-liquid as the matrix. The calibration curves were constructed by plotting the ratio of the peak areas of the analytes to the peak area of the IS against the concentration in the concentration range specific to each compound. A weighted linear regression was used to improve the accuracy. Naphtalene-d_8 was used as the IS due to its indubitable absence in e-liquid samples.

The LOD and LOQ values were estimated using the regression parameters of the calibration curves and were calculated according to the following dependence: $LOD = 3.3 * S_b/a$ where S_b is the standard deviation of the intercept of the calibration curve, and a is the slope of the calibration curve, Additionally, $LOQ = 3*LOD$.

The accuracy of the method was determined by spiking the laboratory-made e-liquid with an appropriate amount of analyte at three different concentration levels, 5, 25 and 200 μg/mL, which correspond to 50, 250 and 2000 ng/mL in the samples with a 100 x dilution factor. In the case of five compounds, leaf aldehyde, *cis*-3-hexen-1-ol, *trans*-hexen-2-ol, 1-amyl alcohol and limonene, different spiking levels were applied and were equal to 10, 50 and 200 μg/mL. Each analysis was performed three times. Accuracy was expressed as the percent (%) difference between the measured concentration and the nominal concentration. The intra-day precision of the developed method was evaluated via a replicate (n = 3) analysis of samples spiked at three concentration levels during one day. The inter-day precision was determined by analysing six replicates (n = 6) of a single fortified solution at a medium concentration level (250 or 500 ng/mL) on three consecutive days. The precision was expressed in terms of the coefficients of variation (CVs).

3. Results and discussion

3.1. Separation and detection conditions

To achieve a high sensitivity and selectivity, optimisation of the GC–MS parameters was performed. The temperature of the injection port, gradient temperature programme, splitless injection time, carrier gas flow rate and source temperature were extensively tested. The GC–MS was first operated in a full scan mode

Table 1
Monitored ions for compounds investigated in this study.

Lp.	Compound name	CAS number	SIM ions, *m*/*z* (1 quantitative (bold), 1 qualitative)
1	1-amylalcohol	71-41-0	**70**
2	Furfural	98−01-1	**96**,95
3	Leaf aldehyde	6728-26-3	**83**,69
4	Furfuryl alcohol	98-00-0	**81**,69
5	Leaf alcohol (*cis*-3-hexen-1-ol)	928-96-1	**67,**82
6	trans-2-hexenol	928-95-0	**82**,67
7	N-hexanol	111-27-3	**56**,69
8	Isopentyl acetate	123-92-2	**70**,55
9	γ-butyrolactone	96-48-0	**86**,42
10	Benzaldehyde	100-52-7	**106**,105
11	Ethyl caproate	123-66-0	**70**
12	Cis-3-hexenylacetate	1708-82-3	**67**,82
13	Hexyl acetate	142-92-7	**69**,84
14	Limonene	138-86-3	**93**,68
15	Benzyl alcohol	100-51-6	**79**,108
16	Isoamyl butyrate	106-27-4	**70**,89
17	Melonal	106-72-9	**82**,67
18	Ethyl heptanoate	106-30-9	**113**,88
19	Geranyl propionate	105-90-8	**67**,82
20	Tetrahydrolinalol	78-69-3	**69**,129
21	Isoamylisovalerate	659-70-1	**70**,85
22	2-isopropyl-5-methyl-2-hexenal	35158-25-9	**97**,139
23	Phenethylalcohol	78-69-3	**91**,122
24	Benzyl acetate	140-11-4	**108**,150
25	Styralyl acetate	93-92-5	**104**,164
26	α-terpineol	98-55-5	**121,**136
27	Decanal	112-31-2	**112**,82
28ab	a) *cis* - Citral (neral)	106-26-3	**69,**134
	b) *trans* - Citral (geranial)	141-27-5	**69**,134
29	Citronellol	106-22-9	**69**,123
30	cis-3-hexenyli-valerate	35852-46-1	**82**,67
31	Linalyl acetate	115-95-7	**93**,121
32	L-menthyl acetate	79-20-9	**138**,82
33	Theaspirane	36431-72-8	**138**,82
34	Eugenol	97-53-0	**164**,149
35	γ-nonanolactone	104-61-0	**85**
36	Hexyl hexanoate	6378-65-0	**117**,99
37	3,4-dihydrocoumarin	119-84-6	**148,**120
38	Anisyl acetate	104-21-2	**121**,180
39	γ-decalactone	706-14-9	**85**,128
40	α,α-dimethylphenethyl butyrate	10094-34-5	**71**,132
41	Phenethylisovalerate	140-26-1	**104**,105
42	Δ-decalactone	705-86-2	**99**,71
43	Raspberry ketone	5471-51-2	**107**,164
44ab	a) *trans*-Hedione	24851-98-7	**83**,156
	b) *cis*-Hedione	80450-69-7	**83,**156
45	γ-dodecalactone	2305-05-7	**85**
46	Δ-tetradecalactone	2721-22-4	**99**,71
IS	Naphthalene-d_8	1146-65-2	**136,**108

(30–250 *m*/*z*) to find appropriate retention times for the analytes and to select ions for the SIM mode. For this purpose, a 2 μL aliquot of a mixture of the investigated analytes and the IS (50 μg/mL) in acetonitrile was injected into the GC–MS system. Finally, the two most abundant ions were selected for quantification. In the case of a few compounds, γ-dodecalactone, γ-nonanolactone, ethyl-caproate and 1-amyl alcohol, only one ion was monitored due to extensive fragmentation of these compounds with the EI technique. Therefore, it was not possible to select additional high abundance ions. The investigated analytes are low-molecular weight compounds (<200 Da), and the main challenge during optimisation was to choose high abundance and selective ions. Meanwhile, the exclusion of ions coming from propylene glycol and glycerine was necessary. Based on an analysis performed in the SCAN mode, propylene glycol and glycerine produced broad, overloaded peaks (45, 43 and 61 *m*/*z*) between 4 and 6 min and 10 and 13 min. To avoid these drawbacks, the undesired matrix ions were excluded from the MS detection.

Two pairs of compounds, geranial and neral as well as *cis*- and *trans*-Hedione, are geometric isomers. In these two cases, the quantification results were expressed as the sum of both isomers. For some analytes, i.e., ethyl heptanoate and geranyl propionate, coelution was observed. However, the selective SIM ions were monitored, and complete chromatographic separation was not required. The chromatograms of the mixture of analytes in the matrix-matched calibration solution and the blank reference e-liquid sample are presented in Fig. 1A.

3.2. Assay validation

3.2.1. Evaluation of matrix effect

The matrix effects were assessed to avoid under- and overestimation of the quantification results. The comparison of the slopes of the calibration curves in the solvent and in the matrix provided information about possible matrix effects. The evaluation of the matrix effects for the investigated compounds is presented in Table 2.

Negative values for the matrix effect signify suppression of the signal, and positive values indicate enhancement. Matrix effects were considered soft and insignificant at values of −20%<ME < 20%,

Table 2
Quantification parameters for matrix-matched calibration curves and evaluation of matrix effects.

Lp.	Compound name	Concentration range [ng/mL]	Calibration curve equations (7 points, n = 3)	Weight	S_a	S_b	r	LOD [ng/mL]	LOQ [ng/mL]	ME [%]
1	1-amylalcohol	100–2000	y = 0.000044x + 0.0014	$1/x^2$	0.000004	0.0009	0.9960	68	204	−29
2	Furfural	25–2000	y = 0.000410x + 0.0003	1/x	0.000008	0.0018	0.9950	14	43	−17
3	Leaf aldehyde	100–2000	y = 0.000051x + 0.001	1/x	0.000003	0.001	0.9980	87	261	−7
4	Furfuryl alcohol	25–2000	y = 0.000120x − 0.0003	$1/x^2$	0.000003	0.0002	0.9936	5	15	17
5	Leaf alcohol (*cis*-3-hexen-1-ol)	100–5000	y = 0.000294x − 0.003	1/x	0.000007	0.005	0.9958	54	163	−10
6	*trans*-2-hexenol	100–5000	y = 0.000059x − 0.00002	$1/x^2$	0.000002	0.00054	0.9978	30	91	−7
7	N-hexanol	50–5000	y = 0.000206x + 0.0087	$1/x^2$	0.000008	0.0009	0.9910	15	45	−16
8	Isopentyl acetate	50–5000	y = 0.000083x − 0.0011	1/x	0.000001	0.0006	0.9960	22	66	5
9	γ-butyrolactone	25–2000	y = 0.00006815x + 0.0021	1/x	0.00000009	0.0002	0.9979	10	30	2
10	Benzaldehyde	25–5000	y = 0.00014x + 0.0027	$1/x^2$	0.00001	0.0008	0.9939	19	57	−0.2
11	Ethylcaproate	50–5000	y = 0.000092x + 0.0008	1/x	0.000001	0.0005	0.9963	19	57	−11
12	*cis*-3-hexenylacetate	25–2000	y = 0.000566x − 0.0007	1/x	0.000003	0.0008	0.9982	4	13	2.0
13	Hexyl acetate	50–5000	y = 0.000111x − 0.0006	1/x	0.000002	0.0008	0.9925	25	75	3
14	Limonene	100–10000	y = 0.000133x − 0.002	1/x	0.000002	0.002	0.9968	56	168	−18
15	Benzyl alcohol	25–2000	y = 0.00047x + 0.032	1/x	0.00001	0.002	0.9930	16	48	34
16	Isoamyl butyrate	50–5000	y = 0.000436x − 0.0005	1/x	0.000008	0.0040	0.9967	30	91	−9
17	Melonal	25–2000	y = 0.000521x − 0.002	1/x	0.000009	0.002	0.9974	13	40	12
18	Ethyl heptanoate	25–2000	y = 0.00056x + 0.0003	1/x	0.00001	0.0022	0.9969	13	38	5
19	Geranyl propionate	50–10000	y = 0.000694x + 0.016	1/x	0.000006	0.004	0.9988	17	50	15
20	Tetrahydrolinalol	25–2000	y = 0.000504x − 0.004	1/x	0.000008	0.002	0.9964	12	37	11
21	Isoamylisovalerate	25–2000	y = 0.00068x − 0.002	1/x	0.00001	0.002	0.9974	12	36	4
22	2-isopropyl-5-methyl-2-hexenal	50–10000	y = 0.0000612x + 0.0007	$1/x^2$	0.0000009	0.0006	0.9979	31	93	41
23	Phenethylalcohol	50–5000	y = 0.00094x + 0.019	1/x	0.00001	0.006	0.9985	20	61	12
24	Benzyl acetate	25–2000	y = 0.00077x − 0.0026	$1/x^2$	0.00002	0.0009	0.9964	4	11	20
25	Styralyl acetate	50–5000	y = 0.000499x + 0.003	1/x	0.000008	0.004	0.9974	27	80	15
26	α-terpineol	25–2000	y = 0.000292x + 0.0003	1/x	0.000007	0.0016	0.9959	18	53	17
27	Decanal	25–2000	y = 0.000065x − 0.0002	1/x	0.000001	0.0003	0.9961	13	38	18
28 ab	*trans*- and *cis*- citral (neral + geranial)	25–10000	y = 0.00114x − 0.0006	1/x	0.00002	0.0079	0.9974	23	69	61
29	Citronellol	25–10000	y = 0.000357x − 0.0005	1/x	0.000003	0.0013	0.9993	12	36	42
30	*cis*-3-hexenyli-valerate	25–2000	y = 0.000617x − 0.006	1/x	0.000005	0.001	0.9987	6	19	13
31	Linalyl acetate	25–5000	y = 0.000264x − 0.0006	$1/x^2$	0.000008	0.0005	0.9910	6	18	87
32	L-menthyl acetate	25–5000	y = 0.00030x + 0.007	1/x	0.00001	0.004	0.9957	25	74	13
33	Theaspirane	25–2000	y = 0.000537x + 0.0021	$1/x^2$	0.000007	0.0004	0.9974	3	8	0.2
34	Eugenol	50–2000	y = 0.000163x − 0.001	1/x	0.000003	0.001	0.9964	20	61	133
35	γ-nonanolactone	50–10000	y = 0.00118x + 0.001	$1/x^2$	0.00002	0.002	0.9945	5	16	13
36	Hexyl hexanoate	25–2000	y = 0.00058x − 0.0029	$1/x^2$	0.00001	0.0007	0.9961	4	12	11
37	3,4-dihydrocoumarin	50–2000	y = 0.00045x + 0.031	$1/x^2$	0.00002	0.002	0.9912	17	51	45
38	Anisyl acetate	50–5000	y = 0.00076x − 0.008	$1/x^2$	0.00002	0.002	0.9945	9	27	97
39	γ-decalactone	25–2000	y = 0.00115x + 0.006	1/x	0.00003	0.008	0.9987	22	67	71
40	α,α-dimethylphenethyl butyrate	25–5000	y = 0.00057x + 0.009	1/x	0.00001	0.004	0.9961	23	70	190
41	Phenethylisovalerate	25–2000	y = 0.00184x − 0.003	$1/x^2$	0.00003	0.002	0.9975	3	8	10
42	Δ-decalactone	25–2000	y = 0.00060x + 0.013	$1/x^2$	0.00005	0.003	0.9962	15	45	111
43	Raspberry ketone	50–2000	y = 0.00065x + 0.004	$1/x^2$	0.00005	0.005	0.9909	26	77	117
44 ab	*trans*- and *cis*-Hedione	25–2000	y = 0.000331x + 0.001	1/x	0.000007	0.002	0.9974	16	49	14
45	γ-dodecalactone	25–2000	y = 0.00100x − 0.011	1/x	0.00002	0.005	0.9950	16	48	17
46	Δ-tetradecalactone	25–2000	y = 0.000309x − 0.003	1/x	0.000005	0.001	0.9974	12	37	131

S_a – standard deviation of the slope, S_b – standard deviation of the intercept. *r* – correlation coefficient, *LOD* – limit of detection. *LOQ* – limit of quantitation, *n* – number of measurements, ME –matrix effect.

Table 3
Concentration of flavouring compounds in investigated e-liquid samples.Only detected compounds are listed in Table.

E-liquid sample	Compound detected	Concentration found [mg/mL ± SD (n = 3)]	Concentration found [%w/v ± SD (n = 3)]	Total determined flavour compounds (except nicotine) by GC–MS and LC–MS methods [mg/mL]
Raspberry	Citronellol	0.0060 ± 0.0003	0.00060 ± 0.00003	0.07
	Heksan-1-ol	0.0132 ± 0.0009	0.00132 ± 0.00009	
	cis-3-hexenylvalerate	0.00320 ± 0.00007	0.000320 ± 0.000007	
Devil_1	2-izopropyl-5-metyloheks-2-enal	0.026 ± 0.002	0.0026 ± 0.0002	0.62
	Furfural	0.0122 ± 0.0007	0.00122 ± 0.00007	
	γ-butyrolactone	0.041 ± 0.002	0.0041 ± 0.0002	
Devil_2**	2-isopropyl-5-methyl-2-hexenal	0.0205 ± 0.0003	0.00205 ± 0.00003	0.68
	Furfural	0.0160 ± 0.0005	0.00160 ± 0.00005	
	γ-butyrolactone	0.032 ± 0.002	0.0032 ± 0.0002	
Vanilla	Furfural	0.012 ± 0.006	0.0012 ± 0.0006	4.7
Strawberry	Hedione (*cis* and *trans*)	0.365 ± 0.003	0.0365 ± 0.0003	3.1
	Isoamyl butyrate	0.088 ± 0.009	0.0088 ± 0.0009	
	Ethyl caproate	0.202 ± 0.002	0.0202 ± 0.0002	
	Leaf alcohol (*cis*-3-hexen-1-ol)	1.35 ± 0.01	0.135 ± 0.001	
	γ -decalactone	0.3458 ± 0.0002	0.03458 ± 0.00002	
	Furfuryl alcohol	0.045 ± 0.006	0.0045 ± 0.0006	
	2-izopropyl-5-metyloheks-2-enal	0.021± 0.003	0.0021 ± 0.0003	
Apple	1-amylalcohol	0.07052 ± 0.0008	0.007052± 0.00008	19
	Leaf alcohol (*cis*-3-hexen-1-ol)	0.163 ± 0.005	0.0163 ± 0.0005	
	cis-3-hexenylacetate	0.149 ± 0.002	0.0149 ± 0.0002	
	Hexyl acetate	0.2727 ± 0.0002	0.02727 ± 0.00002	
	Isoamyl butyrate	0.17 ± 0.01	0.017 ± 0.001	
	Isoamyl isovalerate	0.0040 ± 0.0004	0.00040 ± 0.00004	
	Isopentyl acetate	0.1178 ± 0.0006	0.01178 ± 0.00006	
	Leaf aldehyde	0.066 ± 0.001	0.0066 ± 0.0001	
	n-Hexanol	1.52 ± 0.02	0.152 ± 0.002	
	2-izopropyl-5-metyloheks-2-enal	0.22 ± 0.03	0.022 ± 0.003	
	Citronellol	0.00428 ± 0.00007	0.000428 ± 0.000007	
Black currant	α,α-dimethylphenethyl butyrate	0.2573 ± 0.0005	0.02573 ± 0.00005	1.7
	Benzyl acetate	0.0394 ± 0.0006	0.00394 ± 0.00006	
	Furaneol	0.019 ± 0.001	0.0019 ± 0.0001	
	γ -nonalactone	0.365 ± 0.003	0.0365 ± 0.0003	
	Isoamyl butyrate	0.071 ± 0.004	0.0071 ± 0.0004	
	Leaf alcohol	0.28 ± 0.01	0.028 ± 0.001	
	Linalyl acetate	0.0794 ± 0.0002	0.00794 ± 0.00002	
	Raspberry ketone	0.0352 ± 0.0005	0.00352 ± 0.00005	
	α-terpineol	0.0169 ± 0.0002	0.00169 ± 0.00002	
	Tetrahydrolinalool	0.074 ± 0.002	0.0074 ± 0.0002	
	Isoamyl isovalerate	0.00584 ± 0.00009	0.000584 ± 0.000009	
	Limonene	0.0315 ± 0.0003	0.00315 ± 0.00003	
	Furfural	0.0075 ± 0.0007	0.00075 ± 0.00007	
	Geranyl propionate	0.0330 ± 0.0005	0.00330 ± 0.00005	
	2-isopropyl-5-methyl-2-hexenal	0.0173 ± 0.0004	0.0173 ± 0.0004	
	Anisyl acetate	0.00485 ± 0.00004	0.000485 ± 0.000004	
Camel	Decanal	0.15 ± 0.05	0.015 ± 0.005	4.0
	Anisyl acetate	0.0077 ± 0.0003	0.00077 ± 0.00003	
Elem_1	No compounds detected			0.19
Elem_2**	No compounds detected			3.1
Standard_1	Anisyl acetate	0.0397 ± 0.0002	0.00397 ± 0.00002	1.4
Standard_2**	Anisyl acetate	0.0420 ± 0.0002	0.00420 ± 0.00002	8.3
Banana	Benzyl acetate	0.045 ± 0.003	0.0045 ± 0.0003	3.9
	cis-3-hexenylvalerate	0.0097 ± 0.0003	0.00097 ± 0.00003	
	Citral (*cis* and *trans*)	0.0099 ± 0.0002	0.00099 ± 0.00002	
	Eugenol	0.026 ± 0.004	0.0026 ± 0.0004	
	Isoamyl butyrate	0.292 ± 0.008	0.0292 ± 0.0008	
	Isopentyl acetate	1.16 ± 0.06	0.116 ± 0.006	
	Ethyl caproate	0.238 ± 0.006	0.0238 ± 0.0006	
	Limonene	1.65 ± 0.04	0.165 ± 0.004	
	α - terpineol	0.0088 ± 0.0004	0.00088 ± 0.00004	
	Tetrahydrolinalool	0.0271 ± 0.0007	0.00271 ± 0.00007	
	Hexyl acetate	0.01410 ± 0.00006	0.001410 ± 0.000006	
	Geranium propionate	0.0161 ± 0.0005	0.00161 ± 0.00005	
	3,4-dihydrocoumarin	0.013 ± 0.002	0.0013 ± 0.0002	
Black tea	Theaspirane	0.0550 ± 0.0003	0.00550 ± 0.00003	5.2
	Geranium propionate	0.060 ± 0.004	0.0060 ± 0.0004	
	Benzaldehyde	1.22 ± 0.03	0.122 ± 0.003	
	Benzyl alcohol	0.037 ± 0.001	0.0037 ± 0.0001	
	Leaf alcohol	0.053 ± 0.004	0.0053 ± 0.0004	
	Hexyl hexanoate	0.07614 ± 0.00007	0.007614 ± 0.000007	
	n-Hexanol	0.034 ± 0.003	0.0034 ± 0.0003	

Table 3 (*Continued*)

E-liquid sample	Compound detected	Concentration found [mg/mL ± SD (n = 3)]	Concentration found [%w/v ± SD (n = 3)]	Total determined flavour compounds (except nicotine) by GC–MS and LC–MS methods [mg/mL]
	Citronellol	0.023 ± 0.002	0.00225 ± 0.0002	
	α - Terpineol	0.0281 ± 0.0002	0.00281 ± 0.00002	
	Tetrahydrolinalool	0.11787 ± 0.00009	0.011787 ± 0.000009	
	trans-2-Hexenol	0.0061 ± 0.0009	0.00061 ± 0.00009	
	cis-3-hexenylacetate	(0.00475)*	(0.000475)*	
	Limonene	0.14 ± 0.01	0.014 ± 0.001	
	2-izopropyl-5-metyloheks-2-enal	0.026 ± 0.002	0.0026 ± 0.0002	
	L-menthyl acetate	0.085 ± 0.004	0.0085 ± 0.0004	
	Isopentyl acetate	(0.006147)*	(0.0006147)*	
Ice mint	Limonene	0.12 ± 0.01	0.012 ± 0.001	11
	L-menthyl acetate	0.135 ± 0.003	0.0135 ± 0.0003	
	Leaf alcohol (*cis*-3-hexen-1-ol)	0.052 ± 0.005	0.0052 ± 0.0005	
	α,α-dimethylphenethyl butyrate	0.495 ± 0.006	0.0495 ± 0.0006	
	α - terpineol	(0.00860)*	(0.000860)*	
Cappucino	Decanal	0.043 ± 0.003	0.0043 ± 0.0003	20
	γ-butyrolactone	0.008 ± 0.001	0.0008 ± 0.0001	
Tobacco	Decanal	0.0110 ± 0.0008	0.00110 ± 0.00008	5.8
Strong mint	Leaf alcohol (*cis*-3-hexen-1-ol)	0.36 ± 0.01	0.036 ± 0.001	26
	L-menthyl acetate	6.4 ± 0.2	0.64 ± 0.02	
	Phenenthyl alcohol	0.430 ± 0.003	0.0430 ± 0.0003	
	α - terpineol	0.0221 ± 0.0001	0.00221 ± 0.00001	
	Decanal	0.0173 ± 0.0002	0.00173 ± 0.00002	
	cis-3-hexenylvalerate	(0.00712)*	(0.000490)*	
	Benzyl acetate	0.034 ± 0.003	0.0034 ± 0.0003	
	Limonene	1.0 ± 0.1	0.10 ± 0.01	
	Isopentyl acetate	0.023 ± 0.001	0.0023 ± 0.0001	
	Geranium propionate	0.0408 ± 0.0006	0.00408 ± 0.00006	
	Tetrahydrolinalool	0.0059 ± 0.0003	0.00059 ± 0.00003	
Menthol_1	L-menthylacetate	1.56 ± 0.01	0.156 ± 0.001	14
	Limonene	0.59 ± 0.03	0.059 ± 0.003	
	α-terpineol	0.0446 ± 0.0008	0.00446 ± 0.00008	
	Benzyl acetate	0.157 ± 0.006	0.0157 ± 0.0006	
	Benzyl alcohol	0.135 ± 0.006	0.0135 ± 0.0006	
	cis-3-hexenylvalerate	0.08 ± 0.02	0.008 ± 0.002	
Menthol_2**	L-menthylacetate	1.54 ± 0.03	0.154 ± 0.003	9.7
	Limonene	0.36 ± 0.02	0.036 ± 0.002	
	α - terpineol	0.0415 ± 0.0004	0.00415 ± 0.00004	
	cis-3-hexenylvalerate	0.055 ± 0.005	0.0055 ± 0.0005	
	Benzyl acetate	0.065 ± 0.004	0.0065 ± 0.0004	
	Benzyl alcohol	0.0428 ± 0.0002	0.00428 ± 0.00002	
Peach	Phenenthyl isovalerate	0.0105 ± 0.0001	0.00105 ± 0.00001	0.39
	γ -decalactone	0.0156 ± 0.0008	0.00156 ± 0.00008	
	Δ-decalactone	0.032 ± 0.001	0.0032 ± 0.0001	
	Tetrahydrolinalool	0.114 ± 0.008	0.0114 ± 0.0008	
	Isoamyl isovalerate	0.0110 ± 0.0007	0.00110 ± 0.00007	
	Limonene	0.023 ± 0.002	0.0023 ± 0.0002	
	α,α-dimethylphenethyl butyrate	0.0065 ± 0.0002	0.00065 ± 0.00002	
	γ - dodecalactone	0.0514 ± 0.0005	0.00514 ± 0.00005	
Watermelon	Melonal	0.084 ± 0.004	0.0084 ± 0.0004	0.89
	Leaf alcohol	0.32 ± 0.03	0.32 ± 0.03	
	cis-3-hexenylacetate	0.024 ± 0.001	0.0024 ± 0.0001	
	Citronellol	0.0164± 0.0008	0.00164 ± 0.00008	
	α-terpineol	0.03122 ± 0.0009	0.003122 ± 0.00009	
	Isopentyl acetate	0.0169 ± 0.0002	0.00169 ± 0.00002	
Cherry	Isopentyl acetate	0.617 ± 0.009	0.0617 ± 0.0009	2.1
	Benzaldehyde	0.89 ± 0.02	0.089 ± 0.002	
	Benzyl acetate	0.03133 ± 0.00009	0.003133 ± 0.000009	
	Anisyl acetate	0.06099 ± 0.00006	0.006099 ± 0.000006	
	Citronellol	0.0059 ± 0.0001	0.00059 ± 0.00001	
Lemon	Citral (*cis* and *trans*)	12.3 ± 0.7	1.23 ± 0.07	13
	Limonene	0.69 ± 0.09	0.069 ± 0.009	
	α - terpineol	0.0431 ± 0.0002	0.00431 ± 0.00002	
	cis-3-hexenylvalerate	0.02468 ± 0.00006	0.002468 ± 0.000006	
	γ-nonalactone	0.00640 ± 0.00003	0.000640 ± 0.000003	
Orange	Limonene	4.1 ± 0.3	0.41 ± 0.03	6.4
	Benzyl acetate	1.196 ± 0.002	0.1196 ± 0.0002	
	cis-3-hexenylacetate	0.543 ± 0.002	0.0543 ± 0.0002	
	α - terpineol	0.350 ± 0.006	0.0350 ± 0.0006	
	Tetrahydrolinalool	0.077 ± 0.002	0.0077 ± 0.0002	
	Ethyl caproate	0.089 ± 0.006	0.0089 ± 0.0006	

* value $C_{min} < x < LOQ$.
** samples with declared zero-level of nicotine.

medium for ME > ±20% and ME < ±50%, and high for the ranges of ME < −50% and ME > 50%. Most of the compounds (approximately 70%) exhibited negligible MEs lower than >±20%, 11% showed a medium ME with values ranging from −29% to 42%, and 9 compounds showed a high ME. The matrix effects for the majority of compounds included ion enhancement rather than suppression. This effect comes from blocking active sites (silanol groups and metal ions) in the GC system (column and injector), which means more particles can reach the detector [30]. Therefore, to overcome these drawbacks, a matrix-matched calibration, instead of an external calibration, was used for quantification purposes.

3.3. Selectivity, linearity, LOD and LOQ

No interfering peaks were observed in the SIM chromatograms (Fig. 1B) for the retention times of the investigated flavouring compounds; therefore, the method was selective for quantification.

Weighted least squares linear regressions with an appropriate factor ($1/x$ or $1/x^2$) were adopted for the calibration to improve the accuracy. All correlation coefficients were greater than 0.990 for the matrix-matched calibration curves within the concentration ranges. The summary of the obtained data are shown in Table 2.

The calculated LODs fulfilled the following criteria: $10 \times LOD > C_{min}$, and $LOD < C_{min}$ [31]. The values of the LODs of the analytes were in the range of 3–87 ng/mL, whereas the LOQ values ranged from 8 to 261 ng/mL. The obtained LOD and LOQ values suggest that the method is sensitive and suitable for the determination of trace amounts of flavouring compounds in e-liquids.

3.3.1. Accuracy and precision

The obtained results indicate that the developed method is accurate within 84.0–112.7%, regardless of the spiking level, and the acceptable range of 80–120% was fulfilled. The intra-day precision results were between 0.1 and 10%, and the inter-day precision ranged from 1 to 11%. Thus, the acceptance criteria (CV values are within ±20% at the lowest concentration levels and ±15% for the other levels) for the analytical method validation was met [23–25]. The intra- and inter-day accuracy and precision data are summarized in Table S1 (Supplementary Electronic Material in the online version at DOI: 10.1016/j.chroma.2017.08.057).

4. Analysis of real samples

To demonstrate the applicability of the developed method, the 25 e-liquids previously examined in Part I were examined to determine 46 compounds. This supplemented and extended the information about the composition of the investigated samples. The obtained results are listed in Table 3. The calculations were performed in the same manner as in Part I.

A relatively high percentage (56%) of the tested e-liquids contained at least five flavouring compounds at a total concentration up to 12.3 mg/mL. Of the analysed e-liquid samples, the lowest total flavour concentration determined by the developed GC–MS method was in the e-liquid "Raspberry" at 0.022 mg/mL, and the highest was in "Lemon" at 13.1 mg/mL (Fig. S1, Electronic Supplementary Material in the online version at: DOI 10.1016/j.chroma.2017.08.057). Limonene (44% of the samples; concentration range: 0.02–4.06 mg/mL) and benzyl acetate (28% of the samples; 0.03–1.12 mg/mL) were the two most frequent flavouring substances. The following substances: *cis*-3-hexenyl valerate, isopentyl acetate, tetrahydrolinalool and α-terpineol in the concentration ranges of 0.003–0.081 mg/mL, 0.02–1.16 mg/mL, 0.006–0.118 mg/mL, and 0.009–0.350 mg/mL, respectively, occurred with a high (24%) frequency as well.

Considering the results obtained from Part I and Part II, the total flavour chemicals levels did not exceed 26 mg/mL (2.6% w/v) in all the samples(Table 3).

The lowest total flavour concentrations were in the e-liquid "Raspberry" at 0.07 mg/mL, and the highest was in "Strong mint" at 26 mg/mL. Summarised results obtained with the use of LC–MS/MS and GC–MS methods revealed that the information regarding the content of flavour chemicals in e-liquids is incomplete (Table S2, Electronic Supplementary Material in the online version at: DOI 10.1016/j.chroma.2017.08.057). For zero-nicotine e-liquids (except "Menthol"), the total amount of the flavour compounds was higher than those with nicotine, probably to enhance the taste. The majority of the analytes are artificial and natural food fragrances and flavour substances. The most commonly detected substances were ethyl maltol (detection frequency 68%), limonene, ethyl maltol, vanillin and methyl cyclopentenolone (detection frequency of each: 46%). Moreover, methyl cyclopentenolone, ethyl acetoacetate, menthol, citral (neral and geranial), L-menthyl acetate and limonene occur at concentrations ranging from 4.1 mg/mL (for limonene, e-liquid "Orange") to 18.1 mg/mL (for methyl cyclopentenolone, e-liquid "Cappuccino"). Additionally, menthol, L-menthyl acetate and menthone were the main ingredients of mint flavoured samples. Pyrazine and pyridine-related compounds, 2,3,5-trimethylpyrazine, 2,3,5,6-tetramethylpyrazine and pyridine, which are responsible for the flavour and aroma of tobacco and tobacco smoke, were characteristic of tobacco-flavoured e-liquids.

5. Conclusions

In this paper, a method based on a GC–MS technique was developed and validated. The method used simple dilution with ACN for the simultaneous determination of 46 flavouring substances in e-liquids.

The methods presented in Part I and Part II can be treated as complementary methods, depending on the need, to determine the content of flavouring compounds in e-liquids. The use of the developed LC–MS/MS and GC–MS methods allow for comprehensive screening and quantification of 88 volatile and semi-volatile flavouring substances, including nicotine. Both methods are accurate, reproducible and combine specificity and selectivity, which are crucial in the implementation of a multicomponent analysis. Examining the presence of a few to dozens of flavouring compounds in e-liquid samples stresses the need for monitoring their levels in e-liquid samples for consumer intake and related hazards.

Currently, e-liquid manufacturers and distributors must abide by the Tobacco Directive 2014/40/EU and Classification, Labelling and Packaging Regulations (CLP). According to these requirements, refillable e-cigarettes and their refill containers should have a list of ingredients and be labelled to provide information about possible health warnings and risks to consumers. Some of the flavour chemicals used in e-liquids are classified as hazardous to health under the CLP Regulation (may cause allergic skin reaction, eye irritation, allergy or asthma symptoms or breathing difficulties if inhaled and may be harmful if swallowed). However, the majority of e-liquid manufacturers often do not comply with the currently enforced regulations [32], which was proven in the present study. Insufficient label information about the content of e-liquids may expose consumers to a health risk. The authors are aware that the number of e-liquids chosen for this study only represent a small part of the total amount of the commercially available products. Nonetheless, the presented results confirmed the wide range of flavouring compounds in e-liquids and provided insights into flavour constituents in the thousands of products being sold on the market.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.chroma.2017.08.057.

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