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Improvement of the quality of analytical results generated by GC/MS method for characterization of *Helichrysum italicum* (Roth) G. Don essential oil

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DATA AVAILABILITY

The data used to support the findings of this study are included within the article.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

ABSTRACT

Most of the papers that treat the composition of essential oils for this purpose use GCMS or GCMS and GC-FID techniques for the identification and/or quantification of individual compounds. Therewithal, papers usually treat the composition as semi-quantitative (percentage composition as the area of the peak of a particular compound in relation to the total area of the peaks on the chromatogram, given as 100%). At the same time, the quality of the results is not treated in metrological terms. A GCMS method for the quantification of components present *Helichrysum italicum* hydro-distilled essential oil samples from Bosnia and Herzegovina with a similar composition was developed and optimized. The stability of the method set-up and adequacy of sample storage and manipulation was assessed. Calibration method and assessment of measurement uncertainty was established for neryl acetate, one of the major compounds with known health benefits.

KEY WORDS: GCMS, semi-quantitative composition, calibration, measurement uncertainty, *Helichrysum italicum* essential oil

1. Introduction

Helichrysum italicum (Roth) G. Don is a subshrub from genus *Helichrysum* (fam. Asteraceae) native to Mediterranean region [1]. The genus name originates from two Greek words “*helios*” meaning sun and “*chryos*” meaning gold, which refers to the inflorescence color of the most species from the genus, including *H. italicum* [2]. In recent years, the research on *H. italicum* essential oil, present in all green parts of the plant, has been a topic of interest for many scientist and many of them confirmed its traditional use and also found new potential benefits of use in medicine and industry [3]; [4]. In the Eastern European Mediterranean countries (Croatia and Bosnia and Herzegovina) there was a significant increase of the commercial exploitation of the wild *H. italicum* populations, due to its wide application. A great demand for the *H. italicum* essential oil resulted in the huge expansion of its plantation cultivation in: France, Croatia, Bosnia and Herzegovina, Italy, Bulgaria and Serbia [5]. Authors determined high variability in complex chemical composition of *H. italicum* essential oils and attributed that variability to the influence of geographical origin, ecological condition, the genotype, the plant growth stage as well as the plant part used for preparation of essential oils [6]. Nowadays, research also focuses on the methods of isolation and extraction of essential oil that also gave great impact on the complex composition [7]. Since the high variability in chemical composition of *H. italicum* essential oils significantly affects their quality, it was a reason more to conduct this type of analysis and ensure high quality of analytical results. This study provided semiquantitative GC/MS (gas chromatography coupled with mass spectrometry) analysis of the volatile constituents of *H. italicum* essential oils with estimation of precision for 6 selected compounds and accuracy for one of the most prominent compound – neryl acetate. Laboratory for chemistry within Institute of Metrology of Bosnia and Herzegovina has developed a testing method for determination of chemical compounds present in *H. italicum* essential oil on GC/MS measuring system. The method was developed due to the increased need to assess the

quality of the *H. italicum* essential oil present on the Bosnian market. The main objective of this study was to identify selected chemical constituents in several essential oil samples and to evaluate the precision, accuracy and measurement uncertainty of the measurement method established on GC/MS system. The study was conducted in order to improve the quality assurance of the method and analytical results.

2. Materials and methods

2.1 Essential oil

Samples of *H. italicum* essential oil used in this research were previously prepared in several refineries across Bosnia and Herzegovina by hydro-distillation process. Samples were submitted to our laboratory for chemical analysis and quality control for the purpose of further placement on local and international market. Samples were received in tinted glass vials with a volume from 5mL to 20mL with dropper screw caps to ensure sample integrity. Samples are stored in laboratory refrigerator at a temperature of 4°C (recommended temperature for storage of such samples is from 2-8°C). In order to ensure the quality of the analytical result, upon receiving and before the GC/MS analysis samples of essential oils are tested for organoleptic and physical parameters. Testing is conducted in terms of confirmation of adequate distillation process. The sample of essential oil must be transparent, slightly colored with very intense scent that comes from volatile compounds which make up most of the essential oil composition. Small amount of oil was placed on a paper towel and evaporated to determine if there is a greasy deposit left on the towel, which indicates the possible presence of fatty acids, suggesting improper composition of oil or mixture with other vegetable oils after distillation.

2.2 Sample preparation for GC/MS analysis

After organoleptic and physical control, concentrated essential oil samples were prepared for analysis on GC/MS system by dilution in *n*-hexane (C₆H₁₄, HPLC grade, Honeywell) with optimized dilution. Diluted samples were placed on GC/MS autosampler system (ALS) and the analysis was carried out according to the previously established and optimized method described below.

2.3 Analytical technique/method

GC/MS is standard and most commonly used technique for analysis of volatile chemical components present in essential oils [8]. Compounds analyzed by gas chromatography must meet the precondition of volatility in order to elute with carrier gas based on their relative vapor pressure but also affinity for the stationary phase. When mass spectrometer is used as a detector with a gas chromatograph, component identification is possible by comparing the mass spectra of the compounds in the sample with those contained in the database [9-10]. GC/MS analysis of the essential oil samples was carried out on an Agilent Technologies, Inc. gas chromatograph (7820A) with a capillary HP5-ms ultra-inert column (-60 to 325°C, 30m × 250µm and film thickness 0.25µm). Gas chromatograph is equipped with an Agilent mass selective detector (MSD-5977E). Helium gas (5.0 purity) was used as the carrier gas at constant flow rate 1.02 ml/min, pressure of 8.4599 psi, and average velocity of 36.987cm/sec. Injection volume of 1 µL was employed. Sample injection was carried in splitless injection mode at 220 °C and pressure of 8.4599 psi. The oven temperature was programmed from 60 °C (1 min hold) to 246 °C (0 min hold) at a rate of 3 °C/min, and then to 280 °C at a rate of 10 °C/min. Equilibration time was 3 minutes, and solvent cut-off time was 4 minutes in order to avoid the peak from solvent. Before and after each injection three washes of needle with solvent (*n*-hexane) were employed. The program resulted in a total run time of 86.40 minutes. The mass selective detector (MSD) was operated using electron ionization at 70eV in the scan mode and in the *m/z* range of 40-400. The MSD transfer line temperature was 250°C, ion source temperature was 230°C, and quadrupoles 150°C. ChemStation software was used for instrument control and data analysis.

2.4 Identification and semi-quantitative analysis

After the analysis performed, the generated chromatograms are subjected to RTE mode integration. RTE integration is done according to previously determined parameters (minimum peak area 0.1% of largest peak, baseline drop else tangent integration). These parameters enable further identification and integration of peaks. Individual peaks of chemical components of *H. italicum* essential oil were identified by comparison of their retention indices (RI) with

those of authentic compounds or literature data and computer matching between samples mass spectra and mass spectra from spectrometer database libraries (Wiley7NIST05 and NIST14). Different monoterpene and sesquiterpene hydrocarbons and oxygen-containing compounds were identified and six (6) representatives of characteristic molecule classes present in *H. italicum* essential oil were selected for further processing. The selected compounds are presented in **Table 1**.

Table 1 Selected compounds for further analysis

Chemical name	IUPAC name	Molecular formula	RI (on HP-5 column)	Molecular class
D-limonene	(4R)-1-methyl-4-prop-1-en-2-ylcyclohexene	C ₁₀ H ₁₆	1030	monoterpene
neryl acetate	3,7-Dimethyl-2,6-octadien-1-yl acetate	C ₁₂ H ₂₀ O ₂	1364	monoterpenic ester
α -copaene	(1R)-1,3-dimethyl-8-propan-2-yltricyclo[4.4.0.0 ^{2,7}]dec-3-ene	C ₁₅ H ₂	1376	sesquiterpene
italicene	(1R,2S,5R,7R)-2,6,6,9-tetramethyltricyclo[5.4.0.0 ^{1,5}]undec-8-ene	C ₁₅ H ₂₄	1403	sesquiterpene
γ -curcumene	1-methyl-4-[(2R)-6-methylhept-5-en-2-yl]cyclohexa-1,3-diene	C ₁₅ H ₂₄	1480	sesquiterpene
Italidione (MW=224)	2,4,6,9-tetramethyldec-8-ene-3,5-dione	C ₁₄ H ₂₄ O ₂	1493	diketone

3. Statistical analysis

3.1 Assessment of sample and method stability and estimation of overall precision

Ten selected samples with representative yet somewhat different relative composition were analyzed in 16 replicates as two sets of 8 replicates under reproducibility conditions (time variable). Time difference between two sets was approximately 4 months in order to demonstrate the adequacy of sample storage (sample stability) as well as the stability of analytical method.

The composition of the samples was computed from the GC peak areas. Each peak is expressed as a percent share of the total measured area. Results were expressed for each selected compound (**Table 1**) of each sample and reported as % concentration.

Data was then subjected to statistical evaluation by Grubbs outlier test and statistically significant outliers were determined using $\alpha=0.05$ as level of significance. After exclusion of outliers, standard deviation of both sets was calculated and F test was conducted comparing variances and comparing the experimental F value to the tabulated F value for given degrees of freedom at 95% confidence level. Average values of data and pooled standard deviations for two sets were also calculated and compared by means of two-tailed T test for given degrees of freedom at 95% confidence level. Both tests showed satisfactory results indicating the stability of the samples and analytical methods during the given time (**Table 2**).

Table 2 Values of experimental F and T values for selected compounds

Sample/ compound	α -copaene		D-limonene		italidione (MW=224)		italicene		neryl acetate		γ -curcumene	
	F _{exp} *	T _{exp} **	F _{exp} *	T _{exp} **	F _{exp} *	T _{exp} **	F _{exp} *	T _{exp} **	F _{exp} *	T _{exp} **	F _{exp} *	T _{exp} **
1	1.103	0.385	0.147	1.539	1.013	1.566	1.375	0.735	1.455	1.837	1.205	0.539
2	3.637	1.460	1.104	0.059	1.239	1.019	1.963	0.574	3.241	1.268	1.093	0.348
3	1.300	0.170	0.993	0.359	2.999	0.368	1.652	0.048	1.332	0.101	1.096	0.222
4	1.571	1.637	1.400	0.528	1.182	0.941	1.154	0.981	1.416	0.738	1.676	0.854
5	2.699	0.587	1.241	0.102	1.704	0.896	1.785	1.091	2.001	0.874	1.377	2.042
6	3.192	0.839	2.104	1.124	1.349	0.024	2.209	1.352	1.205	0.786	1.137	1.606
7	1.681	0.015	0.533	0.736	1.049	0.143	1.939	0.935	0.583	1.449	3.431	1.433
8	1.814	0.867	2.123	1.753	1.412	1.697	1.353	1.955	1.345	1.608	1.417	1.154

9	2.304	0.493	1.957	0.586	0.957	1.020	2.960	1.538	2.859	0.030	1.133	1.969
10	1.479	0.257	1.610	0.379	1.188	0.320	1.547	0.754	3.129	1.284	1.808	1.166
* One tailed, $\alpha=0.05$, 95% confidence $df_1=7$ $df_2=7$ ($n-1$), $F_{tab} = 3.787$												
** One tailed, $\alpha=0.05$, 95% confidence $df=14$ (n_1+n_2-2), $T_{tab} = 2.145$												

3.2 Assessment of bias for selected compound – neryl acetate

For the purpose of testing the accuracy of the results, several experiments were performed by adding pure neryl acetate to two essential oil matrices in several concentration intervals. Neryl acetate was chosen as a compound with proven health benefits [14-16] and whose concentration in immortelle essential oil is a significant indicator of the oil quality.

The spiking was done by adding the accurate amounts of pure substance to the same volume of essential oil. 0.5 mL of oil was accurately pipetted to GC vials and spiked with 20 – 80 μ L of pure neryl acetate (Neryl acetate – analytical standard with purity 98.2% and 97.8% Sigma-Aldrich). One essential oil (sample 5) sample was spiked with 20, 40, 60 and 80 μ L of pure compound and the other one (sample 7) with 20, 30, 40, 50, 60 and 70 μ L. Spiked samples, as well as the oil without addition of pure neryl acetate, were subjected to further dilution, i.e. preparation for GC/MS analysis in accordance with the 2.2 and the analysis was carried out in accordance with 2.3 in three replicates. The GC method was previously optimized for potential carry-over due to the fact that unknown samples of essential oil are routinely analyzed in random order. For the purpose of confirming lack of carry-over the spiked samples were analyzed in the increasing and decreasing order.

Two essential oil matrices subjected to spiking were also previously tested for stability (3.1) – samples 5 and 7. Zero reference concentrations of neryl acetate (concentration of neryl acetate contained in oil without spiking) were calculated as mean average of mean values obtained for two sets of measurements in the previous experiment and mean value of three replicates for selected oils in spiking experiment. After that, the reference concentrations in spiked samples was calculated by adding the spiked amount to the amount of neryl acetate contained in oil, taking the purity of the analytical standard into account as well as the error of automatic pipette from the certificate for the volumes of interest. Measured concentrations were given as mean values of instrument readings for three replicates on each concentration level. Mathematical models for calculation of each stated quantity are given in 3.3.

Recoveries for each sample and concentration level were calculated

$$R_{Xi} = \frac{X_i - X_{0ref}}{X_{ref} - X_{0ref}} \times 100\% \quad (1)$$

Where:

R_{Xi} – recovery in %

X_i – mean measured concentration of sample i

X_{0ref} – ref concentration for unspiked oil

X_{ref} – ref concentration for selected conc. level

Measurement uncertainty of recovery [17] for unspiked oil was calculated according to equation (2):

$$(R_{X0}) = \bar{R}_{X0} \times \sqrt{\frac{s_{X_i}^2}{N \times \bar{X}_i^2} + \left(\frac{u(X_{0ref})}{X_{0ref}} \right)^2} \quad (2)$$

Where:

$\bar{R}_{X0}$ – recovery for unspiked oil in %

$\bar{X}_i$ – mean measured concentration of sample i

s_{X_i} – standard deviation of replicate measurements for X_i

N – number of replicate measurements for X_i

$u(X_{0ref})$ – standard measurement uncertainty of X_{0ref}

Recoveries ranged from 83.7-101.8% (recovery data given in Table 6) with RSD not exceeding 2.2%.

3.3 Calibration model and estimation of measurement uncertainty for selected compound – neryl acetate according to GUM [11]

Recognized sources of measurement uncertainty that have contributed in the budget of combined measurement uncertainty are: the measurement uncertainty and resolutions of the used automatic pipettes on the volumes used (source: calibration certificates for pipettes), purity of the analytical standard (source: certificate of analysis from the manufacturer), resolution of the GCMS instrumental method, standard deviation of measurements used for calibration, pooled standard deviation under reproducibility conditions, uncertainty of calibration curve fit. Contribution of each source of uncertainty was calculated as a product of standard uncertainty and sensitivity coefficient in accordance with the functional dependence. Distributions of standard uncertainty for analytical standard purity and instrument resolution were taken as rectangular, while other distributions were taken as normal. Expanded mathematical model including corrections used for calculation of reference value of neryl acetate in pure oil:

$$X_{ref0} = \bar{X}_w + \delta_{X_{S_0}} + \delta_{X_{S_0}} + \delta_{X_{rec}} + \delta_{X_{res}} \quad (3)$$

Where:

$\bar{X}_w$ – concentration of neryl acetate in pure oil as weighted mean of three sets of measurements with corresponding standard deviations (s_i) calculated as:

$$\bar{X}_w = \frac{\sum_{i=1}^n \left(\bar{X}_i \times \frac{1}{s_i} \right)}{\sum_{i=1}^n \frac{1}{s_i}} \quad (4)$$

$\delta_{X_{S_0}}$ – correction due to repeatability of measurements (This correction is assumed to be zero within uncertainty $u(\delta_{X_{S_0}})$ with normal distribution)

$\delta_{X_{S_0}}$ – correction due to reproducibility of measurements (This correction is assumed to be zero within uncertainty $u(\delta_{X_{S_0}})$ with normal distribution)

$\delta_{X_{rec}}$ – correction due to recovery measurements according to Equation 1 (This correction is assumed to be zero within uncertainty $u(\delta_{X_{rec}})$ with normal distribution)

$\delta_{X_{res}}$ – correction due to instrument resolution (This correction is assumed to be zero within uncertainty $u(\delta_{X_{res}})$ with rectangular distribution)

Expanded mathematical model including corrections used for calculation of reference value of neryl acetate in spiked samples:

$$X_{ref} = X_{ref0} + \frac{V_{cor\ pip2} + \delta_{V_{pip2\ cal}} + \delta_{V_{pip2\ res}}}{V_{cor\ pip1} + \delta_{V_{pip1\ cal}} + \delta_{V_{pip1\ res}} + V_{cor\ pip2} + \delta_{V_{pip2\ cal}} + \delta_{V_{pip2\ res}}} \times (NA_{pur} + \delta_{NA_{pur}}) \quad (5)$$

Where:

X_{ref} – reference concentration

$V_{cor\ pip2}$ – corrected volume for pipette 2 (from calibration certificate)

$\delta_{V_{pip2\ cal}}$ – correction due to measurement uncertainty of volume for pipette 2 from calibration certificate (This correction is assumed to be zero within uncertainty $u(\delta_{V_{pip2\ cal}})$ with normal distribution)

$\delta_{V_{pip2\ res}}$ – correction due to resolution for pipette 2 (This correction is assumed to be zero within uncertainty $u(\delta_{V_{pip2\ res}})$ with rectangular distribution)

$V_{cor\ pip1}$ – corrected volume for pipette 1 (from calibration certificate)

$\delta_{V_{pip1\ cal}}$ – correction due to measurement uncertainty of volume for pipette 1 from calibration certificate (This correction is assumed to be zero within uncertainty $u(\delta_{V_{pip1\ cal}})$ with normal distribution)

$\delta_{V_{pip1_{res}}}$ – correction due to resolution for pipette 1 (This correction is assumed to be zero within uncertainty $u(\delta_{V_{pip1_{res}}})$ with rectangular distribution)

NA_{pur} – purity of analytical standard (from certificate of analysis)

$\delta_{NA_{pur}}$ – correction due to purity of analytical standard from certificate of analysis (This correction is assumed to be zero within uncertainty $u(\delta_{NA_{pur}})$ with rectangular distribution)

Pipette 1 – used for measuring 0.5 mL of oils samples (adjustable volume 0.5-5 mL, resolution 5 μ L)

Pipette 2 – used for spiking (adjustable volume 10-100 μ L, resolution 1 μ L)

Expanded mathematical model of measured value of neryl acetate in spiked samples:

$$X_i = \bar{X}_i + \delta_{X_{res}} + \delta_{X_s} \quad (6)$$

Where:

X_i – measured concentration of neryl acetate in spiked samples –instrument reading

$\bar{X}_i$ – mean concentration of three replicates of neryl acetate in spiked samples –instrument reading

$\delta_{X_{res}}$ – correction due to instrument resolution (This correction is assumed to be zero within uncertainty $u(\delta_{X_{res}})$ with rectangular distribution)

δ_{X_s} – correction due to repeatability of measurements (This correction is assumed to be zero within uncertainty $u(\delta_{X_s})$ with normal distribution)

Examples of the measurement uncertainty budget according to quantified contributions for oil – sample 7 and oil - sample 7 spiked with 30 μ L of neryl acetate, are given bellow in **Table 3** and **Table 4** respectively. The same approach for uncertainty budget was used for other measurements.

Table 3 Example of uncertainty budget for Sample 7 – unspiked oil

Quantity	Value	Standard uncertainty	Probability distribution	Sensitivity coefficient	St. uncertainty contribution	Degree of freedom
$\bar{X}_w$	7.500 %	-	-	-	-	-
$\delta_{X_{S_0}}, u(\delta_{X_{S_0}})$	0 %	3.31E-02 %	normal	1 %	3.31E-02 %	2
$\delta_{X_{res}}, u(\delta_{X_{res}})$	0 %	2.89E-04 %	rectangular	1 %	2.89E-04 %	∞
$\delta_{X_{rec}}, u(\delta_{X_{rec}})$	0 %	1.72E-02 %	normal	1 %	1.72E-02 %	2
$\delta_{X_{S_0}}, u(\delta_{X_{S_0}})$	0 %	1.23E-01 %	normal	1 %	1.23E-01 %	14
X_{ref0}	7.500 %			$u_c(E)$	0.129 %	
Recovery (%)	99.72 %			v_{eff}	16.049	
				$k_{95\%, v_{eff}}$	2.120	
				$U_{measured}(X_0), k=95\%$	0.273%	

Table 4 Example of uncertainty budget for Sample 7 – spiked with 30 μ L of neryl acetate

Quantity	Value	Standard uncertainty	Probability distribution	Sensitivity coefficient	St. uncertainty contribution	Degree of freedom
$\bar{X}_{measured}$	12.788 %	-	-	-	-	-
$\delta_{X_s}, u(\delta_{X_s})$	0 %	9.77E-02 %	normal	1 %	9.77E-02 %	2

$\delta_{x_{res}}, u(\delta_{x_{res}})$	0 %	2.89E-04 %	rectangular	1 %	2.89E-04 %	∞
$V_{cor\ pip1}$	499.91 μ L	-	-	-	-	-
$\delta_{V_{pip1_{cal}}}, u(\delta_{V_{pip1_{cal}}})$	0 μ L	7.50E-01 μ L	normal	-1.06E-04 μ L ⁻¹ %	-7.95E-05 %	9
$\delta_{V_{pip1_{res}}}$	0 μ L	1.44E-01 μ L	rectangular	-1.06E-04 μ L ⁻¹ %	-1.53E-05 %	∞
$V_{cor\ pip2}$	29.74 μ L	-	-	-	-	-
$\delta_{V_{pip2_{cal}}}, u(\delta_{V_{pip2_{cal}}})$	0 μ L	7.50E-02 μ L	normal	1.78E-03 μ L ⁻¹ %	1.34E-04 %	9
$\delta_{V_{pip2_{res}}}, u(\delta_{V_{pip2_{res}}})$	0 μ L	2.89E-01 μ L	rectangular	1.78E-03 μ L ⁻¹ %	5.14E-04 %	∞
NA_{pur}	98.2 %	-	-	-	-	-
$\delta_{NA_{pur}}, u(\delta_{NA_{pur}})$	0 %	1.04E+0 %	rectangular	5.62E-02 μ L/ μ L	5.84E-02 %	∞
$X_{ref\ 0}, u(X_{ref\ 0})$	7.500 %	1.29E-01 %	normal	1 %	1.29E-01 %	16
X_{ref}	13.014 %			$u_c(E)$	0.172 %	
$E = \bar{X}_{measured} - X_{ref}$	-0.226 %			v_{eff}	13.924	
Recovery (%)	95.90 %			$k_{95\%, v_{eff}}$	2.160	
$U_{measured}(Xi), k=95\%$					0.371%	

Uncertainty contribution of each component listed in uncertainty budget is calculated by multiplying each standard uncertainty with its sensitivity coefficient which is obtained as partial derivatives of mathematical models (equations 3, 5 and 6) given above with respect to their input variables. Combined standard uncertainty was calculated as square root of sum of squares of each individual contribution stated above, while expanded uncertainty for each measurement/calibration point was calculated by multiplying combined standard uncertainty with corresponding coverage factor k obtained from calculated effective degrees of freedom using the Welch–Satterthwaite approximation according to GUM [11]. The main contributions to combined measurement uncertainty come from repeatability for a given measurement, reproducibility for a given oil sample and the purity of the standard - neryl acetate. **Table 5** contains data for all measured and reference values with associated expanded uncertainty for all analyzed points.

Table 5 Data use for plotting calibration curve (Figure 1)

Calibration point	Reference value (% of neryl acetate)	Measured value (% of neryl acetate)	$\pm U_{measured}$ (% of neryl acetate)	$\pm U_{fir\ pred}$ (% of neryl acetate)	$\pm U_{expanded}$ (% of neryl acetate)	Recovery (%)
1.	0	0.440	0.273	0.658	0.712	n/a
2.	5.483	5.581 ^a	0.420	0.536	0.681	101.79
3.	7.500	7.478 ^b	0.273	0.508	0.577	99.72
4.	9.192	9.196 ^a	0.591	0.493	0.770	100.10
5.	11.224	10.762 ^b	0.416	0.485	0.639	87.61
6.	12.693	12.127 ^a	0.897	0.489	1.022	92.16
7.	13.014	12.788 ^b	0.371	0.497	0.620	95.60
8.	14.738	13.844 ^b	0.308	0.513	0.598	87.65
9.	15.942	14.303 ^a	0.850	0.534	1.004	84.33
10.	16.399	14.948 ^b	0.327	0.554	0.643	83.69
11.	18.001	16.832 ^b	0.546	0.595	0.808	88.87
12.	18.966	17.003 ^a	0.528	0.644	0.833	85.44
13.	19.546	17.709 ^b	0.387	0.718	0.815	84.85

^a oil sample 5; ^b oil sample 7

Measured value of zero concentration was calculated as an absolute difference between the reference value of unspiked oil (sample 7) and the value for neryl acetate present in the same oil calculated from the known amount of neryl acetate added (20 µL). Uncertainty for zero point was taken as the uncertainty of the measurement with corresponding spike.

In order to functionally represent bias and its uncertainty, a simple linear model $Y = BX + A$ between measured and reference concentrations have been assumed. The parameters of linear model A and B as well as their standard uncertainties are obtained by means of Ordinary Least Square algorithm which is integral part of EXCEL (LINEST function). Statistical test (F test) for checking the adequacy of proposed linear model have been conducted and confirmed as well. Then uncertainty contribution due to curve fit was calculated for both confidence and prediction intervals (see Table 5) as [12-13]:

$$u_{fit\ conf} = \sigma_Y \sqrt{\frac{1}{n} + (X_i - \bar{X})^2 \left(\frac{\sigma_B}{\sigma_Y} \right)^2} \quad (7)$$

$$u_{fit\ pred} = \sigma_Y \sqrt{\frac{1}{N} + \frac{1}{n} + (X_i - \bar{X})^2 \left(\frac{\sigma_B}{\sigma_Y} \right)^2} \quad (8)$$

Where:

$u_{fit\ conf}$ – standard uncertainty of calibration curve fit for confidence interval

$u_{fit\ pred}$ – standard uncertainty of calibration curve fit for prediction interval

σ_Y – standard uncertainty of Y

N – number of replicate measurements

n – number of measurement points taken for curve approximation

X_i – chosen value of X

$\bar{X}$ – mean of reference values (central point of regression)

σ_B – standard uncertainty of slope B

Predicted expanded uncertainty of the regression line at 95% confidence level is calculated as:

$$U_{fitpred} = t_{95, v-2} u_{fit\ pred} \quad (9)$$

Then overall expanded uncertainty of calibration line at 95% confidence level is calculated as RSS of estimated expanded uncertainty for measured value and expanded predicted uncertainty of regression line:

Overall expanded uncertainty with 95% confidence level is calculated as:

$$U(X_i)_{cal-line} = \sqrt{U(X_i)_{measured}^2 + U(X_i)_{fit\ pred}^2} \quad (10)$$

Where:

$U(X_i)_{cal-line}$ – Overall expanded uncertainty of - calibration line at sample X_i

$U(X_i)_{measured}$ – Expanded uncertainty for measured values of sample X_i at 95% confidence level

$U(X_i)_{fitpred}$ – Expanded predicted uncertainty of the regression line at 95% confidence level for sample X_i

$t_{95, v-2}$ – two-tailed Student's t value for n-2 degrees of freedom at 95% confidence level

Overall expanded uncertainty of all individual measurements is calculated in the same way. The values are given in **Table 5**.

Coverage factors for $U(X_i)_{measured}$ and $U(X_i)_{fitpred}$ were calculated for corresponding effective degrees of freedom.

Reference values given in **Table 5** were plotted against measured values and linear fit was obtained with $R^2 = 0.996$. Confidence and prediction intervals were calculated with corresponding coverage factors [12-13] and overall expanded uncertainty values were plotted as presented in the **Figure 1** below.

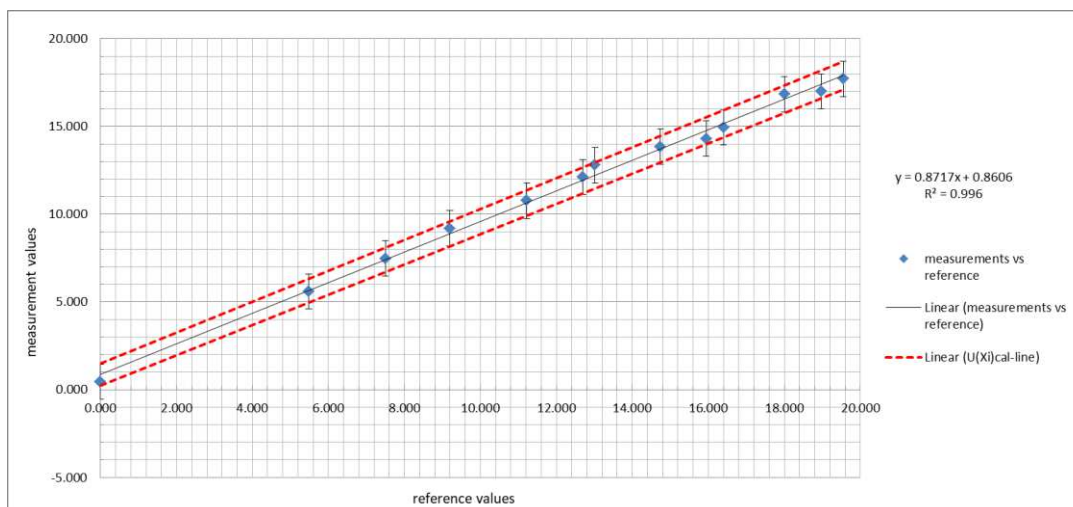


Figure 1 – Calibration curve with uncertainty bars and intervals (data from **Table 5**)

The calibration curve can be used to correct the measured value y (instrument reading) by simply reading the value on the X axis for a given Y value (measurement). In order to read measurement uncertainty interval of any measurement as a projection on the X-axis on which the corrected measurement value (Y) is also read, it is necessary to divide the expanded measurement uncertainties given in equation (10) by the slope value (B). The confidence interval includes all sources of uncertainty except the factor related to the number of replicates for a given measurement, while the prediction interval includes the additional prediction value for N number of readings of unknown sample which is expressed in equation (8) with parameter $1/N$. The use of prediction interval enables a shorter analysis time (replicate measurements are not necessary), but it can potentially be overestimated for a specific measurement result. Uncertainty can be reduced if higher number of replicate measurements is employed.

4. Conclusions

For the purposes of this study, 10 samples of immortelle essential oil with a composition that is typical and representative for samples that are routinely analyzed in the laboratory were selected. In these samples, six compounds were selected that are characteristic for the essential oil of immortelle of the *italicum* species, which is native and/or cultivated in the southern part of Bosnia and Herzegovina. The compounds were selected to represent different percentage concentration levels that are routinely determined by semiquantitative GCMS analysis. Based on the performed F and T test on the mentioned samples for the selected compounds under reproducibility conditions, it can be concluded that the samples are adequately stored and preserved, and that the analytical set-up is also stable. This fact served as the basis for conducting the next experiment, which entails creating a calibration curve and estimating the measurement uncertainty for the selected compound of interest - neryl acetate. Neryl acetate was chosen due to the great health benefits [14-16] of this compound and the method of forming the value of the essential oil, which is based on the percentage composition of this compound. The concentration interval covered by calibration curve is also related to the typical percentage content of neryl acetate in samples that are routinely processed in the laboratory based on many years of experience.

On the basis of the conducted experiment, it can be concluded that different matrices of essential oil of immortelle do not influence, that is, they influence to the same extent the recovery of the added analytical standard, considering that the calibration curve was created by spiking two different matrices of essential oil with external standard. It is assumed that recovery can be influenced by the preparation of the reference concentrations, i.e. the addition of the volatile component by means of pipette, so this aspect was given special attention. During the study, it was demonstrated that the best results were shown for the reverse pipetting mode with blow-out. Based on the generated calibration curve it is possible to both correct the values of readings for selected compound by comparing the value of reading with the reference that includes recovery data, as well as to report corrected values with associated measurement uncertainty, for the selected compound. For other compounds that were subjected only to F and T test, calculated pooled standard deviations under reproducibility conditions can be used as a measure of precision of the

reported results. This allows generation and reporting of an additional parameter of the quality of the test result. Periodic performance of the above tests for all components reported enables the monitoring of the stability of the compounds in the samples, i.e. the adequacy of storage, as well as the stability of the analytical set-up and its performance over time.

5. References

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