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Determination of phytochemical compounds in chicken breast by gas chromatography-tandem mass spectrometry

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Abstract

A gas chromatography-tandem mass spectrometry (GC-MS/MS) method was developed for the simultaneous detection and quantification of five phytochemical compounds (carvone, menthol, thymol, carvacrol and methyl salicylate) in chicken breast. Chicken breast samples were analyzed using a QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) approach using acetonitrile as extraction solvent, followed by a d-SPE (dispersive-solid phase extraction) clean up step. The linearities for the 5 substances were studied in the range between 2 and 100 µg/L and the coefficients of determination (R²) were always > 0.995. Matrix effects were also assessed by comparing the slopes obtained in solvent and chicken breast matrix. The recoveries for all the substances at 3 different spike levels (5, 10 and 50 µg/kg) were in the range 80-102% with RSDs < 15%. The instrumental limits of quantification were in the range 2.7-4.8 µg/kg, while the reporting level of the method was 5 µg/kg for all the aforementioned compounds. The method was successfully applied to 10 chicken breast samples from the local market.

Keywords: Chicken breast, GC-MS/MS, Phytochemical compounds, QuEChERS

1. Introduction

Antibiotics and antimicrobials are not only used as veterinary drugs to prevent and control diseases in animal rearing but also as growth promoters [1]. For this latter purpose they are added to animal feed – a practice that can lead to residues in edible animal products that are potentially dangerous for human health or induce antibiotic resistance of microbes even at small doses [2]. This led to the ban of antibiotics as growth promoters by the European Union since 2006 [3].

Global meat consumption has seen an increase by 58% in the past two decades and in particular chicken meat consumption has increased, as it is not only a source of high-quality protein, important vitamin and minerals, but also the cheapest of all livestock meats [4].

The aforementioned ban of antibiotic growth promoters has fostered the research for alternative

substances: phytochemical feed additives (PFA) are suggested to be among the most promising ones [5]. PFA are composed of plant-derived natural materials with positive effects on animal growth and health, and this definition applies to preparations of ground herbs and spices, essential oils (EOs), extracts and/or oleoresins. PFA contain secondary plant metabolites (phytochemicals) and encompass a wide range of chemical compound classes, including phenols, terpenes, alkaloids, lectins, aldehydes and ketones. The various mechanisms of action are not yet fully understood but involve antimicrobial/antiviral, antioxidative and anti-inflammation activity [6].

Several companies offer feed additives containing PFA in their portfolio and so it is important to determine their residues in edible animal products within the scope of consumer safety assessment [7]. We hence developed a method for the quantitative determination of the following five phytochemicals in chicken breast: carvone, menthol, thymol, carvacrol and methyl salicylate.

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Food matrices are still among the most challenging ones due to their complexity. In addition, the usual sample preparation methods for these matrices are labor intensive [8]. A valid alternative to these methods is the QuEChERS technique [9], although this method was originally developed for pesticide analysis in fruits and vegetables its scope of application has been extended to other analytes and a great variety of different matrices [10,11]. Therefore, we decided to apply this approach as a sample preparation method. A generic QuEChERS extraction method involves a first step in which the analytes of interest are extracted by using an organic solvent (normally acetonitrile) in presence of inorganic salts (like MgSO_4 or NaCl) to ensure a salting out effect. The extract is then cleaned with dispersive sorbents to remove matrix interferences [12].

This method involves so, a simple QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) sample preparation procedure followed by detection by gas chromatography coupled with tandem mass spectrometry (GC-MS/MS).

2. Materials and methods

2.1. Reagents and solutions

Ultra-pure water ($18.2 \text{ M}\Omega \text{ cm}^{-1}$) was obtained in-house using a Millipore water purification system (Cork, Ireland). Acetonitrile LC-MS grade was purchased from Chem-Lab NV (Zedelgem, Belgium).

QuEChERS original method (4 g MgSO_4 and 1 g NaCl) and QuEChERS d-SPE (dispersive Solid Phase Extraction) Animal Origin Food 5982-4950 (50 mg PSA, 150 mg C18EC and 900 mg Na_2SO_4) were obtained from Agilent technologies.

2.2. GC-MS/MS instrumentation and settings

The GC-MS/MS analyses were conducted with a Shimadzu GC 2010 gas chromatograph coupled with a Shimadzu TQ-8050 tandem mass spectrometer (Shimadzu, Kyoto, Japan). The system was equipped with a PTV (Programmed Temperature Vaporization) injection inlet and an AOC-5000 autosampler. GC-MS Real Time Analysis and GC-MS Postrun Analysis software (Shimadzu, Kyoto, Japan) were used for instrument control and data analysis, respectively. The GC analysis was performed on a J&W DB5-MS+DG column (length 30 m, id 0.25 mm, film thickness $0.25 \mu\text{m} + 10 \text{ m}$ Guard Column), the chromatographic conditions were the following: carrier gas helium (minimum purity 99.9995%) in constant linear velocity mode at

30 cm/sec, PTV Injector 60–200 °C at 240 °C/min 1 min then 330 °C for 19 min, septum purge 6 mL/min, Split 1:1; GC oven temperature program of 40 °C for 1 min, ramp 20 °C/min to 130 °C, hold 7.0 min then 40 °C/min until 280 °C (held for 3 min), injection volume 1 μL .

The mass spectrometer was operated in MRM mode (MRM transitions of all five analytes and the internal standard (IS) in Table 1) with the following conditions: electron impact ionization at 70 eV, MS transfer line temperature 280 °C, MS source temperature 200 °C, solvent delay 9.6 min, dwell time 300 ms, collision gas argon (minimum purity 99.9999%) with a collision cell pressure of 200 kPa and detector gain fixed at 1.4 kV.

2.3. Phytogetic compounds standards, internal standard solution preparation

All high purity phytogetic compounds standards were purchased from Sigma-Aldrich.

Mixed standard solutions of the analytes were prepared in acetonitrile by diluting the stock solutions to concentrations of 50, 400, 800, 1200, 1600 and 2000 $\mu\text{g}/\text{mL}$. The internal standard (IS; Butyrophe none) solution was prepared at a concentration of 200 $\mu\text{g}/\text{mL}$ in acetonitrile as well.

The calibration solutions were prepared in triplicate ($n = 3$) by spiking 240 μL of phytoгенics-free chicken breast extract with 30 μL of the appropriate standard solutions of the analytes to obtain concentrations of 2, 5, 10, 20, 50 and 100 $\mu\text{g}/\text{mL}$. The concentration of the IS in each sample was maintained at 10 ppb by adding 30 μL of the IS stock solution. In this way each matrix matched standard solution contained the same amount of matrix and pure solvent.

2.4. Samples

Samples of chicken breast were purchased from the local market in Austria. Each sample was homogenized with the use of a Retsch Mixer Mill MM 400 and stored at $-20 \text{ }^\circ\text{C}$ before analysis.

2.5. Sample preparation

One g of homogenized chicken breast was weighed in a Retsch stainless steel jar, 1 mL of ultrapure water, 2 mL of acetonitrile and 0.7 g of Agilent QuEChERS original method salts were added; the jar was closed and the sample was extracted using a Retsch Mixer Mill MM 400 for 2 min at 30 Hz. Hereafter 1.5 mL of the solution were taken, transferred in a 1.5 mL glass vial and

Table 1. MRM Transitions of the investigated phytochemical compounds and an internal standard.

Compound	Retention Time (mins)	MRM Transition 1 (Quantifier)	Collision Energy (eV)	MRM Transition 2 (Qualifier)	Collision Energy (eV)	MRM Transition 3 (Qualifier)	Collision Energy (eV)
Menthol	9.202	95 > 55.1	16	95 > 67.1	10	81 > 79.1	12
Methyl salicylate	9.545	105 > 77.1	16	105 > 51.1	24	77 > 51.1	16
Carvone	10.721	120 > 92.1	12	152 > 120.1	8	120 > 64.1	22
Butyrophenone (IS)	10.910	82 > 54.1	6	108 > 93.1	10	93 > 77.1	16
Thymol	11.793	135 > 91.1	16	150 > 135.1	12	135 > 115.1	16
Carvacrol	12.113	135 > 91.1	16	150 > 135.1	12	135 > 115.1	16

centrifuged for 5 min at 3234 rcf. An aliquot of 700 μ L of the supernatant was transferred in a 1.5 mL glass vial containing 105 mg of QuEChERS Dispersive SPE, the content of the vial was vortexed for 1 min and then centrifuged for 5 min at 14000 rcf. The sample was then transferred into a GC vial and only at this point the IS was added, to avoid losses during the sample preparation. The IS was used to compensate e.g. for detector and injection volume fluctuations [13].

2.6. Method validation

The following performance characteristics were evaluated for the validation of the method: selectivity, identification, linearity, matrix effects, limits of quantification (LOQ), recoveries and precision.

2.7. Selectivity

The selectivity was verified by analyzing chicken breast samples that were free of the compounds of interest and the presence of peaks that could interfere with those substances were assessed.

2.8. Identification

The criteria used for the identification of analytes were retention time (Rt) and the presence and the relative intensities of three MRM transitions (one quantifier and two qualifiers). According to SANTE 2019 criteria, the Rt of a compound of interest in a sample should not vary more than ± 0.1 min compared to a calibration standard and, the relative intensities for the samples should be within $\pm 30\%$ (relative) of average of calibration standards from the same sequence [14].

2.9. Linearity

Linearity was assessed by measuring six points calibration curves in triplicate (2, 5, 10, 20, 50 and 100 ppb – corresponding to a range of 4 to 200 μ g/kg in chicken breast) prepared in acetonitrile and in blank chicken breast extracts as well.

2.10. Matrix effects

In order to assess the suppression or enhancement of the signal of the analytes due to the matrix, the slopes obtained from the linearity study were used to calculate the percentage of the matrix effect (%ME), according to the following formula:

$$\%ME = \left(\left(\frac{\text{Slope Matrix matched standards}}{\text{Slope Standards in solvent}} \right) - 1 \right) \times 100$$

Negative values indicate signal suppression while positive values indicate signal enhancement: matrix effects in the range $-/+ 20\%$ are permissible [15].

2.11. LOQ

The instrumental LOQ were determined following [16]: the standard deviation of a number of samples at a low concentration was determined. Multiplying this standard deviation (SD) with $3.3 \cdot t_{\alpha}$ (t_{α} the Student's t correlating to the number of samples the standard deviation is based on) gives the LOQ.

$$\text{LOQ} = \text{SD} \cdot 3.3 \cdot t_{\alpha}$$

The reporting level of the method was set at the spike level (see Recoveries and precision) in which the SANTE 2019 criteria (70-120% recoveries range with a 20% RSD) were fulfilled [14].

2.12. Recoveries and precision

Recoveries and precision were evaluated by spiking chicken breast samples that were free of the compounds of interest and preparing them accordingly to the procedure outlined in the Sample preparation. The recoveries were determined for six replicates prepared on three different days (day 1, day 2 = day 1 + 24h and day 3 = day 1 + 144h) at three different spike levels: 5, 10 and 50 $\mu\text{g}/\text{kg}$. The average intraday and interday recoveries and the relative standard deviation (RSD) were calculated as the ratio of the analyte-to-IS peak area and the results were evaluated for compliance to the SANTE 2019 criteria, according to which the average recovery should be in the range 70-120% with an RSD less or equal 20%.

3. Results and discussion

3.1. Method development

3.1.1. GC-MS/MS conditions

The GC parameters (initial PTV injector temperature and ramp rate, initial and final column temperatures, as well as the column temperature ramp rate and carrier gas flow rate) were optimized to achieve the best sensitivity and chromatographic separation. It was possible to achieve chromatographic separation for all the compounds of interest and the IS, see Fig. 1.

In order to find the best MRM transitions the Shimadzu MRM Optimization Tool 1.14 (Shimadzu, Kyoto, Japan) software was used: this software automates the process by collecting product ion scan data and finding the optimum collision energy for each transition. Three MRM transitions per analyte were chosen: one quantifier and two qualifiers.

3.2. Sample preparation

The selection of the right sorbent is critical in order to minimize matrix interferences and achieve good and consistent analytes recoveries. Due to the nature of our matrix we decided to use an animal origin food sorbent. We evaluated the efficiency of the dSPE step by measuring extracts which have been treated or non-treated with the dSPE sorbent in Full Scan mode, and then comparing the sum of the areas of the chromatographic peaks in the TIC chromatograms (m/z 50-600) [17]. The reduction for the treated was in the range of 60%.

3.3. Method validation

3.3.1. Selectivity

No interfering peaks that could prevent the identification or the quantification of the compounds of interest were observed in 12 different chicken breast samples that were analyzed. In

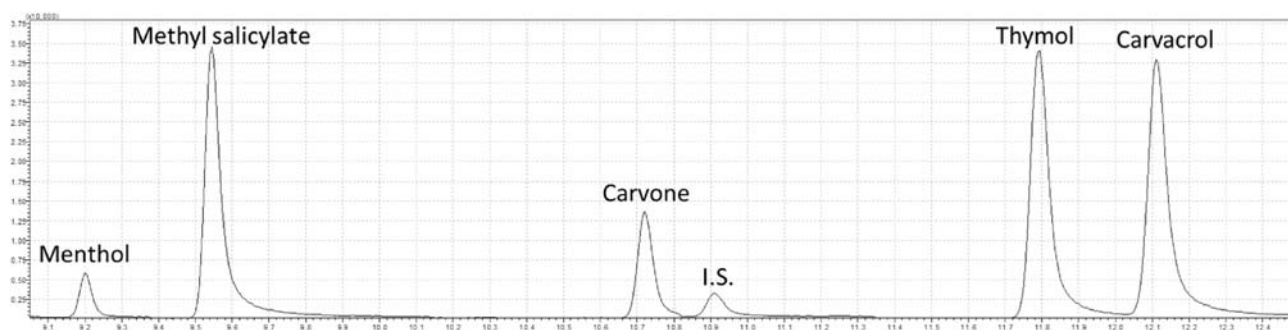


Fig. 1. MRM chromatogram of the chicken breast spiked with target analytes at the concentration of 10 $\mu\text{g}/\text{kg}$ with the assigned peaks.

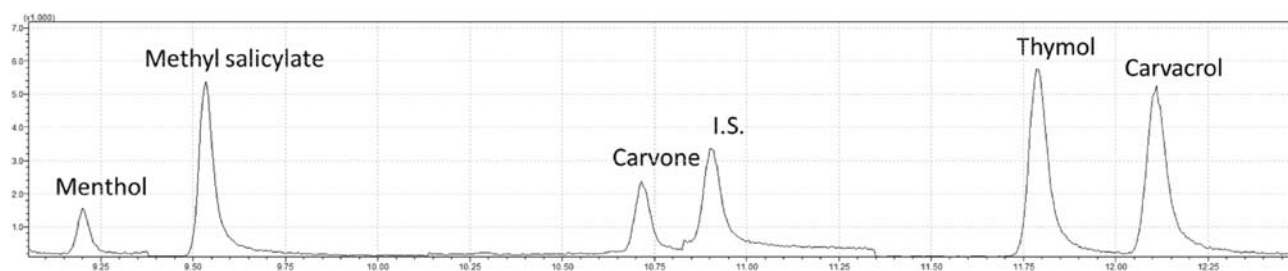


Fig. 2. MRM chromatogram of a real chicken breast sample with the assigned peaks.

Table 2. %ME of investigated analytes, LOQ ($\mu\text{g}/\text{kg}$), accuracy and precision. Conc. in $\mu\text{g}/\text{kg}$ and precision data are given in parenthesis as % coefficient of variation.

Analyte	ME%	LOQ ($\mu\text{g}/\text{kg}$)	Conc.	Intraday			Interday
				Day 1	Day 2	Day 3	
Menthol	12	3.8	5	106 (11)	106 (10)	94 (13)	102 (12)
			10	97 (8)	89 (10)	88 (7)	91 (10)
			50	78 (8)	82 (6)	79 (6)	80 (7)
MES	70	4.3	5	89 (10)	98 (14)	86 (13)	91 (14)
			10	88 (15)	95 (9)	88 (9)	90 (12)
			50	89 (14)	84 (12)	79 (6)	84 (12)
Carvone	26	2.9	5	87 (16)	91 (6)	87 (12)	89 (12)
			10	83 (10)	76 (9)	76 (18)	87 (12)
			50	89 (9)	82 (10)	81 (6)	84 (10)
Thymol	34	2.7	5	99 (10)	103 (11)	100 (14)	100 (12)
			10	87 (7)	99 (7)	99 (9)	95 (11)
			50	81 (10)	92 (12)	95 (6)	89 (12)
Carvacrol	38	4.8	5	97 (9)	82 (11)	94 (14)	91 (14)
			10	88 (9)	91 (14)	112 (4)	100 (13)
			50	81 (11)	89 (14)	100 (8)	90 (14)

addition, 10 procedural blanks were prepared as well [18]. Also, in this case no interfering peaks were detected.

3.3.2. Identification

The SANTE 2019 criteria mentioned before were fulfilled by the matrix matched standards and all the real samples as well. In Fig. 2 a chromatogram of a real sample is shown.

3.3.3. Linearity

Good linearity was achieved for all the compounds of interest in the measured calibration range

with a coefficient of determination (R^2) always > 0.995 in pure solvent and matrix as well.

3.3.4. Matrix effects

The matrix effect results, expressed as %ME are shown in Table 2. Signal enhancement was observed for all investigated substances, this is the most common behavior for GC where the matrix components block the active sites of the column [19]. Only for menthol the %ME is below $\pm 20\%$ so we decided to validate the method with matrix matched calibration. Not surprisingly the %ME for the two isomers thymol and carvacrol are very similar.

Table 3. Concentration of phytochemical compounds in real samples in $\mu\text{g}/\text{kg}$.

Conc. Chicken Breast ($\mu\text{g}/\text{kg}$)	Menthol	Methyl salicylate	Carvone	Thymol	Carvacrol
Sample 1	15,9	104,2	57,1	3,8	6,3
Sample 2	4,9	53,8	18,8	4,2	6,8
Sample 3	7,6	43,8	22,1	6,4	9,0
Sample 4	9,4	90,1	33,8	6,1	8,7
Sample 5	12,6	47,7	24,3	7,5	10,1
Sample 6	5,0	28,0	9,9	6,6	9,2
Sample 7	7,1	81,1	31,0	7,3	9,9
Sample 8	18,8	74,6	36,0	6,1	8,7
Sample 9	15,9	98,8	61,9	7,8	10,5
Sample 10	10,2	100,7	45,8	3,6	6,1

3.3.5. LOQ

Instrumental LOQ were determined by injecting 10 times the lowest matrix matched standard at 4 µg/kg, the instrumental LOQs were always below the lowest calibration level and are reported in Table 2.

The SANTE 2019 criteria were fulfilled at the lowest spike level of 5 µg/kg and so the reporting level for the method was set at this concentration.

3.3.6. Recoveries and precision

The recoveries and the associated accuracy and precision values were within the acceptable interval of the SANTE 2019 criteria for all the three spike levels and both for intraday and interday measurements. The overall results are shown in Table 2.

3.4. Application to real samples

The validated method was applied to 10 samples of chicken breast bought in the local market. As shown in table 3 all the substances of interest were detected in the samples in amounts above the LOQ and with a quite variable range among the different samples, from just above the LOQ for thymol to 104 µg/kg for methyl salicylate.

From a consumer safety point of view, all the analyzed substances have been assessed and currently authorized for food [20] and feed [21] uses. For menthol and carvone an acceptable daily intake (ADI) is established, and it is equal to 4 mg/kg body weight for menthol [22] and 60 mg/kg body weight for carvone [23]. Even taking the highest concentrations found in the real samples the chronic exposure is more than 5 orders of magnitude below the aforementioned ADIs.

4. Conclusions

A simple analytical method involving a QuEChERS extraction followed by a dSPE cleanup step coupled with GC-MS/MS determination was demonstrated to be suitable for the quantification of phytochemical residues in chicken breast, a complex food matrix. The method has been validated according to the SANTE 2019 criteria for accuracy and precision. It was applied to real chicken breast samples and revealed the presence of the compounds of interest in all of them, however well below any level of concern for the consumer safety. This is possibly an indication of the widespread use of phytochemical feed additives after the ban of antibiotics as growth promoters by the European Union in 2006.

References

- [1] Dibner JJ, Richards JD. Antibiotic growth promoters in agriculture: history and mode of action. *Poult Sci* 2005;84: 634–43. <https://doi.org/10.1093/ps/84.4.634>.
- [2] Landers TF, Cohen B, Wittum TE, Larson EL. A review of antibiotic use in food animals: perspective, policy, and Potential. *Public Health Rep* 2012;127:4–22.
- [3] Castanon JIR. History of the use of antibiotic as growth promoters in European poultry feeds. *Poult Sci* 2007;86: 2466–71. <https://doi.org/10.3382/ps.2007-00249>.
- [4] Sebola NA, Mlambo V, Mokoboki HK, Hugo A, Muchenje V. Comparison of meat quality parameters in three chicken strains fed Moringa oleifera leaf meal-based diets. *Journal of J Appl Poult Res* 2018;27:332–40. <https://doi.org/10.3382/japr/pfy001>.
- [5] Yang C, Chowdhury MAK, Huo Y, Gong J. Phytochemicals as alternatives to in-feed antibiotics: potentials and challenges in application. *Pathogens* 2015;4:137–56. <https://doi.org/10.3390/pathogens4010137>.
- [6] Adaszyńska-Skwirzyńska M, Szczerbińska D. Use of essential oils in broiler chicken production – a review. *Ann Anim Sci* 2017;17:317–35. <https://doi.org/10.1515/aoas-2016-0046>.
- [7] Rychen G, Aquilina G, Azimonti G, Bampidis V, Bastos M de L, Bories G, et al. Guidance on the assessment of the safety of feed additives for the consumer. *EFSA J* 2017;15:e05022. <https://doi.org/10.2903/j.efsa.2017.5022>.
- [8] Wilkowska A, Biziuk M. Determination of pesticide residues in food matrices using the QuEChERS methodology. *Food Chem* 2011;125:803–12. <https://doi.org/10.1016/j.foodchem.2010.09.094>.
- [9] Anastassiades M, Lehota SJ, Stajnbauer D, Schenck FJ. Fast and easy multiresidue method employing acetonitrile extraction/partitioning and “dispersive solid-phase extraction” for the determination of pesticide residues in produce. *J AOAC Int* 2003;86:412–31.
- [10] Lehota SJ, Son KA, Kwon H, Koesukwiwat U, Fu W, Mastovska K, et al. Comparison of QuEChERS sample preparation methods for the analysis of pesticide residues in fruits and vegetables. *J Chromatogr A* 2010;1217:2548–60. <https://doi.org/10.1016/j.chroma.2010.01.044>.
- [11] Rejczak T, Tuzimski T. A review of recent developments and trends in the QuEChERS sample preparation approach. *Open Chem* 2015;13:980–1010. <https://doi.org/10.1515/chem-2015-0109>.
- [12] Perestrelo R, Silva P, Porto-Figueira P, Pereira JAM, Silva C, Medina S, et al. QuEChERS - Fundamentals, relevant improvements, applications and future trends. *Anal Chim Acta* 2019;1070:1–28. <https://doi.org/10.1016/j.aca.2019.02.036>.
- [13] Woźniak MK, Banaszkiwicz L, Wiergowski M, Tomczak E, Kata M, Szpiech B, et al. Development and validation of a GC-MS/MS method for the determination of 11 amphetamines and 34 synthetic cathinones in whole blood. *Forensic Toxicol* 2020;38:42–58. <https://doi.org/10.1007/s11419-019-00485-y>.
- [14] Analytical quality control and method validation procedures for pesticide residues and analysis in food and feed. Document N° SANTE/12682/2019. Available at, https://www.eurl-pesticides.eu/userfiles/file/EurlALL/AqcGuidance_SANTE_2019_12682.pdf. [Accessed 7 January 2020].
- [15] Rajski Ł, Lozano A, Uclés A, Ferrer C, Fernández-Alba AR. Determination of pesticide residues in high oil vegetal commodities by using various multi-residue methods and clean-ups followed by liquid chromatography tandem mass spectrometry. *J Chromatogr A* 2013;1304:109–20. <https://doi.org/10.1016/j.chroma.2013.06.070>.
- [16] Signal, Noise and Detection Limits in Mass Spectrometry. Agilent technologies Technical Note 5990-7651EN. Available at <https://www.agilent.com/cs/library/technicaloverviews/public/5990-7651EN.pdf>. [Accessed 7 January 2020].
- [17] Walorczyk S. Validation and use of a QuEChERS-based gas chromatographic–tandem mass spectrometric method for multiresidue pesticide analysis in blackcurrants including studies of matrix effects and estimation of measurement

- uncertainty. *Talanta* 2014;120:106–13. <https://doi.org/10.1016/j.talanta.2013.11.087>.
- [18] Blanks in Method Validation Supplement to Eurachem Guide The Fitness for Purpose of Analytical Methods. Eurachem; 2019. Available at, [MV_Guide_Blanks_supplement_EN.pdf](#). [Accessed 7 January 2020].
- [19] Poole CF. Matrix-induced response enhancement in pesticide residue analysis by gas chromatography. *J Chromatogr A* 2007; 1158:241–50. <https://doi.org/10.1016/j.chroma.2007.01.018>.
- [20] Commission Implementing Regulation (EU) No 872/2012 of 1 October 2012 adopting the list of flavouring substances provided for by Regulation (EC) No 2232/96 of the European Parliament and of the Council, introducing it in Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council and repealing Commission Regulation (EC) No 1565/2000 and Commission Decision 1999/217/EC. OJ L 267, 2.10. 2012. p. 1 . [Accessed 7 January 2021].
- [21] European Union Register of Feed Additives pursuant to Regulation (EC) No 1831/2003. Available online: https://ec.europa.eu/food/sites/food/files/safety/docs/animal-feed-eu-reg-comm_register_feed_additives_1831-03.pdf. [Accessed 7 January 2021].
- [22] Joint FAO/WHO Expert Committee on Food Additives. Meeting. (57th: 2001: Rome I. Safety evaluation of certain food additives and contaminants. World Health Organization; 2002.
- [23] Scientific Opinion on the safety assessment of carvone, considering all sources of exposure. *EFSA J* 2014;12:3806. <https://doi.org/10.2903/j.efsa.2014.3806>.