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Liquid chromatography–mass spectrometry analysis of carvacrol in chicken tissues

Tomasz Śniegocki

Department of Pharmacology and Toxicology,
National Veterinary Research Institute, 24-100 Puławy, Poland
sniego@piwet.pulawy.pl

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Abstract

Introduction: Carvacrol is an essential oil derived from oregano that is used as a natural additive to improve the efficiency of livestock nutrition. Residues of natural additives such as carvacrol should be monitored in food of animal origin to ensure consumer safety. The aim of this study was to appraise the quick, easy, cheap, effective, rugged and safe (QuEChERS) approach coupled with liquid chromatography and mass spectrometry as a means of carvacrol analysis in chicken tissue. **Material and Methods:** A 5 ± 0.05 g portion of plasma, lung, muscle and liver was mixed for 15 min with 5 mL of 1-butanol and 20 mL of water, then centrifuged. A 0.5 mL volume from the top layer was transferred, then 60 mg of octadecylsilane sorbent, 30 mg of primary and secondary amine and 200 mg of $MgSO_4$ were added. The extract was mixed and centrifuged. The top layer was filtered and then transferred to an autosampler vial for analysis by high-performance liquid chromatography–tandem mass spectrometry. **Results:** The limit of detection was calculated at 0.06 µg g^{-1} and the limit of quantification was 0.2 µg g^{-1}, with relative standard deviation repeatability and reproducibility below <20%. **Conclusion:** The validation results showed that this method could be a good alternative to determination of carvacrol by gas chromatography and is suitable for carvacrol analysis in different matrices.

Keywords: carvacrol, essential oils, QuEChERS, mass spectrometry–liquid chromatography, poultry.

Introduction

In recent years, various types of feed additives have become more and more popular, and are used both to improve the efficiency of nutrition and to improve the health status and condition of animals. The growing interest in essential oils as additives to feed is also the result of the 2006 ban on the use of feed antibiotics in the European Union (5, 11). The lack of antibiotics in poultry feed mix leads to a higher incidence of inflammation and diarrhoea, reduces weight gain and increases bird mortality, which makes production costs higher (4). Therefore, new substances are being sought that have a comparable positive impact to the banned antibiotics, especially additives from natural sources best meeting the expectations of consumers, who are increasingly looking for foodstuffs produced with the use of organic methods (29).

Carvacrol, a monoterpene phenol found in oregano, has been widely used as a food additive to preserve and enhance flavour, a cosmetic additive, and a disinfectant in dentistry (21). Recent studies have shown that carvacrol exhibits antibacterial, antiviral, antifungal, antioxidant, antimicrobial, and anti-inflammatory properties (21). Carvacrol is recognised by the US Food and Drug Administration and by the European Food Safety Authority (EFSA) as a substance generally safe for consumption (10, 21). However, EFSA also state that its presence should be monitored to ensure consumer safety (10). The calculated maximum safe concentration of this essential oil in complete feed is 22 mg kg^{-1} for chickens for fattening and 33 mg kg^{-1} for laying hens (9). No limit for muscle, liver, plasma, or lung tissue has been established yet, but Avila Ramos *et al.* (3) pointed out that feeding oregano oil to broilers increases carvacrol accumulation in breast meat. Therefore, carvacrol residues should be monitored in foods of animal origin.

Because of the properties of essential oils, the quantitative analyses of this and similar substances are typically performed using the extraction techniques such as steam distillation (30), pressurised liquid extraction (15), liquid–liquid extraction (2, 3, 15, 30), and microextraction such as solid-phase microextraction (19). The most popular detection methods are mass spectrometry or flame ionisation detection combined

with gas chromatography (2, 3, 7, 15, 19). For the determination of these substances, liquid chromatography with ultraviolet–visible spectrophotometry and fluorimetric and electrochemical detection (1, 13, 16, 27, 32) are also used, but to the best of the author's knowledge, there are no methods reported for carvacrol analysis in animal tissue using mass spectrometry combined with liquid chromatography.

That is why a new method using QuEChERS (quick, easy, cheap, effective, rugged and safe) extraction combined with mass spectrometry and liquid chromatography was developed. This is the first report on the determination of carvacrol in tissues with the use of this technique.

Material and Methods

Reagents. Ultrapure water was filtered through a Millipore Milli-Q system (Burlington, MA, USA). Methanol, isopropanol, 1-butanol, formic acid, anhydrous magnesium sulphate ($MgSO_4$), carvacrol and fipronil-$13C_4$ were obtained from Sigma Aldrich (St. Louis, MO, USA). Pre-heated magnesium sulphate ($MgSO_4$) was prepared in our laboratory for heating at 400°C overnight. Primary and secondary amine (PSA) and octadecylsilane sorbent (C18) were purchased from Supelco (Bellefonte, PA, USA). Formic acid was purchased from Avantor Performance Materials Poland (Gliwice, Poland). Nanosep MF 0.22 µm filters were supplied by Pall (DeLand, FL, USA). All reagents were of analytical grade or higher.

Individual stock standard solutions of carvacrol and fipronil-$13C_4$ as internal standard solutions at a concentration of 1 mg mL^{-1} were prepared in methanol and stored in the dark at below −18°C for no longer than six months. The working standard and internal standard solutions at a concentration of 0.01 mg mL^{-1} were prepared in methanol and stored in the dark at <6°C for no longer than three months.

High-performance liquid chromatography–tandem mass spectrometry. An ExionLC ultra-high performance liquid chromatography (UHPLC) system was connected to an API 5500 Qtrap mass spectrometer (both products of AB Sciex, Concord, ON, Canada). Analyst 1.6.3 software controlled the UHPLC-MS/MS system and Multiquant 3.2 software (both products of AB Sciex, Concord, ON, Canada) was used to process the data. The mass spectrometer was operated in the negative electrospray ionisation (ESI) mode with a capillary voltage of −4.5 kV. The temperature of desolvation was set at 300°C, nebuliser gas (N_2) at 40 psi; curtain gas (N_2) at 40 psi, collision gas (N_2) at high, gas 1 (air) at 40 psi and gas 2 (air) at 40 psi. The multiplier was set at 2100 V. The flow rate of the mobile phase was 600 µL min^{-1} and the injection volume was 10 µL. The chromatography was performed in a Kinetex XB-C18 column of 50 mm × 2.1 mm × 2.6 µm connected to a C18 precolumn of 4 mm × 2 mm × 4 µm (Phenomenex, Torrance, CA, USA). The mobile phase for liquid chromatography analysis consisted of two solutions: A of 0.5% isopropanol in 0.1% formic acid in water and B of methanol. The mobile phase gradient program started at 20% B, progressed to 95% B from 3 min to 6 min, then was 20% B at 6.2 min and was held for 2.8 min. The column was equilibrated for 2 min. The column operated at 35°C and the ions were monitored in multiple reaction monitoring (MRM) mode (Table 1).

Sample preparation. For the method optimisation and validation process, samples of muscle, plasma, liver and lung were obtained from a poultry farm. A 5 ± 0.05 g portion of plasma, lung, muscle and liver tissue sample was mixed with 100 µL of internal standard for 15 min on a Stuart STR4 General Rotator from Cole-Parmer (Vernon Hills, IL, USA) at minimum rotation speed (6 rpm) (0.05 × rcf) with 5 mL of 1-butanol and 20 mL of water, then centrifuged at 2,930 × rcf for 10 min at approximately 0°C. A 0.5 mL volume from the top layer was transferred to the tube, then 60 mg of C18, 30 mg of PSA and 200 mg of $MgSO_4$ were added. Next, the extract was mixed for 2 min on a vortex mixer at 349 × rcf and centrifuged at 2,930 × rcf for 10 min at approximately 6°C. A 0.3 mL volume of the top layer was transferred to Nanosep MF filters (0.2 mm) and centrifuged at 9,447 × rcf at room temperature for 10 min and then transferred to an autosampler vial for analysis.

Validation. The method was developed according to the International Council for Harmonisation of Pharmaceutical Requirements for Human Use (ICH) Q2 (R1) methodology and similarly to previously described methods (6, 12, 14, 17, 18, 25, 26). The following validation parameters were estimated: selectivity, limit of detection (LOD), limit of quantification (LOQ), working range, repeatability, reproducibility, matrix effect and uncertainty of the method. The matrix effect was calculated for each matrix at the second concentration level (1.0 µg g^{-1}) using the same method as previously described (24). In the selectivity study, possible interferences encountered in the method were checked for by analysing 20 blank samples for each matrix from different sources; no interferences were found (Fig. 1). Analyte standard solutions at concentrations 0.2, 1.0, 5.0, 20.0 and 50.0 µg g^{-1} were added to the blank sample containing an internal standard (5.0 µg g^{-1}) and then subjected to the QuEChERS extraction and HPLC-MS^2 procedure. The LOD and LOQ were estimated by calculations based on the signal-to-noise ratio, which was determined by comparing the measured signals from samples with known low concentrations of analyte with those from blank samples and establishing the minimum concentration at which the analyte could be reliably detected or quantified. A typical signal-to-noise ratio is 3:1 for LOD and 10:1 for LOQ (6, 8, 14, 17, 25, 31). Spiked blank samples were prepared as follows: standard solutions concentrations of 0.2, 1.0, 5.0, 20.0, 50.0 µg g^{-1} and internal standard (fipronil–$13C_4$) solutions at 5.0 µg g^{-1} were added to 5.0 g of sample. These spiked blank samples were analysed

according to the previously described procedure. The repeatability and reproducibility were determined at the same five concentration levels of 0.2, 1.0, 5.0, 20.0 and 50.0 µg g^{-1} for six samples at each level. The samples were analysed by the same operators on the same day with the same instrument, and this made for a valid repeatability parameter calculated as the relative standard deviation (RSD, %). For within-laboratory reproducibility, another two sets of blank samples were fortified and analysed by different operators on two different days with the same instrument, and reproducibility was also calculated as the RSD (%). The recovery was calculated by comparing the mean measured concentration with the fortified concentration of the samples. Any matrix effect was assessed by analysing five different samples at the second concentration level (1.0 µg g^{-1}) applying the equation proposed previously by Matuszewski *et al.* (20). The expanded uncertainty was calculated at the second concentration level (1.0 µg g^{-1}) by applying a coverage factor of 2, which gave a level of confidence of approximately 95% (31).

Results

The presented procedure is selective and able to detect carvacrol in various matrices such as muscle, lung, plasma and liver tissue. All required validation parameters, namely repeatability, reproducibility, LOD, LOQ, working range, matrix effect and uncertainty of the method are evaluated and presented in Tables 2 and 3.

Table 1. Parameters for the multiple reaction monitoring of carvacrol

Compound	Precursor ion (m/z)	Product ion (m/z)	DP (V)	EP (V)	CE (V)	CXP (V)
Carvacrol	149.0	134.0	−55	−10	−22	−9
		133.0	−55	−10	−38	−17
Fipronil-13C_4 (IS)	438.8	333.8	−130	−10	−22	−37

IS – internal standard; DP – declustering potential; EP – exit potential; CE – collision energy; CXP – collision cell exit potential. The quantifier ion is in bold

Table 2. Parameters obtained for the calibration curves during validation

Concentration (µg g^{-1})	Repeatability (RSD_r,%) (n = 6)	Within-lab reproducibility (RSD_{wR},%) (n = 18)	Expanded uncertainty (µg g^{-1})	Apparent recovery (%)
Lung				
0.2	3.8 ± 4.6	5.6 ± 4.4	-	103.9 ± 4.3
1.0	3.7 ± 3.6	4.8 ± 4.1	1.0 ± 0.27	107.7 ± 2.4
5.0	2.8 ± 2.9	3.2 ± 3.6	-	101.1 ± 2.5
20.0	3.2 ± 3.4	4.7 ± 3.6	-	104.9 ± 4.3
50.0	2.9 ± 3.2	4.9 ± 3.1	-	103.1 ± 4.3
Plasma				
0.2	4.1 ± 3.6	5.1 ± 4.1	-	102.6 ± 5.1
1.0	3.6 ± 3.0	4.3 ± 4.2	1.0 ± 0.23	102.7 ± 3.4
5.0	3.8 ± 2.9	3.4 ± 3.3	-	103.1 ± 2.7
20.0	3.2 ± 3.7	4.0 ± 3.6	-	102.6 ± 5.1
50.0	4.1 ± 2.9	4.7 ± 3.2	-	103.6 ± 2.3
Liver				
0.2	5.8 ± 4.3	6.3 ± 4.6	-	106.2 ± 7.8
1.0	4.9 ± 3.7	5.2 ± 4.3	1.0 ± 0.34	104.7 ± 4.1
5.0	3.8 ± 3.9	3.8 ± 3.7	-	104.1 ± 4.3
20.0	4.2 ± 3.0	4.7 ± 4.3	-	106.2 ± 3.8
50.0	3.7 ± 3.6	4.2 ± 3.1	-	103.2 ± 4.3
Muscle				
0.2	3.8 ± 4.5	4.6 ± 4.0	-	98.9 ± 3.0
1.0	3.6 ± 3.3	4.1 ± 3.7	1.0 ± 0.26	103.2 ± 3.4
5.0	2.8 ± 3.8	3.7 ± 3.2	-	105.1 ± 3.1
20.0	3.2 ± 3.4	4.0 ± 3.2	-	99.9 ± 3.9
50.0	3.7 ± 2.9	4.1 ± 3.6	-	103.6 ± 3.5

RSD_r – relative standard deviation for repeatability; RSD_{wR} – relative standard deviation for within-lab reproducibility

Table 3. Validation report for carvacrol

Matrix	LOD (µg g^{-1})	LOQ (µg g^{-1})	Matrix effect (%)	Working range (µg g^{-1})	Determination coefficient	Calibration curve
Lung	0.06	0.2	4.5 ± 1.9%	0.2–50.0	0.985	y = 0.025x + 0.04
Plasma	0.06	0.2	4.3 ± 2.7%	0.2–50.0	0.982	y = 0.065x + 0.03
Liver	0.06	0.2	6.4 ± 3.6%	0.2–50.0	0.980	y = 0.355x + 0.01
Muscle	0.06	0.2	3.3 ± 2.4%	0.2–50.0	0.991	y = 0.048x + 0.03

LOD – limit of detection; LOQ – limit of quantification

The analysis of 20 blank samples of the matrices did not reveal any interference (Fig. 1), which confirmed the good selectivity of the method.

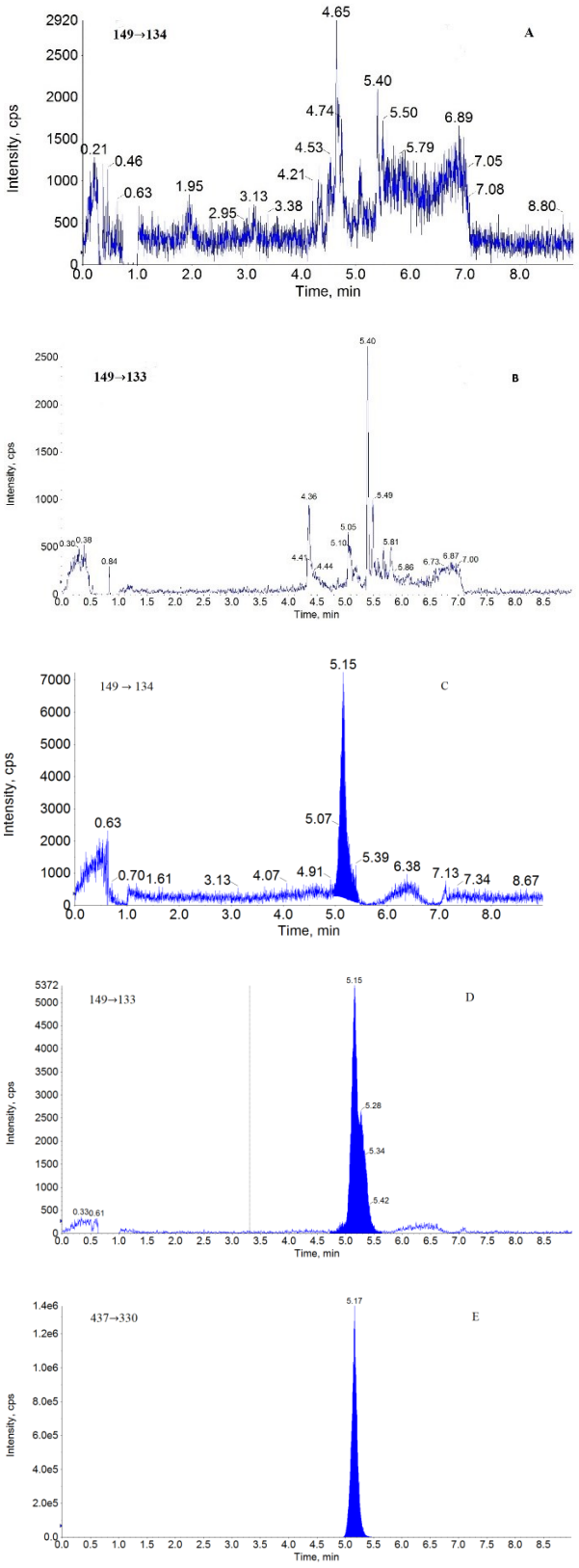

Fig. 1. Chromatograms obtained during validation. cps – counts per second; A and B – blank muscle sample; C and D – blank muscle sample containing carvacrol at the limit of quantification; E – internal standard

The linearity was evaluated based on the matrix-matched calibration curves, which were prepared by fortifying blank samples of different matrices before the extraction procedure at five concentration levels in the range of 0.2–50 μg g^{-1}. The repeatability was calculated as the relative standard deviation (RSDr, %) after the analysis of six samples spiked with carvacrol at the same five concentrations as described above. The samples were analysed on the same day with the same instrument. The spiked samples were analysed on two subsequent days with the same instrument and the same operators. The within-laboratory reproducibility was also calculated as the relative standard deviation (RSDwR, %) of the results obtained after fortifying another two sets of blank samples of the analysed compounds at the same concentration levels as for the repeatability and analysing them on two days with the same instrument and different operators. Similarly to how LOD and LOQ were estimated in other publications, they were arrived at by calculations based on the signal-to-noise ratio. The expanded uncertainty was calculated for each matrix at the second concentration level and is presented in Table 2. The matrix effect was determined for each matrix at 1.0 μg g^{-1} concentration and is presented in Table 3. The calculated ion suppression of the matrix effects for carvacrol in all matrices did not exceed 15%.

The correlation coefficient of the standard curves prepared with the carvacrol-fortified samples was ≥0.98. Depending on the matrix assayed, the coefficients of variation of repeatability for the fortified samples were in a 2.8–5.8% range, while the range for reproducibility was 3.2–6.3%. The average apparent recovery was 99.9–106.2%. Determination of LOD and LOQ were according to ICH Q2 (R1) and were respectively estimated at 0.06 μg g^{-1} and 0.2 μg g^{-1} for all matrices (14).

Discussion

Carvacrol is an essential oil the presence of which is usually determined by gas chromatography coupled with mass spectrometry or a flame ionisation detector (2, 3, 15, 17, 30). Another group of detection methods are voltammetry techniques (13, 14). The last group are methods for the determination of carvacrol using liquid chromatography coupled with classical UV-Vis or fluorescence detectors (1, 7, 32). The reasons for the recognised difficulty in the determination of carvacrol using the LC-MS technique are the size of the molecule, its volatility and the difficulty in obtaining a mass spectrum. Therefore, in order to optimise the conditions for the determination of this compound, both ESI and atmospheric pressure chemical ionisation (APCI) methods had to be tested in both positive and negative ionisation modes.

To achieve the maximum sensitivity, the mass spectrometry parameters, including the ionisation method (ESI or APCI), ionisation mode (negative or

positive), the capillary and voltages, source and desolvation gas temperatures, desolvation gas, collision energy (CE) and declustering potential (DP) were first optimised by direct flow infusion. In the case of positive ionisation, very weak spectra were obtained in both ESI and APCI modes. However, in the case of negative ionisation, both ESI and APCI modes achieved ions that were selected for further analysis (Table 1).

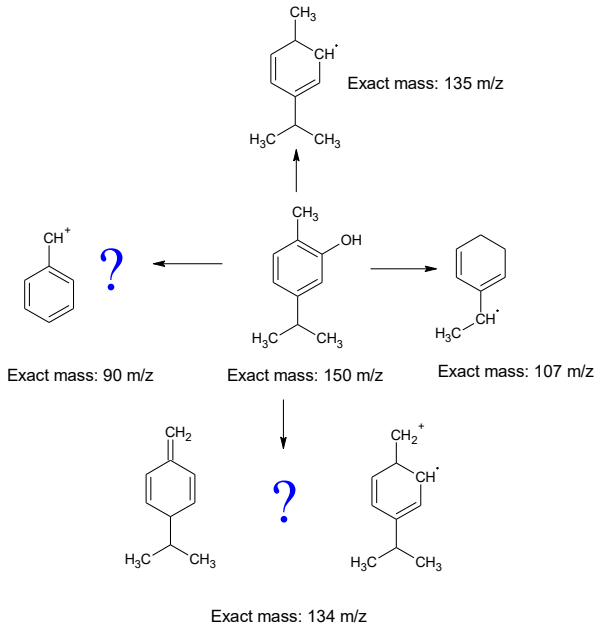

Fig. 2. The proposed carvacrol fragmentation path

Unfortunately, there is no information available on the fragmentation path for this compound in the negative ionisation mode using the electrospray technique. The scheme presented in Fig. 2 is a proposal and may be different from the real fragmentation path. The main ions obtained in this experiment in negative ionisation are 134, 133, 106 and 89 m/z. Ion 106 and ion 89 m/z have a relatively low signal, so they were omitted due to the sensitivity of the method. Figure 3 shows the fragmentation spectra at different collision energies (−15 V, −30 V and −45 V). The energies were chosen to show the differences in the fragmentation spectra and to confirm that ion 134 m/z is not an isotopic variant of ion 133 m/z. In Fig. 3a, both ions 134 m/z and 133 m/z are similar at a collision energy of −15 V. In Fig. 3b, the dominant ion 133 m/z can be seen at a collision energy of −45 V (ion 134 m/z is not present). In Fig. 3c, the dominant ion is the 134 m/z ion at a collision energy of −15 V. These spectra are evidence that ion 134 m/z is not an isotope of ion 133 m/z because it should be present at different collision energies. Additional evidence for this can be found in a publication on thymol an isomer of carvacrol (28). In that paper, the authors analyse the isotope ratios of thymol. Assuming analogies to thymol in carvacrol, the 134 m/z ion should be much smaller and according to Trivedi *et al.* (28) should not exceed 8% of the abundance of ion 133 m/z. In this case, ion 133 m/z is also the dominant ion, but the ratio of ion 133 to ion 134 m/z is about 70 ± 12% (Fig. 1c and d). Additionally, in the presented method, ion 134 m/z has a higher abundance and better repeatability and that is why the decision was made to make it a quantifier ion.

The results indicated that the ESI negative mode was more favourable than the APCI negative ion mode in yielding a better signal-to-noise ratio in real samples (Fig. 4). The ion shown in Fig. 4 is the ion with 133 m/z, because the most abundant ion in APCI mode is this ion, not the 134 m/z ion, which is less intense in the APCI mode.

Another problem was the selection of the internal standard. The ideal internal standard of carvacrol, which would be the isotope of the analyte, is unfortunately not available. Therefore, fipronil–$13C_4$ was chosen. Its advantage is that it ionises in both positive and negative modes. In addition, it is soluble in alcohols.

According to the published literature, the chromatographic columns typically used for the analysis of carvacrol are C18 (1, 13), a RP-amide C16 column and a monolithic column (7). The length of the column ranged from 150 mm to 250 mm, and the average retention time ranged from 7.9 (7) to 13.2 min (1). Therefore, based on the literature data, a C18 column was selected in this study, which provided a relatively short retention time of 5.2 min and very good chromatographic separation of carvacrol from interferences (Fig. 1). The most commonly used phase was ACN:H_2O (1, 7), but some authors used MeOH:H_2O:THF (32). Most researchers used the isocratic mode (1, 7, 32). In the current study, the decision was made to use 0.5% isopropanol in 0.1% formic acid in water as phase A and methanol as phase B in a gradient flow. Methanol was used because it is much cheaper and gives better separation than acetonitrile and reduces tailing of peaks. Additionally, based on our previous experience, a small amount of isopropanol was added because we have observed that the weaker isopropyl alcohol shows a smaller front and slightly higher peaks than methanol (22–24) (Fig. 1). The LOQ for this method is 0.2 µg g^{-1} for all matrices and after recalculation to mL the limits are 0.195 µg mL^{-1} for plasma, 0.193 µg mL^{-1} for liver tissue, 0.197 µg mL^{-1} for lung tissue and 0.194 µg mL^{-1} for muscle tissue and are similar to those achieved applying other methods, when the LOQ was generally in the range of 0.14–3.8 µg mL^{-1} (7, 13, 15, 27). Ares *et al.* (2) obtained an LOD of less than 15 µg/L for carvacrol, but because the Food and Drug Administration and the European Food Safety Authority have recognised carvacrol as a substance generally safe for consumption (10, 21) such a low limit is not necessary for the determination of carvacrol residues in tissues.

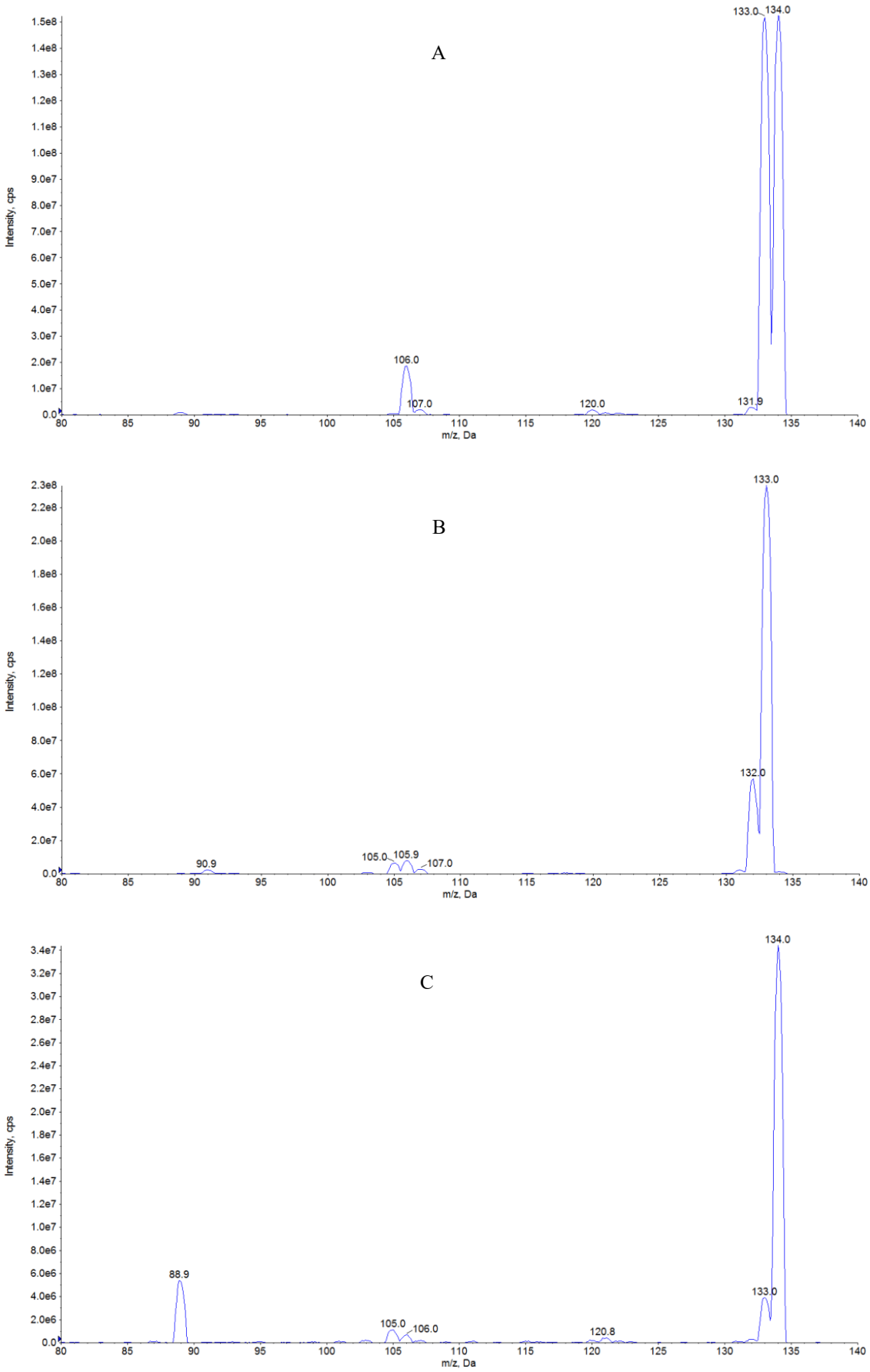

Fig. 3. Mass spectra of carvacrol at different collision energies. A – collision energy −30 V; B – collision energy −45 V; C – collision energy −15 V

Chromatography conditions were optimised; nevertheless the quality of results was also dependent on the optimisation of sample preparation. The most common extraction techniques are steam distillation (30), pressurised liquid extraction (15), liquid–liquid extraction (2, 3), and microextraction such as solid-phase microextraction (19). The aim of this study was to develop a fast, easy, inexpensive, effective, robust and safe method without using an evaporation step or additional equipment during the extraction step. Therefore, methods in which additional equipment was used were not considered (15, 19, 30). According to the literature, the most suitable solvents for carvacrol extraction in different matrices were methanol, hexane and chlorinated solvents (2, 3, 15, 30). The choice was made to avoid the use of chlorinated solvents because of their potential harm to health, which is greater than alcohols can inflict, for example. Injecting a sample dissolved in an immiscible solvent such as hexane can lead to possible precipitation and errors in analysis to which a procedure using methanol/water is not prone. Methanol is water-soluble and therefore also could not be taken under consideration.

Considering that carvacrol is a very volatile compound with low molecular mass (150.217) and soluble in alcohols but insoluble in water, different alcohols that dissolve poorly in water were tested to determine the most efficient extraction solvent. One of the alcohols taken into consideration was 1-butanol, which is the member of the butanol group of alcohols least soluble in water. The other isomers mix with water very well or without restriction, which can cause loss in recovery and significantly reduce extraction efficiency or even prevent extraction completely. The next group were alcohols of the pentanol group (1-, 2- and 3-pentanol), which are much less miscible with water. The other alcohols in this group mix well with water because of their row nature, which can cause a loss in recovery and significantly decrease the extraction efficiency. Based on the exploratory tests, the best results were obtained with 1-butanol extraction (Fig. 5). Further purification steps were designed based on previous experience with QuEChERS methods (24) and involved PSA and C18 sorbent. Amounts of C18 sorbent from 30 to 120 mg were used for the isolation of undesirable co-extracted compounds from 1-butanol extracts. The results of the experiments demonstrated that 60 mg of C18 sorbent was enough to remove the matrix compounds for 5 g of sample. In the QuEChERS technique, the addition of PSA is very common, because it removes polar compounds. For this reason, amounts of PSA sorbent from 0 to 120 mg were tested for the isolation of co-extracted matrix compounds from 1-butanol extracts. The results demonstrated that 30 mg PSA was enough to remove polar compounds. Additionally adding 200 mg $MgSO_4$ removed the residual water.

To the best of the author's knowledge, this is the first time a method coupling selective liquid chromatography with mass spectrometry with electrospray in negative ionisation mode has been developed for carvacrol. The same applies to a method based on QuEChERS with 1-butanol extraction without an evaporation step. The validation outcome indicated high repeatability and reproducibility, with an average recovery between 99.9% and 106.2%. The proposed method has proven to be linear, highly selective, and sensitive. This has shown that the determination of carvacrol is reliable with this method in all validated matrices and is a good alternative to the determination of carvacrol in samples analysed by other techniques with a similar LOD. It is the hope of the author that this work will help to spark a discussion on the determination of essential oils by liquid chromatography mass spectrometry – a field that still lacks enough attention in the scientific literature.

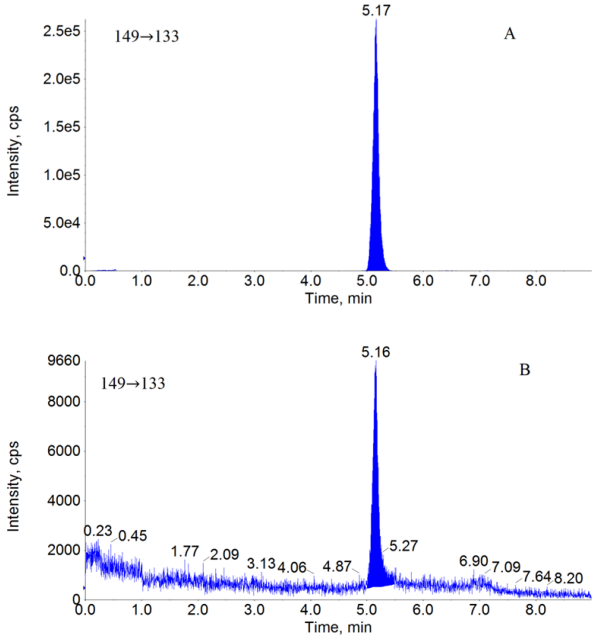

Fig. 4. Chromatograms obtained during optimisation. A – Muscle sample containing carvacrol at a 10 µg g^{-1} concentration in negative-ion electrospray ionisation mode; B – Muscle sample containing carvacrol at a 10 µg g^{-1} concentration in negative-ion atmospheric pressure chemical ionisation mode

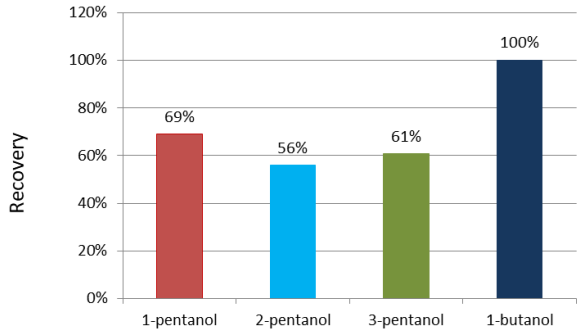

Fig. 5. Comparison of suitability of different extraction solvents for carvacrol

Conflict of Interests Statement: The author declares that there is no conflict of interests regarding the publication of this article.

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