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An improved extraction method of rapeseed oil sample preparation for the subsequent determination in it of azole class fungicides by gas chromatography

Mikhail F. Zayats [a,*], Sergey M. Leschev [b], Marina A. Zayats [b]

[a] Pesticide Dynamics Laboratory, RUE "Institute of Plant Protection", 2 Mira St., 223011 Priluki, Minsk Distr., Belarus
[b] Analytical Chemistry Department, Faculty of Chemistry, Belarusian State University, 14 Leningradskaya St., 220006 Minsk, Belarus

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ABSTRACT

The distribution of 19 azole class pesticides in hexane/aqueous–organic mixtures systems and rapeseed oil (or oil solution in hexane)/organic solvents has been studied at 20 ± 1 °C. The distribution constants (P) and coefficients (D) between hydrocarbon and polar phase are calculated. It is found that all the studied pesticides are hydrophobic, i.e., in hexane–water system $\log P \gg 0$. Replacement of water by organic solvents results in sharp $\log P$ falling, and their values become negative. It is revealed that solutions of strong inorganic acids in anhydrous acetonitrile extract azole class pesticides from hexane and vegetable oils most fully and selectively. In particular, the acidification of acetonitrile causes a drop of D values in 50–2000 times for the majority of the studied pesticides. This phenomenon was used for the development of the improved technique for the quantitative analysis of a widely used azole class pesticides, which can be presented at trace levels in rapeseed oil. The proposed methodology is based on dissociation extraction (DE) of azoles using perchloric acid in anhydrous acetonitrile, with following clean-up of acetonitrile extract from organic impurities by hexane and aqueous solution of dipotassium hydrogen orthophosphate, and final GC–ECD (gas chromatography with electron capture detection) determination of azole fungicides. The values of obtained recoveries were between 85% and 115% with RSD values below 10%. The obtained limits of quantitation, ranged from 3.0 to 300 μg kg^{-1}, are below the maximum residue levels (MRLs) set by the European Union for the majority of pesticides. The developed method was successfully applied to different rapeseed oil samples.

1. Introduction

One of the key prerequisites for obtaining high yields of rapeseed is an effective chemical plant protection against a complex of pests, diseases and weeds [1,2]. Compounds of conazole class (triazoles and imidazoles) are widely used as fungicides in large amounts at rape cultivation [2]. Although they are usually decomposed before harvesting, some trace amounts of pesticide residues can contaminate the final production [1,2]. Monitoring of pesticide residues in food is of great interest and is regulated in many countries by setting maximum residue levels (MRLs) for pesticides in food [3,4].

The analysis of pesticide residues in vegetable oils is very complicated, because of the inherent complexity of the matrix, mainly comprising triglycerides (98–99%). Solvent extraction is probably the main strategy used for the sample preparation of oily matrices, at least at the first stage [3]. At the same time it is very difficult to avoid co-extraction of fatty material and other matrix components, especially taking into account that the majority of the pesticides are lipophilic compounds [5]. Despite advances in the development of highly efficient chromatographic instrumentation for the determination of pesticides, sample preparation remains an important part of analysis. Since even small amount of matrix components can harm the different parts of the instruments used and interfere with analytes, the extraction step is usually followed by purification of extracts.

The choice of sample treatment is also related to the detection method. The more sensitive and specific detection method is used, the less stages of sample treatment will be required. Thus, a 10-fold dilution of sample in methanol acidified with 1% acetic acid was enough to determine 70 pesticides covering a wide range of polarity using the scheduled selected reaction monitoring mode in analysis by liquid chromatography/tandem mass spectrometry

* Corresponding author. Tel.: +375 292763835; fax: +375 175092367.
E-mail address: mikhail_zayats@tut.by (M.F. Zayats).

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[6]. The on-line coupling of reversed-phase liquid chromatography and gas chromatography through oven transfer adsorption–desorption interface allowed to inject olive oil directly with no sample pre-treatment step other than filtration [7]. In spite of absence of any sample preparation stages, the recoveries of 6 from 8 studied pesticides were lower than 46%. Spoiling of chromatographic instruments makes the usage of such techniques in routing analysis in both cases very problematic.

Some simple methods of determination of organophosphorus pesticide residues in olive oil were published. They consisted in a single [8] or 2-times [9] extraction with acetonitrile from undiluted [9] or diluted with hexane [8] olive oil. No cleanup was necessary because there were no interferences in the chromatogram at GC–FPD [9] or GC–NPD [8] determination. The advantages of these methods of sample preparation are good recoveries of analytes (74–118%) and absence of interference peaks on chromatograms. From the other hand, a large amount of matrix components can reduce the life time of the chromatographic instruments. In most of the papers cited further clean-up of the solvent extracts was necessary to improve quantitative results in subsequent chromatographic analysis.

Low-temperature lipid precipitation is a simple low-cost method of purification of acetonitrile extracts from vegetable oils [10,11]. This simple clean-up procedure was applied for organophosphorus and triazine pesticides determination in olive oil, soybean oil, peanut oil and sesame oil. Recoveries of studied pesticides were between 51% and 112%. Low temperature purification technique was enough for the chromatographic system to maintain its separation efficiency for at least 100 sample injections. From the other hand the clean-up stage is time consuming (acetonitrile extracts were stored at −20 °C overnight) and often followed by further purification steps [3].

Gel permeation chromatography (GPC) is one of the more widely spread techniques in the analysis of pesticides residues in vegetable oil. It is generally used after a preliminary liquid–liquid partition with acetonitrile for the subsequent GC analysis with electron-capture (ECD), thermoionic specific (TSD), nitrogen–phosphorus (NPD) and mass-spectrometric (MS) detection [3,12,13]. The recoveries in most cases were satisfactory (>84%). The main drawback of GPC is the partial overlapping between the pesticide fraction and the components from the matrix (mainly triglycerides). For this reason, an additional clean-up on Florisil is usually included after GPC step [3]. The need of additional instrumentation and relatively long duration of sample preparation should be noted as a disadvantage also.

Solid-phase extraction is one of the most popular techniques in sample preparation of vegetable oils [14]. It has been used mainly for clean-up purposes and is usually performed in a column/cartridge. Amongst the advantages of SPE over liquid partitioning procedures are higher precision and throughput, and lower solvent consumption, avoiding also the formation of emulsions, which are often time-consuming. Typical sorbents for SPE are Florisil [15,16], alumina [15,16], graphitized carbon black [16], ENVI-Carb [15], C18 [15,16], silica [17]. New materials as multi-walled carbon nanotubes (MWCNTs) and single-walled carbon nanotubes (SWCNTs) are also successfully applied for clean-up purposes [18]. Recent sample treatment protocols focus on the use of either a unique sorbent or the combination of two or more commercially available SPE sorbents for clean-up procedure [17]. SPE method is characterized by satisfactory recoveries in most cases and clean extracts with lower consumption of organic solvents. By contrast, these methods are time- and labour-consuming, with several manual stages.

The QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) sample preparation approach for the analysis of pesticide residues became very popular nowadays [19–23]. It was used in different modifications, when some steps or reagents were excluded [19,20] or changed [21]. The QhEChERS methodology for vegetable oils ordinary comprises the following steps: addition of water, acetonitrile, NaCl, anhydrous $MgSO_4$, and shaking for pesticide extraction. The extract is then centrifuged and cleaned-up by dispersive SPE (d-SPE) with primary secondary amine (PSA) sorbent, anhydrous $MgSO_4$, C18 and graphitized carbon black (GCB). The next steps include again shaking, centrifugation, evaporation, reconstituted in the appropriate solvent and filtration. Despite the multistage character of procedure, the recoveries were satisfactory in most cases [19–23].

Matrix solid-phase dispersion (MSPD) is a relatively new extraction and clean-up technique for pesticide multiresidue analysis based on SPE principle [24,25]. The main difference between MSPD and classic SPE is the physical state of samples applied to the column. In SPE samples are liquid (solution) before application to the column whereas in MSPD samples are applied to the column in solid form (dispersed mixture of sample and solid support). Such approach was used in multi-residue method for clean-up of extract obtained from olive oil by liquid-liquid partitioning with acetonitrile saturated with petroleum ether for further GC–MS and LC–MS/MS determination [24,25]. The drawback of this method is the multistage and need for an additional clean-up on SFE column [24,25].

In general, the methods, which allow to obtain relatively clean extracts, are multistage, time- and solvent-consuming and expensive [3]. The main reason for this is low selectivity of extraction of pesticides from vegetable oils, and the interference of the matrix components at final determination, which leads to errors. Moreover, the majority of methods, mentioned above, are directed to removal of main matrix components (triglycerides) while minor compounds (acids, steroids, tocopherols, etc.) are not taken into account. At the same time carboxylic acids, for example, may present in oil in significant amounts [26–27], and can cause such adverse effects as formation of foams and emulsions at extraction process. Unsaturated carboxylic acids are thermally unstable and decompose in GC injector, which leads to its contamination or even spoiling.

This work is focused on the development and evaluation of an improved and simple sample preparation strategy of rapeseed oil for the subsequent GC–ECD determination of azole fungicides, usually used at rape cultivation.

2. Materials and methods

2.1. Chemicals and reagents

19 analytical standards of the active ingredients (AI) of pesticides (Fig. 1) the residues of which are controlled in agricultural products in the Pesticide Dynamics Laboratory of the Institute of Plant Protection (Priluki, Minsk distr., Belarus) were used in this study. Pesticide standards of analytical grade quality (⩾98%) were supplied by «Cheminova A/S» (Lemvig, Denmark), «Bayer CropScience AG» (Frankfurt-on-the-Main, Germany), «Syngenta Crop Protection Münchwilen AG» (Münchwilen, Switzerland), «BASF Agricultural Center Limburgerhof» (Limburgerhof, Germany), "Dow AgroSciences LLC" (Indianapolis, USA), Crompton Co. (Guelph, Canada), Isagro S.p.A. (Milan, Italy). Stock solutions of pesticides with concentration 100 mkg mL^{-1} were prepared in acetonitrile.

HPLC-gradient grade acetonitrile was purchased from Panreac (Barcelona, Spain). Analytical grade acetone and hexane, reagent grade dimethyl sulfoxide, sulfuric acid, hydrochloric acid and dichloromethane were purchased from EKOS-1 (Moskow, Russia). 85% orthophosphoric acid was purchased from Belreahim (Minsk, Belarus). Analytical grade ethylene glycol was purchased from

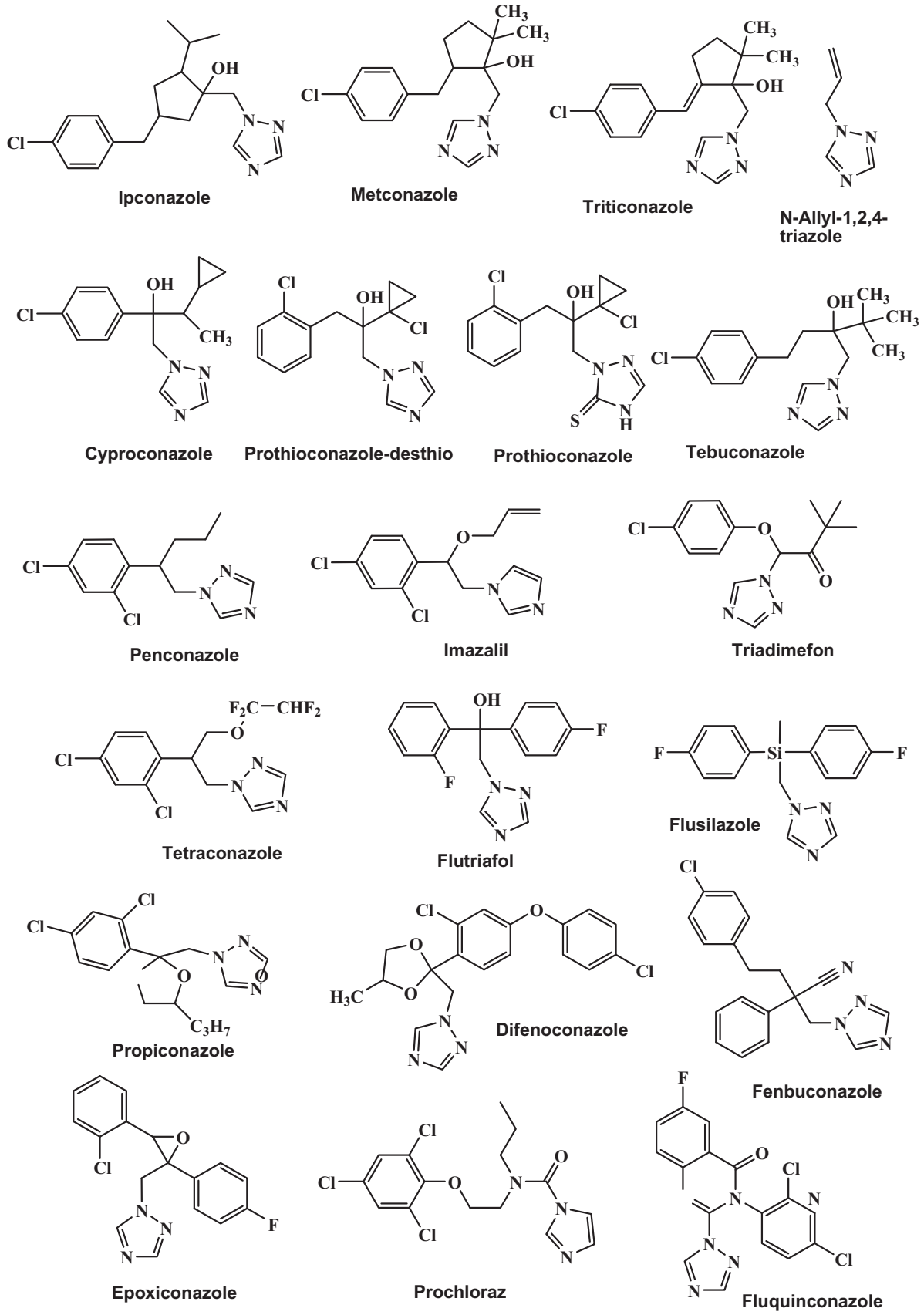

Fig. 1. Structure formulas of azole pesticides and N-allyl-1,2,4-triazole.

MERCK (Darmstadt, Germany). Analytical grade 65% perchloric acid and reagent grade dipotassium hydrogen orthophosphate were purchased from firm "Five Oceans" (Minsk, Belarus). N-allyl-1,2,4-triazole was synthesized, purified and kindly provided by Organic Chemistry Department of Belarusian State University (Minsk, Belarus). Rapeseed oil was isolated from seeds by hexane extraction with the subsequent extract evaporation using rotor evaporator at 40 °C.

2.2. HPLC analysis

LC analysis was performed with the «HP 1100» (Hewlett Packard, Germany) HPLC instrument comprising degas-unit, pump, manual injector (Rheodyne) with 20 μl loop, column oven and diode array detector. The LC was equipped with a 250 mm × 2.1 mm i.d. LC column (XBridge BEH300, 5 μm C18, Waters, Milford, USA). The Chemstation software was used for the instrument control and the data handling.

The compounds were separated by gradient elution. The eluent A was an aqueous solution of phosphoric acid (0.02 mol L^{-1}) and the eluent B was acetonitrile. The composition was maintained at 40% of eluent B at 1 min then changed from 40% B to 80% B during 17 min and then remained isocratic for 25 min. Then it was changed back to 40% B during 0.5 min and the column was re-equilibrated for 7 min before the next injection. The flow rate was 0.15 mL min^{-1}. The column temperature was maintained at 35°C. Determination of allyltriazole concentrations was carried out by HPLC separately from other pesticides in isocratic elution conditions – 5% (by volume) solution of acetonitrile in water.

Detection of allyltriazole was performed at a wavelength of 194 nm; triticonazole, fluquinconazole, prothioconazole – at 260 nm, other azoles – at 220 nm.

2.3. GC analysis

GC analysis of oil extracts was carried out by using a Crystal 5000.2 GC with an electron capture detector (Chromatec, Russia). The GC was equipped with a 30 m × 0.32 mm i.d., 0.5 μm film, HP-5MS column.

2 μL was injected in splitless mode. The split valve was closed for 1 min and the analytes were transferred to the GC column from the liner, after that the split was opened at ratio of 1/80 for 2 min for venting the solvent residues from the liner. In 3.00 min after the injection the split ratio was switched to 1/20. The injector temperature was maintained at 280 °C.

Nitrogen of 5.0 grade quality (99.999%) was used as carrier gas at the constant inlet pressure (135 kPa). The oven temperature was maintained at 70 °C for 2 min after the injection and then programmed first at 25 °C min^{-1} to 170 °C, then at 3 °C min^{-1} to 230 °C, and finally at 10 °C min^{-1} to 280 °C and was maintained for 19 min. The ECD temperature was maintained at 320 °C.

2.4. Determination of partition constants and coefficients of azoles

The experiments on determination of partition constants and coefficients were carried out at 20 ± 1 °C. Azole concentrations were determined by HPLC-DAD. The choice of HPLC-DAD is explained by some advantages of this method under GC–ECD. Better repeatability of obtained results, wider linear range of detection (0.03–60 μg mL^{-1}), similar sensitivity to all the studied compounds and the possibility of analyzing of DMSO, ethylene glycol and acidified acetonitrile phases are the main reasons of it. Moreover, prothioconazole and flusilazole cannot be determined by GC–ECD, as first is non-volatile and second has very weak electron-absorbing properties compared to other azoles. Studied pesticides were analyzed individually and then combined into 5 groups (3–5 substances) in order to reduce the time of experiment and avoid the overlapping of the peaks of substances in the chromatogram. The resolution (Rs) measured between every two adjacent peaks at the chromatograms of every group was above 1.5. Pesticide mix stock solutions of each group (20 mg L^{-1} for each pesticide) were prepared from stock solutions of individual substances. Azole concentrations in investigated water-acetonitrile solutions and organic solvent were determined by HPLC directly or after preliminary 2-time dilution with water. The determination of pesticide concentration in hexane was carried on by preliminary blowing of hexane in an air stream and dissolution of the dry residue in water–acetonitrile mixture (1–1, by volume). The partition constants of pesticides (P) were calculated according to the following equation:

$$P = \frac{C_{hex}}{C_{pol}}, \quad (1)$$

where C_{hex} and C_{pol} are the steady-state concentrations of the substance in hexane and polar phases, respectively.

Partition coefficients (D) of azoles between hexane and solutions of acids in water or acetonitrile were calculated according to the following equation:

$$D = \frac{C_{hex}}{C_{pol}^{sum}}, \quad (2)$$

where C_{pol}^{sum} is the total concentration of molecular and protonated forms of azole in the polar phase.

Standard deviations in the calculated partition constants (coefficients) did not exceed 10%.

The recoveries of substances (R) from rapeseed oil sample were calculated with the use of following equation:

$$R = \frac{m_{det}}{m_{add}} \times 100\% \quad (3)$$

where m_{add} and m_{det} are pesticide amounts, added to oil and determined in the final extract, respectively.

The recoveries of azoles by sample preparation method of rapeseed oil were calculated at two concentration levels by comparing the chromatograms of samples of rapeseed oil with artificial additives of azoles and without them (n = 5). The quantity of azoles in the extracts was determined by the absolute calibration method.

To explain the dependence of the P values of pesticides on the nature and composition of the polar phase the increments of methylene and functional groups of the logarithm of partition constants were used. The increments of methylene, phenyl, vinyl and hydroxyl groups were taken from [28,29]. The increment of N-triazolyl group was calculated according to the following equation:

$$I_{C_2H_2N_3} = lgP_{all-tr} - I_{CH_2} - I_{C_2H_3} \quad (4)$$

where lgP_{all-tr} is the logarithm of partition constant of allyl-triazole, I_{CH_2} and $I_{C_2H_3}$ are the increments of methylene and vinyl groups, respectively, which were taken from [28,29]. Standard deviations in the calculated $I_{C_2H_2N_3}$ did not exceed ±0.1–0.2.

2.5. Determination of partition coefficients of oleic acid

Determination of the partition coefficients of oleic acid was carried out by titration of steady-state phases with 0.01 mol L^{-1} KOH solution in water in the presence of phenolphthalein. The titration of hexane and oil phases was carried out after preliminary addition of 96% ethanol (2 volumes of 96% ethanol were added to 1 volume of the titratable phase).

3. Results and discussion

3.1. Distribution of pesticides and matrix components

The optimal conditions of extraction and preconcentration of azoles from oily matrices were selected on the basis of experimentally determined distribution constants of azoles between hexane, which can be considered as an approximate model of vegetable oils, and water–organic mixtures (Table 1).

As it seen from Table 1, the replacement of water for water-organic mixtures and organic solvents leads to a dramatic drop of pesticide log$P$ values, reaching 4–5 units for some substances. In most cases the magnitude of log$P$ changes from positive to negative.

This phenomenon is typical for hydrophobic organic substances and is caused by the essential fall of solvophobic effect of the polar phase characterized by the increment of the methylene group when the water is changed to polar organic solvents and aqueous organic mixtures [28].

It is interesting that distribution constants of the majority of pesticides pass through a pronounced minimum at water content about 10% in the aqueous acetonitrile phase despite an increase of I_{CH_2}. At the same time the distribution coefficient of rapeseed oil in general and oleic acid in particular slightly decreases compared with anhydrous acetonitrile. It means that mixture of acetonitrile–water (9–1, by volume) extracts azole pesticides from rapeseed oil more efficiently and selectively than pure acetonitrile.

Similarly, the distribution constants of azole pesticides are reduced by small additives of ethylene glycol. The reason for this phenomenon is described in literature on the example of aliphatic amines, and this phenomenon is also characteristic of water–methanol and water–acetonitrile solutions [29].

Despite the fact that the hexane/DMSO system is characterized by higher I_{CH_2} than the hexane/acetonitrile system, distribution constants of azoles in hexane/DMSO are lower due to several reasons.

Firstly, a significant part of the carbon skeleton of the azole molecules consists of not only the methylene groups but also of aromatic rings, the increments of which are less for the hexane/DMSO system than for the hexane/acetonitrile one [28].

Secondly, significantly lower increments of hydroxyl and N-triazolyl groups are observed for the system hexane/DMSO compared to the hexane/acetonitrile system (Table 2), which leads to a strong decrease of distribution constants of azoles containing OH-groups.

As follows from Table 1, the distribution constants of less hydrophobic pesticides (flutriafol, imazalil, triticonazole, cyproconazole) in the hexane – ethylene glycol system are less than in the hexane – acetonitrile system. It is the result of a greater contribution of increments of hydrophilic groups to the change of distribution constants of these substances (Table 2).

Replacement of hexane for rapeseed oil increases the distribution coefficients due to the solvation of the pesticide molecules by oil ester groups. Thus, for the systems with ethylene glycol and acetonitrile the increase of the distribution coefficients is equal to 0.8–1.5 orders (Table 1). Consequently, the effectiveness of solvent extraction of studied azoles from rapeseed oil decreases significantly in comparison with hexane. From the practical point of view it means that large volumes of solvent must be used for quantitative extraction of analytes. For example, for 95% recovery of prothioconazole–desthio from rapeseed oil a 13-fold volume of acetonitrile must be used at a single extraction.

Dimethylsulfoxide extracts azole class pesticides from oil (such as from hexane) most completely among all organic solvents. At the same time, DMSO extracts from oil quite a large number of components, interfering with further chromatographic analysis.

Ethylene glycol can also be used for the extraction of several azoles but it extracts almost all the studied pesticides worse than the above extractants (Table 1). In general, all polar extractants, mentioned above, are not very effective and selective for the isolation of pesticides from oil matrices.

Acidified solutions of acetonitrile extract azole pesticides from oil (such as from hexane) much more effectively than all above-mentioned extractants (Table 3). This is caused by the presence of 1,3-imidazole or 1,2,4-triazole ring capable to protonation in

Table 1
Logarithms of distribution constants of pesticides, allyl-triazole and logarithms of distribution coefficients of oleic acid, sum of rapeseed oil acids and rapeseed oil in partition systems hexane/aqueous solutions of acetonitrile, hexane/ethylene glycol, hexane/mixture of acetonitrile and ethylene glycol, hexane/DMSO, rapeseed oil/acetonitrile, rapeseed oil/ethylene glycol at 20 °C.

Substance	H_2O	20% AcN[a]	50% AcN	80% AcN	90% AcN	AcN	Ethgl[a]	AcN–Ethgl (9–1, v/v)	DMSO[a]	Oil[a]–AcN	Oil–Ethgl
Cyproconazole	1.07	0.37	−0.92	−1.58	−1.70	−1.30	−1.67	−1.79	−2.50	−0.42	−0.39
Difenoconazole	2.80	1.50	−0.58	−1.16	−1.67	−1.53	−0.84	−1.74	−1.98	−0.38	−0.09
Epoxyconazole	1.84	0.80	−0.91	−1.76	−1.85	−1.88	−1.17	−1.96	−2.28	−0.73	−0.06
Fenbuconazole	1.45	–	−1.58	−2.27	−2.52	−2.38	−1.06	–	−3.18	–	–
Fluquinconazole	1.8	–	−0.77	−1.48	−1.72	−1.76	−0.60	–	−2.58	–	–
Flusilazole	2.13	–	−0.90	−1.49	−1.93	−1.80	−0.66	–	−2.21	–	–
Flutriafol	0.26	−0.46	−1.69	−2.25	−2.34	−2.16	−2.22	−2.39	−3.45	−1.00	−1.11
Imazalil	1.83	–	−0.86	−1.50	−1.69	−1.27	−1.43	–	−3.15	–	–
Ipconazole	2.45	–	−0.63	−1.14	−1.61	−1.34	−0.92	–	−2.10	–	–
Metconazole	1.70	0.81	−0.76	−1.61	−1.64	−1.44	−1.36	−1.68	−2.19	−0.28	0.00
Penconazole	2.14	–	−0.43	−1.02	−1.28	−1.11	−0.37	–	−1.37	–	–
Prochloraz	2.18	–	−0.84	−1.16	−1.75	−1.52	−0.61	–	−1.74	–	–
Propiconazole	2.21	1.28	−0.28	−1.10	−1.23	−1.18	−0.48	−1.28	−1.21	−0.31	0.31
Prothioconazole	−0.19	–	−1.65	–	−2.46	−2.21	−0.87	–	−2.89	–	–
Prothioconazole–desthio	1.87	1.30	−0.21	−0.88	−1.04	−0.92	−0.60	−1.09	−1.80	−0.17	–
Tebuconazole	1.49	0.71	−0.89	−1.63	−1.71	−1.63	−1.45	−1.76	−2.21	−0.33	−0.16
Tetraconazole	1.79	–	−1.19	−1.90	−2.19	−2.01	−1.08	–	−2.59	–	–
Triadimefon	1.95	–	−0.56	−1.45	−1.53	−1.43	−0.16	–	−1.84	–	–
Triticonazole	0.66	0.01	−1.31	−1.94	−2.02	−1.82	−2.14	−2.06	−2.65	−0.39	−0.62
Allyl–triazole	−1.82	−1.95	−2.17	−2.23	−2.11	−1.73	−2.04	−1.94	−2.04	–	–
Oleic acid	–	–	1.10	0.26	0.05	0.01	–	–	–	0.40	–
Sum of rapeseed oil acids[b]	–	–	1.41	0.52	0.30	0.30	–	–	–	–	–
Rapeseed oil	–	–	–	–	1.95	1.80	–	–	–	–	–

[a] Solvents: AcN – acetonitrile, Ethgl – ethylene glycol, DMSO – dimethyl sulfoxide; oil – rapeseed oil.
[b] Distribution coefficients of sum of rapeseed oil acids and rapeseed oil were determined in partition system hexane:rapeseed oil (3:1)/aqueous solutions of acetonitrile; the content of free acids in undiluted oil was ~12 g L^{-1} in terms of oleic acid.

Table 2
Increments of methylene I_{CH_2} [a], phenyl $I_{C_6H_5}$ [a], vinyl $I_{C_2H_3}$ [a], hydroxyl I_{OH} [a] and *N*-triazolyl $I_{C_2H_2N_3}$ groups of distribution constants for extraction systems hexane/aqueous solutions of acetonitrile, hexane/ethylene glycol, hexane/mixture of acetonitrile and ethylene glycol and hexane/DMSO at 20 °C.

Solvents[b]	I_{CH_2}	$I_{C_6H_5}$	$I_{C_2H_3}$	I_{OH}	$I_{C_2H_2N_3}$
H_2O	0.63	2.0	1.1	−3.8	−3.6
20% AcN	0.48	1.6	1.1	−3.0	−3.5
50% AcN	0.29	0.7	0.8	−2.5	−3.3
80% AcN	0.22	0.2	0.2	−2.5	−2.6
90% AcN	0.19	0	0	−2.4	−2.3
AcN	0.13	0	−0.1	−1.8	−1.8
Ethgl	0.27	0.7	0.6	−2.9	−2.9
AcN–Ethgl = 9–1	0.17	−0.1	–	−2.3	–
DMSO	0.22	−0.1	0.3	−2.6	−2.6

[a] Increments of methylene, phenyl, vinyl and hydroxyl groups were taken from [28,29].
[b] Solvents: AcN – acetonitrile, Ethgl – ethylene glycol, DMSO – dimethyl sulfoxide.

molecules of fungicides, and the high activity of hydrogen ions in anhydrous acetonitrile. Even a small addition of water significantly reduces this effect (Table 3). The use of more concentrated 0.1 M solution of perchloric acid in AcN allows to decrease log*D* of pesticides in comparison with 0.01 M solution. Obviously, such low values of log*D* of pesticides are estimative only, but they illustrate the effectiveness of the use of acidified acetonitrile solutions.

When comparing sulfuric and perchloric acids, one should give preference to the latter because distribution coefficients with its use are less. This phenomenon can be explained by greater strength of perchloric acid than sulfuric acid and the differentiating influence of acetonitrile on the strength of acids. It should be noted that even slight acidification of acetonitrile with perchloric acid leads to dramatic fall of logarithms of distribution coefficients of azole class pesticides. This fall is about 2.2–3.4 logarithm units on average (for tetraconazole and penconazole, respectively). The exception is prothioconazole where there is no reduction in log*D*, fluquinconazole, flusilazole and triadimefon where the decrease in log*D* is 0.9–1.8.

A triazole ring, presenting in the molecules of the considered pesticides, is also protonated in aqueous solution, but at much higher concentration of acid. Thus, when reducing the pH from 7 to 0, i.e. when passing from hexane/water extraction system to hexane/1 M hydrochloric acid solution in water the logarithms of distribution coefficients of considered azoles are reduced by 1.6–2.3 units for epoxiconazole and cyproconazole, respectively. This is equivalent to a reduction of distribution coefficients in 40–200 times. Nevertheless, it is not enough for some pesticides for their complete extraction from oil matrices. This phenomenon can be used for the separation of the pesticides from coextractive components of rapeseed oil at a purification step.

From the practical point of view it is more conveniently to carry out the extraction process not from oil, but from oil diluted 3–4 times with hexane. It allows to reduce its viscosity and to reduce the time needed for phase separation and reaching equilibrium.

It can be seen (Tables 1 and 3) that acidification of acetonitrile with perchloric acid (0.1 mol L^{-1} solution) allows to use 50–300 times less volume of solvent for oil sample preparation (for epoxiconazole and prothioconazole–desthio, respectively) to achieve the same recoveries of azole pesticide residues, even taking into account a 3-times dilution of oil with hexane.

So, a one-step extraction with 0.1 mol L^{-1} perchloric acid solution in acetonitrile is enough for quantitative (>95%) extraction of considered azoles (with $\log D \leqslant -2.1$) from the 3 times diluted rapeseed oil with hexane at the ratio of phase volumes diluted oil – acetonitrile equal to 6–1. Thus, it is possible to carry out 2 times preconcentration of pesticides in acetonitrile relatively undiluted oil. The exception is fluquinconazole, the recovery of which under such conditions is about 87%.

The acetonitrile extract can be additionally purified from hydrophobic matrix components (oil and oil acids) by its washing with hexane. To remove most pigments and residual matrix moderate hydrophobic compounds, the extract should be diluted with 1 mol L^{-1} aqueous solution of hydrochloric acid and washed with small amount of hexane (see below). Then the extract should be neutralized to pH = 7–8), using 10% dipotassium hydrogen orthophosphate (K_2HPO_4) solution and the pesticides can be extracted by dichloromethane ($P > 500$). At this stage hydrophilic substances and other remaining substances with acidic properties, including higher carboxylic acids are removed. The use of more alkaline solutions (solutions of potassium or sodium carbonate) should be avoided as prochloraz and fluquinconazole, containing amide groups, can decompose. After that dichloromethane extract should

Table 3
Logarithms of distribution coefficients of pesticides between hexane or hexane solution of rapeseed oil and aqueous–acetonitrile solutions of acids, at 20 °C.

Substance	Hex[a]-1 mol L^{-1} HCl(aq[a])	Hex-(AcN[a]-0.1 mol L^{-1} HCl_{aq}) = 5–1(4–1)	Hex-AcN (0.01 mol L^{-1} H_2SO_4)	Hex-AcN (0.01 mol L^{-1} $HClO_4$)	Hex-oil(2–1)-AcN (0.01 mol L^{-1} $HClO_4$)	Hex-oil(2–1)-AcN (0.1 mol L^{-1} $HClO_4$)
Cyproconazole	−1.22	−2.02	−4.4	<−4.6	−2.7	<−2.9
Difenoconazole	1.05	−1.68	−3.9	−4.6	−2.4	<−2.9
Epoxyconazole	0.29	−1.80	−3.9	<−4.6	−2.4	<−2.9
Fenbuconazole	–	–	–	<−4.6	–	−2.21
Fluquinconazole	–	–	–	−2.68	–	−1.61
Flusilazole	–	–	–	−3.45	–	−2.8
Flutriafol	−1.59	−2.53	−4.3	<−4.6	–	<−3.3
Imazalil	–	–	–	<−4.9	–	<−3.0
Ipconazole	–	–	–	<−4.6	–	−2.6
Metconazole	−0.27	−1.88	−4.4	<−4.6	−2.5	−2.8
Penconazole	–	–	–	−4.6	–	−2.6
Prochloraz	–	–	–	<−4.9	–	<−3.0
Propiconazole	0.60	−1.21	−3.6	−4.0	–	−2.9
Prothioconazole	–	–	–	−2.16	–	−1.18
Prothioconazole–desthio	–	−1.15	–	–	–	<−3.0
Tebuconazole	−0.56	−1.92	−4.1	−4.5	–	−2.9
Tetraconazole	–	–	–	−4.2	–	−2.4
Triadimefon	–	–	–	−3.22	–	−2.5
Triticonazole	−1.47	−2.34	–	−4.5	–	<−3.0

[a] Solvents: hex – hexane, AcN – acetonitrile, Ethgl – ethylene glycol; aq – aqueous solution.

Table 4
Retention times t_R of azole pesticides at GC–ECD analysis; average recoveries R (%) ± RSD (%), n = 5 of pesticides from rapeseed oil samples prepared by method described above; limits of quantitation LOQ, limits of detection LOD and maximum residue levels MRLs of pesticides in rapeseed oil for European Union countries, mg/kg [4].

Substance	t_R (min)	Amount added (mg kg^{-1})	R (%)	Amount added (mg kg^{-1})	R (%)	LOQ (mg kg^{-1})	LOD (mg kg^{-1})	MRL (mg kg^{-1})
Triadimefon	17.23	0.10	91 ± 7	0.01	85 ± 8	0.01	0.003	0.2
Tetraconazole	17.61	0.10	100 ± 8	0.01	88 ± 6	0.01	0.003	0.15
Penconazole	18.77	0.060	95 ± 8	0.006	85 ± 7	0.006	0.002	0.05
Flutriafol	20.98	3.0	85 ± 9	0.3	103 ± 6	0.3	0.1	0.2
Imazalil	21.52	0.30	107 ± 7	0.03	104 ± 8	0.03	0.01	0.05
Prothioconazole–desthio	22.56	0.10	103 ± 4	0.01	112 ± 7	0.01	0.003	0.15
Cyproconazole	23.21	0.30	93 ± 2	0.03	94 ± 5	0.03	0.01	0.4
Propiconazole	25.97; 26.30	0.10	96 ± 3	0.01	90 ± 6	0.01	0.003	0.1
Tebuconazole	26.96	3.0	100 ± 4	0.3	94 ± 6	0.3	0.1	0.5
Epoxyconazole	27.62	0.30	110 ± 5	0.03	114 ± 9	0.03	0.01	0.05
Metconazole	28.99	1.0	108 ± 4	0.1	97 ± 7	0.1	0.03	0.1
Triticonazole	29.53	0.10	102 ± 8	0.01	100 ± 7	0.01	0.003	0.02
Ipconazole	30.32	0.50	99 ± 4	0.05	90 ± 8	0.05	0.02	0.01
Fluquinconazole[a]	31.79	0.030	85 ± 6	0.003	86 ± 7	0.003	0.001	0.05
Prochloraz[a]	31.88	0.050	115 ± 10	0.005	95 ± 10	0.005	0.002	0.5
Fenbuconazole	32.54	0.30	94 ± 5	0.03	100 ± 6	0.03	0.01	0.05
Difenoconazole	36.36; 36.56	0.10	101 ± 5	0.01	85 ± 7	0.01	0.003	0.5

[a] Fluquinconazole and prochloraz must be analyzed separately because of the overlapping of their peaks on chromatogram.

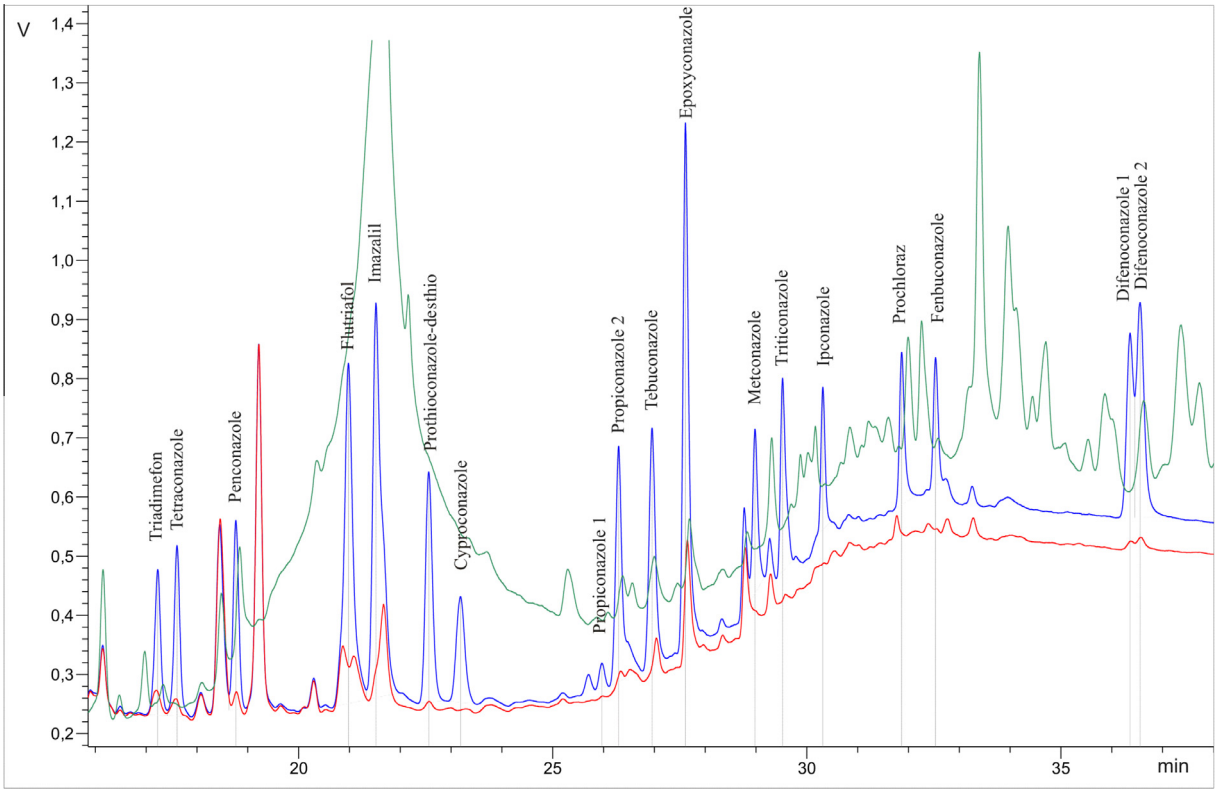

Fig. 2. The overlaid chromatograms of the acetonitrile extract of rapeseed oil without addition of azole pesticides and without clean-up from co-extractants (green) and of the samples of rapeseed oil with the addition (blue) and without addition (red) of azole pesticides, prepared by the proposed technique. The amount of pesticides, added to rapeseed oil (mg kg^{-1}) is following: triadimefon – 0.01; tetraconazole – 0.01; penconazole – 0.01; flutriafol – 1.0; imazalil – 0.02; prothioconazole–desthio – 0.1; cyproconazole – 0.1; propiconazole – 0.02; tebuconazole – 2.0; epoxyconazole – 0.02; metconazole – 1.0; triticonazole – 0.05; ipconazole – 0.5; prochloraz – 0.01; fenbuconazole – 0.3; difenoconazole – 0.05.

be evaporated to dryness and dissolved in a suitable solvent for chromatographic analysis. For example, a mixture of hexane and acetone in a volume ratio of 4–1 may be used for GC.

Based on the data given above, the following technique was developed for rapeseed oil sample preparation for the simultaneous quantitative determination of azole residues in it.

3.2. Sample preparation technique of rapeseed oil

5 g of the sample of vegetable oil is dissolved in 15 mL of hexane saturated with acetonitrile. Then 3 mL of freshly prepared 0.1 mol L^{-1} perchloric acid ($HClO_4$) in acetonitrile is added and is shaken for 2 min. Acetonitrile phase becomes green. After full

phase separation, the upper layer is discarded and the lower layer is washed two times with 10 mL of hexane saturated with acetonitrile, hexane is discarded. After that 4 mL of 1 mol L^{-1} HCl is added to acetonitrile extract and obtained solution is washed one time with 2 mL of hexane for the removing of most of coextractive components, including oil pigments, hexane is discarded. The loss of pesticides at this stage is not more than 3%. Then 15 mL of 10% (wt) dipotassium hydrogen orthophosphate (K_2HPO_4) is added to acetonitrile extract (solution becomes light yellow). Azole pesticides are extracted twice with 5 mL of dichloromethane (CH_2Cl_2). The dichloromethane extracts are combined, evaporated to about 0.5 mL on a rotary vacuum evaporator and blown out to dryness under a stream of air (or nitrogen). The dry residue is practically free of organic acids and hydrophilic impurities. The dry residue is dissolved in 1 mL of hexane–acetone (4–1, v/v) mixture, and analyzed by gas chromatography.

The recoveries of azole pesticides were determined within a range corresponding to the limits of quantitation (LOQ, defined as 10 times the signal-to-noise ratio) and 10× the LOQ [30], or at the maximum residue levels (MRLs) set by the European Union [4] and were 85–115% (Table 4). The LOQs for the majority of pesticides in rapeseed oil were much below the MRLs.

Chromatograms of rapeseed oil samples with and without additives of azole pesticides prepared by the procedure described above are shown in Fig. 2. We can see the effectiveness of the developed procedure while comparing the chromatograms of the samples, prepared by proposed method and more simple technique, based on non-acidified acetonitrile extraction [8,9] without further clean-up from co-extractants (Fig. 2, green chromatogram). The sample without cleaning of extract and acid addition was prepared from the same sample of rapeseed oil without addition of azole pesticides by acetonitrile extraction (10 mL) from rapeseed oil (2 mL), evaporation on rotary vacuum evaporator to about 0.5 mL, blowing out to dryness under a stream of air and dissolution in 1 mL of hexane–acetone = 4–1 (by volume) mixture. It should be noted that final solutions before GC analysis contain 2 g of oil in 1 mL of solution for the sample without clean-up and 5 g of oil in 1 mL of solution for the sample, prepared by the proposed method. At the same degree of preconcentration the chromatogram of sample without clean-up is much more "dirty" (see Fig. 2). In this case nearly half of the conazole class pesticides cannot be determined at MRL concentration due to a very high level of noise.

3.3. Comparison of developed method with published techniques

The method proposed in this paper is very simple and consumes only cheap and rather common reagents that allow to perform such technique in any laboratory regardless its equipment. Thus, there is no need of use of gel permeation chromatography [3,12,13], column chromatography with different expensive sorbents [14–18,24,25], centrifuge [19–23], not to mention such sophisticated instruments as GC–MS/MS [13,18] and LC–MS/MS [1,6,20,22,24] or on-line coupled reversed-phase liquid chromatography and gas chromatography [7]. Only flasks, test-tubes and pipettes are used for sample preparation and simple GC–ECD for analysis. The procedure of sample preparation lasts ~1 h for 4 samples, that is rather quick, especially while comparing with GC analysis of one sample (50 min). The final extracts are much more pure in comparison with obtained by one-stage acetonitrile extraction (Fig. 2, green chromatogram) and are clean enough to obtain good chromatograms without cleaning of injector liner at least at 200 injections.

3.4. Application to real samples

The proposed method was used for the analysis of 17 samples of spring rapeseed oil and 13 samples of winter rapeseed oil. In one sample of spring rapeseed oil the amount of propiconazole residue (0.25 mg kg^{-1}) exceeded the MRL, established in Belarus and EU countries (0.1 mg kg^{-1}). In all other cases the amounts of pesticide residues were below the MRLs.

4. Conclusion

In this study, we have applied, for the first time, dissociation extraction in a water-free medium as a sample treatment strategy for high-effective complete extraction of pesticide residues of conazole group class from rapeseed oil. The proposed methodology was combined with gas chromatography with electron capture detection for quantitation of pesticide residues at limits of quantitation ranged from 3.0 to 300 μg kg^{-1}. The obtained detection limits are below the maximum residue levels (MRLs) set by the European Union for the majority of pesticides. Dissociation extraction offers various attractive advantages compared with ordinary solvent extraction. It provides remarkably higher recoveries while using the same amount of extractant or allows consuming much less volume of solvent to achieve the same recoveries of azole pesticides. The clean-up procedure by hexane from acidified water–acetonitrile mixture with following neutralization and purification step with aqueous solution of dipotassium hydrogen orthophosphate provides removing of acids and hydrophilic substances from extract. The application of this sample preparation strategy, undoubtedly, could be extended to other kinds of vegetable oils and other pesticides with basic properties.

Conflict of interest

The authors declared no conflict of interest.

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