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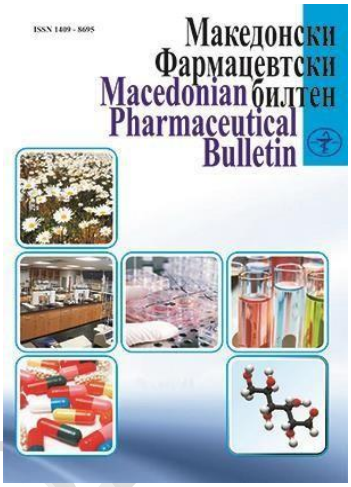

Title: Optimization and evaluation of an LC-MS method for structural characterization of ibuprofen and its related compounds

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Optimization and evaluation of an LC-MS method for structural characterization of ibuprofen and its related compounds

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Abstract

Ibuprofen is a well-established and widely prescribed non-steroidal anti-inflammatory drug that inhibits prostaglandin synthesis and belongs to the profen class of compounds. Under accelerated, stress, or forced-degradation conditions, ibuprofen as an active substance may give rise to several degradation products. The HPLC method for determination of ibuprofen-related compounds and degradation products described in the current European Pharmacopoeia monograph for ibuprofen is using UV detection with non-volatile mobile phase, and is not suitable for mass spectrometric detection. The aim of this study was to transform the existing pharmacopoeial method into a method compatible with mass spectrometric detection and evaluate the analytical performance of the new method. Mass spectrometric detection conditions using atmospheric pressure chemical ionization (APCI) in positive mode were optimized using an orbitrap high-resolution mass spectrometer. The optimized method proved suitable for separation, detection and structural characterization of ibuprofen and selected ibuprofen-related compounds. The developed LC-MS-compatible approach represents a useful analytical tool for impurity profiling and investigations of potential API-excipient interaction products. The results of this study demonstrate the successful optimization of an ibuprofen analytical method that maintains close correspondence with the original pharmacopoeial method and is fully compatible with mass spectrometry for the detection and structural elucidation of ibuprofen and ibuprofen-related compounds.

Keywords
ibuprofen, MS, orbitrap, APCI

Introduction

Ibuprofen ((2RS)-2-[4-(2-methylpropyl)phenyl]propionic acid) is a well-established and widely prescribed non-steroidal anti-inflammatory drug (NSAID) that inhibits prostaglandin synthesis and belongs to the profen class of compounds (2-arylpropionic acids) (Day & Graham, 2014; Douša et al., 2017; Irvine et al., 2018; Toledo et al., 2021; Veloso et al., 2021; Zappaterra et al., 2023). Its chemical structure is presented in Fig. 1 (European Pharmacopoeia, 2017).

Fig. 1

This active pharmaceutical ingredient (API) is extensively used for the treatment of various types of pain, fever, and inflammatory conditions. Ibuprofen is available in multiple dosage forms, including conventional tablets, chewable tablets, soft gelatin capsules, and oral suspensions for oral administration; gels, creams, and sprays for dermal and transdermal delivery; as well as injectable formulations intended for intravenous administration. (Douša et al., 2017; Gee et al., 2014; Hadgraft et al., 2003; Irvine et al., 2018; Zappaterra et al., 2023).

Under accelerated, stress, or forced-degradation conditions, ibuprofen as an active substance may give rise to several degradation products. In addition to those specified in the current European Pharmacopoeia monograph for ibuprofen, numerous ibuprofen-related degradation compounds have been reported in the literature (Caviglioli et al., 2002; Cory et al., 2010; Farmer et al., 2002; European Pharmacopoeia, 2017; Stojanovska Pecova et al., 2020). Each of these compounds is controlled according to predefined acceptance criteria established in the harmonized ICH guidelines Q3A (ICH, 2006a) and Q3B (ICH, 2006b).

Therefore, any newly identified degradation product of ibuprofen or any potential product resulting from a chemical interaction between ibuprofen and any excipient should be considered an ibuprofen-related compound. Early awareness of possible reactions

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occurring within an ibuprofen formulation can facilitate the implementation of strategies to limit or reduce their contents or justify higher thresholds. It requires that the resulting products be appropriately detected, analyzed, and characterized. Mass spectrometry (MS) is the most suitable and widely used analytical technique for this purpose due to its compatibility with liquid chromatography (LC–MS) and possibility of adapting existing chromatographic methods for impurity profiling and structural elucidation of API and finished product degradation products (Chadha & Bhandari, 2014; Douša et al., 2017; Farias et al., 2021; Gullapalli, 2010; Pan et al., 2011).

The HPLC method for the determination of ibuprofen-related compounds and degradation products described in the current European Pharmacopoeia monograph for ibuprofen (Eur, Ph., 2017) is not suitable for mass spectrometric (MS) applications due to the presence of phosphoric acid in both the mobile phase and diluent solutions. The use of non-volatile acidic modifiers is incompatible with efficient ionization and may result in contamination of the MS source, thereby compromising sensitivity and analytical performance. Furthermore, the relatively high flow rate employed in the compendial method (2 mL/min) renders the method less suitable for direct LC-MS coupling and is less compatible with the LC-MS configuration employed in this study. Consequently, modifications to the chromatographic conditions are required to enable MS compatibility while maintaining adequate separation and detection of ibuprofen-related substances and degradation products.

The aim of this study was to transform the existing method described in the European Pharmacopoeia monograph for ibuprofen into an LC–MS compatible method suitable for the detection, characterization, and structural elucidation of ibuprofen-related compounds. Special focus was placed on its potential application as an analytical tool for impurity profiling and API-excipient compatibility studies. Particular emphasis was placed on compounds potentially formed through degradation processes or interactions between ibuprofen and commonly used pharmaceutical excipients. In addition, the performance characteristics of the modified chromatographic method were evaluated and mass spectrometric conditions were optimized using a high-resolution Orbitrap mass spectrometer.

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Materials and methods

Ibuprofen active pharmaceutical ingredient (API) was obtained as a non-commercial sample for research purposes. The following reference standards were also used: ibuprofen isopropyl ester (Pharmaffiliates, India), 2,3-dihydroxypropyl 2-(4-isobutylphenyl)propanoate (TRC, Canada), ibuprofen 1,2-propylene glycol ester (Sigma-Aldrich, Merck KGaA, Germany). In addition, European Pharmacopoeia Chemical Reference Substance (Ph. Eur. CRS) impurity B was obtained as well and British Pharmacopeia reference standard impurity E, while ibuprofen EP related compounds A, J, N and ibuprofen ethyl ester were obtained by LGC Standards, Poland, and ibuprofen related compound O from Aozeal, Hong Kong. Formic acid and acetonitrile, used as analytical-grade reagents, were purchased from Merck KGaA (Darmstadt, Germany).

The test solution was prepared according to the European Pharmacopoeia monograph for ibuprofen. Briefly, 20 mg of ibuprofen (or equivalent amount of ibuprofen containing mixtures) were dissolved in 2.0 mL acetonitrile and diluted to 10.0 mL with mobile phase A, giving a final concentration of 2.0 mg/mL. Reference solution was prepared by diluting 1.0 mL of the test solution to 100.0 mL with mobile phase A, followed by a further dilution of 1.0 mL of this solution to 10.0 mL with mobile phase A.

Calibration solutions were prepared by appropriate dilution of the stock solution with mobile phase A to obtain concentrations in the range 0.0025-2.5 mg/mL.

Individual reference compounds were initially dissolved in acetonitrile to prepare stock solutions and subsequently diluted with mobile phase A to the working concentrations prior to analysis. Unless otherwise stated, all reference compounds were prepared at a concentration of 0.1 mg/mL.

All HPLC–MS analyses were performed using a Vanquish UHPLC system coupled to a DAD detector and an Orbitrap Exploris 120 HRMS, as well as a Dionex Ultimate 3000 HPLC-DAD chromatograph (Thermo Scientific Co.). Chromatographic separation was achieved on a Zorbax Extend-C18 column (150 × 4.6 mm, 5 μm; Agilent Technologies, Inc.) with a flow of 1.0 mL/min at 25 °C. For MS detection, atmospheric pressure chemical ionization (APCI) operated in positive ionization mode was used with vaporizer temperature set at 400 °C and capillary temperature maintained at 300 °C. The sheath, auxiliary, and sweep gas flow rates were set at 50, 5, and 5 arbitrary units,

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respectively. The positive corona discharge current was set at 4 μA. Full-scan mass spectra were acquired over the m/z range of 100–1000. Instrument control and data processing were carried out using Chromeleon 7 and Xcalibur 4.7 software.

Results and discussion

Optimization of HPLC separation with UV-detection

The usual strategy for transforming a non-volatile to a volatile mobile phase involves replacing phosphoric acid with a formic acid in a volume ratio in the range 0.1 to 1% and that was the first step in this work, together with decreasing the flow rate. Gradient elution starting with 0.3% formic acid in mobile phase A was found as optimal with a flow rate of 1 mL/min. In addition, different detection wavelengths (265, 254, and 214 nm) were evaluated in order to select the most suitable wavelength for the detection of the compounds of interest using UV/DAD detection.

Satisfactory chromatographic separation was achieved on a C18 column with dimensions 150 × 4.6 mm, 5 μm, used as the stationary phase, at room temperature. A mixture consisting of 3 mL formic acid and 340 mL acetonitrile, diluted with water to a final volume of 1000 mL, was used as mobile phase A, whereas acetonitrile was used as mobile phase B. Samples were initially dissolved in mobile phase B (acetonitrile) and subsequently diluted with mobile phase A. An injection volume of 20 μL was used for all samples. The flow rate was set at 1.0 mL/min, and elution was performed using the following gradient program: 0–25 min, 100% A (v/v); 25–55 min, linear gradient to 15% A (v/v); 55–70 min, 15% A (v/v); 70–72 min, return to 100% A (v/v); and 72–80 min, 100% A (v/v) for column re-equilibration. The detection wavelength was finally set at 265 nm. The obtained chromatogram together with the UV/Vis absorption spectrum and mass spectrum of ibuprofen are presented in Fig. 2.

Fig. 2

Examination of the UV/Vis spectra (Fig. 2) revealed a characteristic absorption shoulder centered at approximately 265 nm for ibuprofen, as well as for the peaks of the investigated related compounds. This spectral feature is also described in the

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pharmacopoeial method for the identification of ibuprofen using UV/Vis spectrophotometry. Although quantification in the pharmacopoeial method is performed at 214 nm, significant baseline disturbances associated with solvent absorption (formic acid in the mobile phase) were observed under the optimized chromatographic conditions at this wavelength.

Furthermore, when progressively lower concentrations of ibuprofen were evaluated, 0.025 mg/mL represented the lowest concentration at which a well-defined chromatographic peak could be reliably detected at 214 nm (chromatograms at 265 nm, 214 nm and 254 nm presented in Fig. 3). As can be seen, detection at 265 nm produced a clearly distinguishable peak of satisfactory intensity at the same concentration, indicating the potential for reliable detection at even lower concentration levels. The evaluation of sensitivity at different wavelengths is shown in Table 1 with S/N results for LOQ concentration 0.025 mg/mL at 3 wavelengths and in Table 2 with LOQ results at 265 nm for LOQ concentration 0.0025 mg/mL. In addition, monitoring at 265 nm resulted in a stable chromatographic baseline with substantially lower background interference while maintaining adequate sensitivity for the detection of ibuprofen and related compounds. Therefore, a detection wavelength of 265 nm was selected for further method testing and subsequent analyses as it provided the most favorable balance between analytical sensitivity, spectral specificity, and baseline stability. The final chromatogram obtained for ibuprofen is presented in Fig. 2, while comparative chromatograms from the three tested wavelengths (265 nm, 214 nm and 254 nm) from the developed MS-friendly method, along with chromatogram from the original EP method are presented in Fig. 3.

Fig. 3
Table 1
Table 2

The developed chromatographic conditions were intended primarily to support LC-MS investigations of ibuprofen-related compounds, including degradation products and potential API-excipient interaction products, rather than routine quantitative quality-control testing.

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Optimization of mass spectrometric conditions

In the next phase, the MS parameters were optimized in order to achieve favorable ionization conditions and obtain high-quality mass spectra for ibuprofen and its related compounds. During method development, both heated electrospray ionization (HESI) and atmospheric pressure chemical ionization (APCI) were evaluated. Under the investigated conditions, HESI demonstrated inferior performance characterized by elevated background noise and poor spectral reproducibility. In contrast, APCI operated in positive ionization mode was found more suitable providing robust and reproducible ionization with minimal chemical noise for both ibuprofen and the investigated related compounds. The obtained mass spectrum of ibuprofen with APCI in positive mode is shown in Fig. 2.

It was observed that ibuprofen exhibited a stronger response in negative ionization mode, which was expected considering its acidic nature as a carboxylic acid. However, positive ionization mode proved more suitable for characterization of related substances and potential alcohol-derived interaction products i.e. esters of ibuprofen. Furthermore, satisfactory spectral information for ibuprofen could also be obtained in positive-ion mode, whereas all other compounds of interest exhibited excellent ionization efficiency under these conditions. Therefore, APCI operated in positive ionization mode was selected for all subsequent analyses.

Throughout the investigation, a recurring ion species corresponding to a mass increase of +18 Da relative to the protonated molecular ion was consistently observed. Such ions are commonly attributed to ammonia adducts, $[M+NH_4]^+$. Since no ammonia-containing reagent was intentionally added during the analysis, the observed adduct was presumed to originate from residual ammonia present within the LC–MS system and column.

The observation of adduct formation was not unexpected, since adduct generation represent some of the principal ionization pathways in APCI. In APCI, ion–molecule reactions frequently compete with proton-transfer processes often resulting in the formation of adduct ions. Similar phenomena have been reported for the APCI analysis of esters, NSAID derivatives, and structurally related compounds, which exhibit a propensity for ionization through adduct formation rather than direct protonation (Byrdwell, 2001; Honing et al., 2000; Klee et al., 2014; Kostiainen & Kauppila, 2009; Schmit et al., 1985). In addition, cluster ions containing multiple bound ammonia molecules have been observed, further supporting the proposed ionization mechanism.

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The formation of such cluster ions has been extensively described in the literature for APCI ionization. Consequently, the potential generation of both simple adducts and adduct clusters should be taken into consideration during spectral interpretation and structural elucidation of the detected compounds using this proposed method.

From the observed MS spectrum obtained for the peak of ibuprofen (Fig. 2) a peak at m/z 224.1644 was present in the spectrum, corresponding to the quasimolecular ion of ibuprofen with an adduct of 18 Da i.e. $[M+18]^+$. In contrast, a very low-intensity peak at m/z 207.1379 was detected and assigned to the protonated molecular ion $[M+H]^+$. As previously discussed, the formation of this ion is not favored under these ionization conditions, which explains its relatively low abundance in the spectrum.

A highly intense fragment ion at m/z 161.1324 was consistently observed in the spectra of ibuprofen and all investigated ibuprofen-related compounds. MS/MS experiments confirmed this ion as a major product ion of the precursor species detected under the applied APCI conditions. Its exact mass is consistent with the elemental composition $C_{12}H_{17}^+$. Although the exact fragmentation pathway could not be unequivocally established, theoretical fragmentation analysis suggests that its formation may involve decarboxylation and rearrangement processes within the ibuprofen structural framework. The recurring presence of this fragment indicates that it may serve as a characteristic diagnostic ion during structural characterization of ibuprofen-related compounds.

The applicability of this MS-compatible method is therefore of considerable importance, as it enables the characterization of a wide range of both known and unknown ibuprofen-related impurities. The fragment ion at m/z ~161.1323, with high mass accuracy, may serve as a valuable diagnostic indicator in the characterization of such compounds and in the elucidation of their structures, as its presence strongly suggests that the structural modification has occurred at the carboxyl group. Furthermore, it indicates that the unknown compound largely retains the aromatic moiety of the ibuprofen scaffold together with its characteristic alkyl substituents.

Method performance evaluation

The analytical performance of the optimized chromatographic method was evaluated through assessment of selectivity, sensitivity, linearity, precision, and robustness. The

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results of these tests are summarized in Tables 2-5. The analytical performance of the proposed method was assessed through selected characteristics relevant to its intended application, following the general principles of ICH Q2(R1).

Specificity was assessed through the analysis of standard solutions of ibuprofen, known ibuprofen-related substances, and a series of potential ester derivatives that may arise as ibuprofen-related compounds with different excipients. To support the evaluation of chromatographic selectivity, the retention times of ibuprofen and the investigated related compounds obtained using the developed method are presented in Table 3 and the structures of the non-pharmacopoeial ibuprofen-related compounds (esters with alcoholic excipients) are given in Supplementary figure 1. Some structurally related compounds exhibited similar retention behavior, which is in agreement with observations reported for the pharmacopoeial method.

Table 3

Sensitivity was determined as the limit of quantification (LOQ) established as the lowest concentration producing an acceptable signal-to-noise ratio and suitable relative standard deviation (RSD) from six injections (Table 2) and the value was set at 0.0025 mg/mL. Since a satisfactory signal-to-noise ratio (S/N = 12) was obtained at the established LOQ concentration of 0.0025 mg/mL, the LOD was considered to be below this concentration level.

Linearity was evaluated using standard solutions of ibuprofen over the concentration range of 0.0025–2.5 mg/mL. Calibration curve (Fig. 4) was constructed by plotting peak area against concentration, and the linear relationship was assessed by regression analysis and the coefficient of determination of $R^2 > 0.999$.

Fig. 4

The precision of the analytical procedure was evaluated through assessment of both system precision and method precision. System precision was demonstrated by six consecutive injections of a standard ibuprofen solution at a concentration of 0.25 mg/mL. Method precision and intermediate precision were evaluated using representative

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ibuprofen-containing experimental mixtures analyzed on two different days. Although somewhat higher RSD values were observed for the method precision experiments compared with system precision, these results are considered acceptable considering the nature of the investigated laboratory-prepared samples and the relatively high ibuprofen content levels. The results are summarized in Table 4.

Table 4

Method robustness was investigated by varying selected chromatographic parameters, including column temperature, flow and column type. The column temperature was adjusted to 20 °C and 30 °C (±5 to the tested method), while chromatographic performance was additionally examined using an equivalent chromatographic column. Robustness was assessed by calculating the overall relative standard deviation (RSD) of the obtained results and evaluating the influence of these deliberate variations on method performance. Robustness was also assessed by introducing flow rate variations. Compared to the other tested conditions, the obtained overall RSD was less favorable; nevertheless, the chromatographic performance remained satisfactory, demonstrating adequate method robustness. The results for all robustness evaluations are presented in Table 5.

Table 5

It should be here noted that accuracy was not evaluated as a separate analytical performance parameter. Since the present method was primarily developed as an LC-MS compatible analytical tool for the detection, characterization and structural elucidation of ibuprofen-related compounds, a formal accuracy study was not included within the scope of this work. Should the method be further developed for quantitative determination of related substances or routine quality-control applications, appropriate recovery-based accuracy studies would need to be performed.

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Conclusion

The results of this study demonstrated the successful optimization of an ibuprofen analytical method that is fully compatible with mass spectrometric detection while maintaining chromatographic behavior comparable to that of the original pharmacopoeial method and at the same time enabling direct LC–HRMS compatibility.

The developed LC–MS approach therefore represents a valuable analytical tool for the investigation of ibuprofen, degradation products, API/excipient interaction products, and other related compounds, while simultaneously providing a mean for the structural characterization of unknown chromatographic peaks observed during pharmaceutical development and quality control studies using LC-MS. Application to quantitative analysis of specific pharmaceutical products would require additional product-specific optimization and validation.

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Резиме

Оптимизација и евалуација на LC-MS метод за структурна карактеризација на ибупрофен и негови сродни соединенија

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Клучни зборови: ибупрофен, МС, орбитрап, APCI

Ибупрофенот е добро познат нестероиден антиинфламаторен лек кој ја инхибира синтезата на простагландини и спаѓа во класата на профени. При услови на забрзана или форсирана деградација, ибупрофенот како активна супстанца може да доведе до појава на повеќе деградациони продукти и други сродни соединенија. HPLC методот за определување на сродни и деградациони продукти опишан во Европската фармакопеја за ибупрофен користи UV детекција и неиспарлива мобилна фаза, поради што не е погоден за детекција со масена спектрометрија. Целта на оваа студија беше адаптација на постојниот фармакопејски метод во LC-MS-компатибилен метод и евалуација на неговите аналитички перформанси. Оптимизирани се условите за детекција со масена спектрометрија со примена на хемиска јонизација при атмосферски притисок (APCI) во позитивен мод и орбитрап масен анализатор со висока резолуција. Потврдена е погодноста на оптимизираниот метод за детекција и структурна карактеризација на ибупрофен и неговите сродни соединенија и претставува корисна аналитичка алатка за профилирање на сродни и деградациони соединенија и испитување на потенцијални интеракции помеѓу активната супстанца и помошните супстанци. Резултатите од оваа студија покажуваат успешна адаптација на фармакопејскиот метод во LC-MS-компатибилен метод кој е споредлив со оригиналната постапка и

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овозможува детекција и структурна карактеризација на ибупрофен и неговите сродни соединенија.

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Table 1. S/N ratio results for each evaluated wavelength

LOQ	S/N ratio
0.025 mg/mL 214 nm	16
0.025 mg/mL 254 nm	76
0.025 mg/mL 265 nm	123

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Table 2. LOQ results at 265 nm

LOQ 265 nm	S/N ratio	Area	t_r/min
0.0025 mg/mL Average (n=6)	11.8	0.0527	37.04
RSD%	1.78	3.16	0.03

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Table 3. Retention times for ibuprofen and each evaluated impurity

Compound	Retention time (min)
Impurity J	10.29
Impurity N	16.60
Glycerol ester	26.84
Impurity A	36.27
Impurity O	36.05
Ibuprofen	36.80
Impurity E	39.73
Propylene glycol esters*	39.78
Impurity B	40.43
Ethyl ester	49.13
Isopropyl ester	51.73

*the standard was obtained as mixture of impurities co-eluting under one peak

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Table 4. Precision results

System Precision	Area	tr/min
Average (n=6 injections 0.25 mg/mL std)	4.96	36.65
RSD%	0.08	0.01
Method Precision	Area	Content
Average (*n*=6 samples, intraday 1)	54.8569	108.1
RSD%	4.24	2.79
Average (*n*=6 samples, intraday 2)	56.22	103.7
RSD%	9.98	5.60
Overall average (interday)	55.54	105.92
Overall RSD%	7.48	4.70

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Table 5. Method robustness results

Original method	Area	20 °C	Area	30 °C	Area
Average	4.96	Average	4.96	Average	4.97
RSD%	0.08	RSD%	0.08	RSD%	0.13
Overall average	N/A	Overall average	4.9622	Overall average	4.97
Overall RSD%	N/A	Overall RSD%	0.10	Overall RSD%	0.12
Equiv. column type	Area	Flow 1.1 mL/min	Area	Flow 0.9 mL/min	Area
Average	5.07	Average	4.57	Average	5.56
RSD%	0.11	RSD%	0.14	RSD%	0.06
Overall average	5.02	Overall average	4.76	Overall average	5.26
Overall RSD%	1.07	Overall RSD%	4.29	Overall RSD%	5.92

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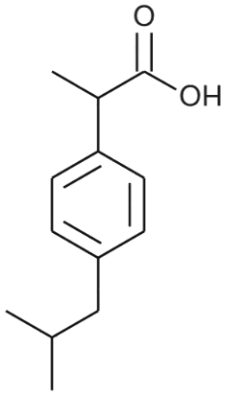

Fig. 1. Ibuprofen structure.

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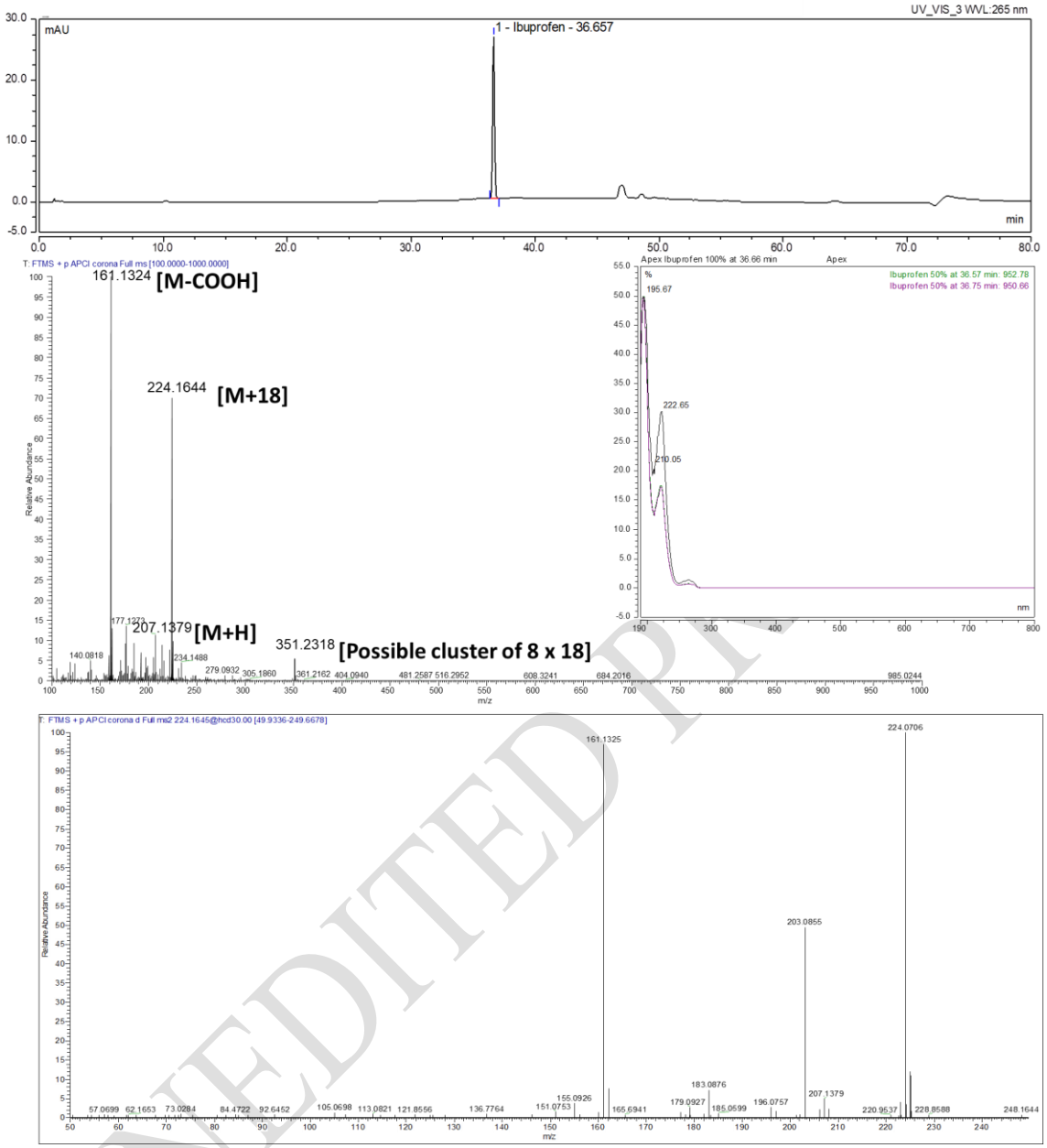

Fig. 2. Chromatogram obtained for ibuprofen with its MS/MS and UV/Vis spectra.

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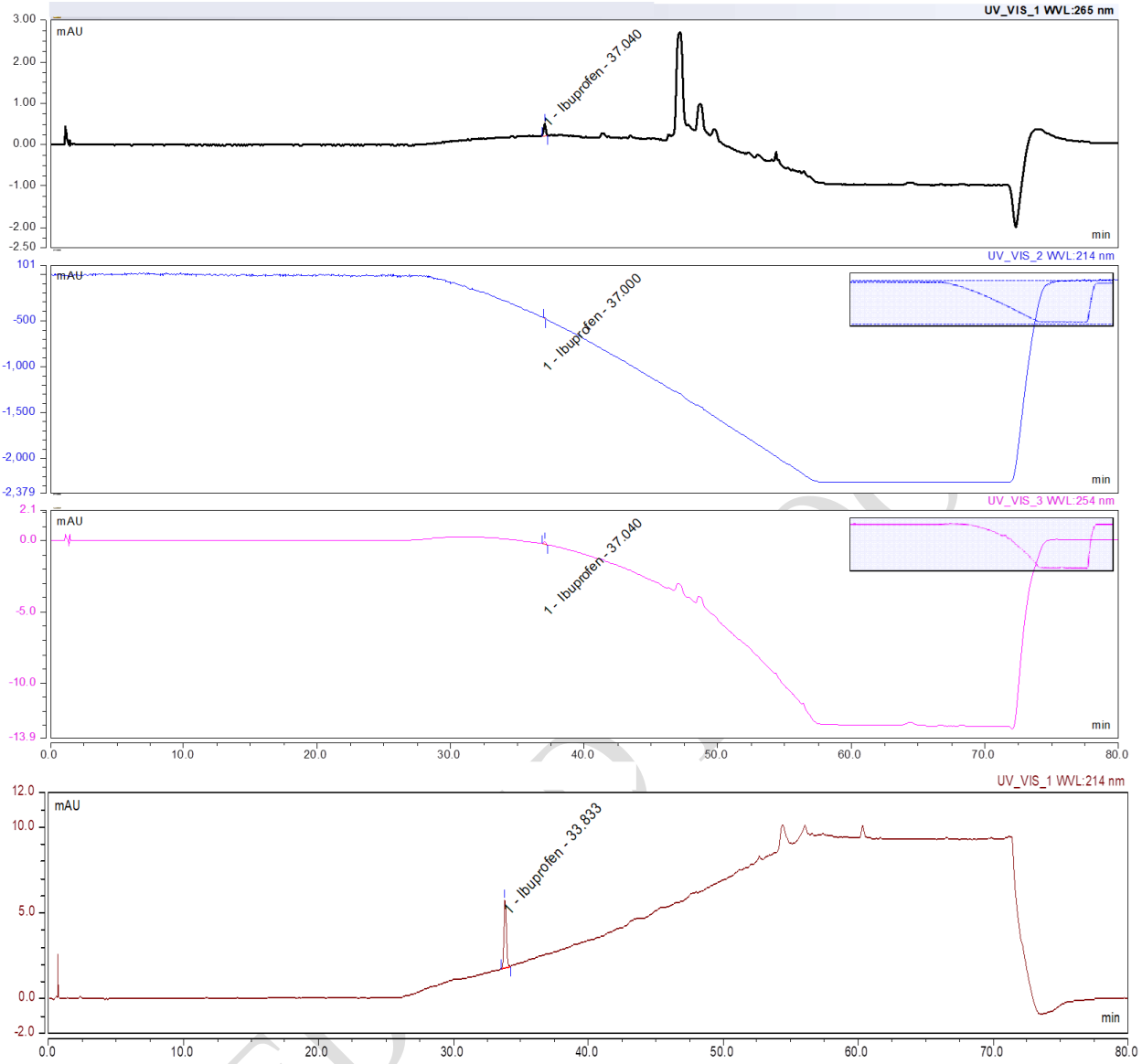

Fig. 3. LOQ chromatograms for the three tested wavelengths: 265 nm – black; 214 nm – blue; 254 nm – pink (from the MS adapted method) and LOQ chromatogram from the original EP method: 214 nm – brown (bottom chromatogram).

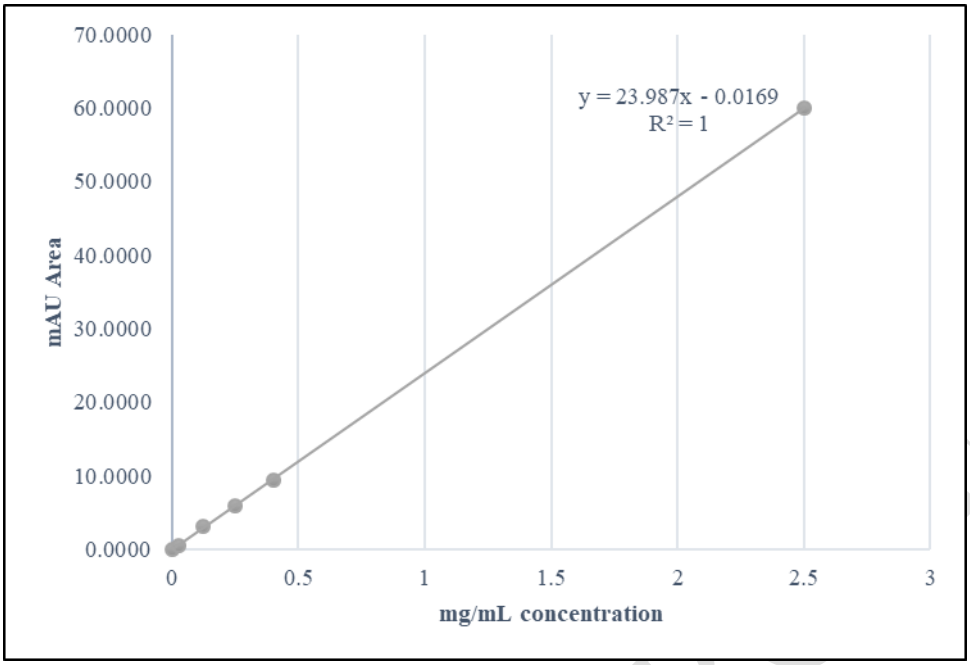

Fig. 4. Calibration curve representing the linearity of the method.

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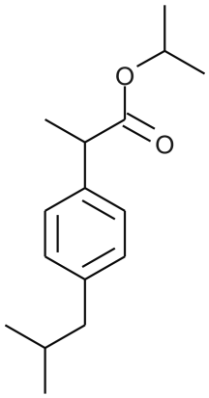
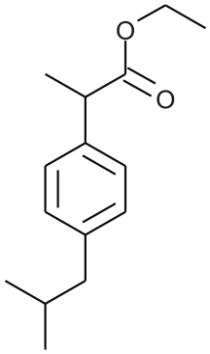
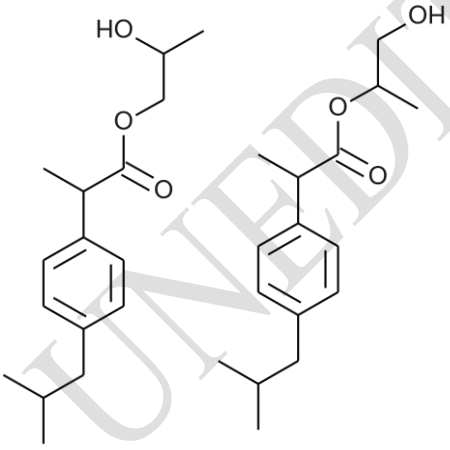
Supplementary figures



Ibuprofen-isopropyl ester



Ibuprofen-ethyl ester



Ibuprofen-propylene glycol esters

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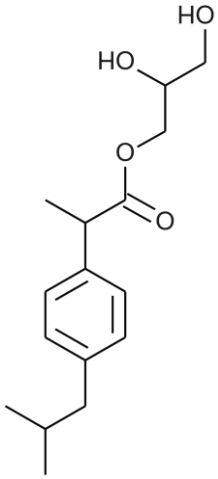

Ibuprofen-glycerol ester

Supplementary Figure S1. Chemical structures of the non-pharmacopoeial ibuprofen-related compounds investigated in this study. Structures of the official ibuprofen-related compounds are described in the European Pharmacopoeia monograph for ibuprofen and are not reproduced here

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