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GC–MS-based metabolomics for the detection of adulteration in oregano samples

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Abstract: Oregano is one of the most used culinary herb and it is often adulterated with cheaper plants. In this study, GC–MS was used for identification and quantification of metabolites from 104 samples of oregano (*Origanum vulgare* and *O. onites*) adulterated with olive (*Olea europaea*), venetian sumac (*Cotinus coggygria*) and myrtle (*Myrtus communis*) leaves, at five different concentration levels. The metabolomics profiles obtained after the two-step derivatization, involving methoxyamination and silanization, were subjected to multivariate data analysis to reveal markers of adulteration and to build the regression models on the basis of the oregano-to-adulterants mixing ratio. Orthogonal partial least squares enabled detection of oregano adulterations with olive, Venetian sumac and myrtle leaves. Sorbitol levels distinguished oregano samples adulterated with olive leaves, while shikimic and quinic acids were recognized as discrimination factor for adulteration of oregano with venetian sumac. Fructose and quinic acid levels correlated with oregano adulteration with myrtle. Orthogonal partial least squares discriminant analysis enabled discrimination of *O. vulgare* and *O. onites* samples, where catechollactate was found to be discriminating metabolite.

Keywords: chromatography; PCA; OPLS; *Origanum vulgare*; *Origanum onites*; *Olea europaea*; *Cotinus coggygria*; *Myrtus communis*.

INTRODUCTION

The global industry that produces herbs and spices is continuously growing, giving a lot of space for motivated adulteration. Thus, developing new analytical methods is highly desirable. Use of spectroscopic techniques combined with

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chemometrics tools, for fraud detection in industry, has increased. Fast, reliable, and efficiant techniques are required to check the authenticity of food.[1,2]

Oregano is one of the most used culinary herbs for pizzas and Mediterranean food.[3] Because of that it is often adulterated with cheaper plants for dilution.[4] Commercially available oregano is usually mixed with other plants, such as cistus, olive tree leaves, myrtle, sumac, and others. Tolerable impurities in oregano, to be sold, of *O. vulgare* and *O. onites* are up to 2 %.

Hitherto, several instrumental techniques utilized to access quality of oregano, such as Fourier transform infrared spectroscopy (FTIR), liquid chromatography–high resolution mass spectrometry (LC–HRMS) and nuclear magnetic resonance spectroscopy (NMR).[5–7] A GC–MS test is also described for detection of oregano adulteration with olive leaves. By injecting of crude ethanolic extracts into GC–MS system, two chromatographic peaks with unknown chemical structures are reported to be markers of the adulteration.[8,9]

In our study, GC–MS was used for identification and quantification of the primary and secondary metabolites, in 104 samples, of oregano adulterated with olive (*Olea europaea*), Venetian sumac (*Cotinus coggygria*) and myrtle (*Myrtus communis*) leaves, at five different concentration levels. The metabolomics profiles obtained after two-step derivatization involving methoxyamination and silanization were subjected to multivariate data analysis to reveal markers of adulteration and to build the regression models on the basis of the oregano-to-adulterants mixing ratio.

EXPERIMENTAL

Plant sample collection

Two grounded samples of *Origanum vulgare*, one of *Origanum onites* and one made of the two mixed species were obtained from commercial sources. Corresponding vouchers of the crude, grounded drugs (BO23ABZOR, BO21ZOR, BO22AAOR and BO6FOR) were deposited in Department of Pharmacy and Biotechnology, University of Bologna (Via Irnerio 42, Bologna, Italy). The leaves of *O. europaea*, *M. communis* and *C. coggygria* were harvested in the Botanical Garden of Bologna (Italy) in September 2019, the samples were identified by Prof. Ferruccio Poli, and voucher specimens (BOLO0602019, BOLO0602020, BOLO0602021) were retained at the Herbarium of Alma Mater Studiorum University of Bologna (SMA, Via Irnerio 42, 40126, Bologna, Italy).

Morphological analysis of the oregano samples was performed following the identification suggested by 9th European Pharmacopoeia[10] and using the oregano taxonomic key presented in Ietswaart, as described in the paper by Mandrone *et al.*[6,11]

Sample preparation

Four samples of oregano were mixed with dried and grounded leaves of three adulterants: Venetian sumac (*C. coggygria*), myrtle (*M. communis*) and olive (*O. europaea*) to make binary mixtures in mass ratios 1, 5, 10 and 20. Two aliquots were taken of each sample, resulting in total of 96 adulterated samples and 8 intact oregano samples, which are all used for further analysis. The samples were then prepared for GC–MS analysis by modified litera-

ture procedure.[12,13] Plant samples were further powdered with electrical mill (IKA, A11 basic, Merck, Italy). Each sample (20 mg) was extracted, with 1 mL mixture of methanol and water (1:1 volume ratio) with addition of 250 μL 2-deoxy-ribose as internal standard (1 mg/mL) on ultrasonic bath (Sonorex Super RK 100, Bandelin, Berlin, Germany) for 5 min. After extraction, samples were centrifuged for 5 min at 15.000 rpm (DLAB D2012 plus, Beijing, China), then 200 μL of supernatant were transferred into eppendorf tube and concentrated under flow of nitrogen up to 100 μL and then stored in the freezer at –20 °C. Samples were lyophilized and then dissolved in pyridine (containing methoxyamine-hydrochloride, concentration 20 mg/mL, that was freshly prepared) and transferred into a glass vial. First step of derivatization was carried out in a sand bath for 2 h at 40 °C. For second step, 100 μL of BSTFA was added, and the mixture was kept for 60 min at 80 °C. Before analyzed on GC–MS, samples were cooled at room temperature.

GC–MS Analysis

The GC–MS analyses of the samples were performed on Agilent 7890A GC system (Agilent Technologies, Santa Clara, CA, USA) with a 5975C mass selective detector (MSD) and a FID connected by capillary flow technology through a two-way splitter. A HP-5MSI, non-polar capillary column (30 m×0.25 mm, 0.25 μm film thickness), was used. Column temperature started from 60 to 270 °C at a rate of 3 °C/min, then heated 20 °C/min to 310 °C with 8 min hold at the end. Carrier, auxiliary and make up gas was helium, inlet pressure was 20.6 psi (flow 1.0 mL/min at 210 °C), auxiliary pressure was 3.8 psi and FID make up flow was 25 mL/min. Mass spectra obtained by electron ionization with 70 eV at 200 °C. Quadrupole temperature was set to 150 °C and MS range was 40–900 amu. FID temperature was 300 °C, split ratio was 5:1 and injection volume was 1 μL for all analyses.

Data processing

Library search and compound identification were performed using the MSD ChemStation software, version E02.02 (Agilent Technologies, Santa Clara, CA, USA), the NIST AMDIS software, version 2.70, and the commercially available libraries Wiley07, NIST17 and Adams04.

All the MS chromatograms were converted to AIA format using the MSD ChemStation software. The peak picking, nonlinear peak alignment, and matching of the retention times were then carried out utilizing XCMS online platform which is based on the R software.[14,15] CentWave feature detection algorithm was applied, with 100 ppm maximal tolerated m/z deviation in consecutive scans, 5 s minimum chromatographic peak width, 10 s maximum chromatographic peak width, 0.01 minimum difference in m/z for peaks with overlapping retention times, and value 6 as signal/noise threshold. For the peak alignment, 10 s used as allowable retention time deviations, 0.5 as minimum fraction of samples necessary in at least one of the sample groups for it to be a valid group and 0.5 as width of overlapping m/z slices to use for creating peak density chromatograms and grouping peaks across samples. A total of 104 chromatograms were used for processing which resulted in 557 features detected, and all used in the multivariate analysis.

The data in the table obtained from XCMS online platform were normalized to the content of internal standard (2-deoxy-ribose), and subjected to multivariate data analysis.

Principal component analysis (PCA), orthogonal partial least squares to latent structures (OPLS), and orthogonal partial least squares to latent structures – discriminant analysis (OPLS-DA) methods were performed using SIMCA software (version 15, Sartorius, Göttingen, Germany). The data were mean centered and scaled to unit variance.

RESULTS AND DISCUSSION

Analysis of GC–MS chromatograms of oregano samples

The metabolomes of the intact and adulterated oregano samples were analyzed by GC–MS after two-step derivatization involving methoxyamination and silanization. On this way, even polar metabolites can be introduced in GC–MS system, enabling identification of broad spectrum of the primary and secondary metabolites present therein.

The most abundant compounds in the intact oregano samples were sugars, primarily disaccharide sucrose, and monosaccharides such as glucose and fructose. Next, organic acids such as quinic, citric, malic, γ-aminobutyric, shikimic, rosmarinic, caffeic, succinic acid, ester catechollactate, phosphoric acid, sugar acids such as threonic, erythronic, and glucaric acid, alcohols were present, mainly myo-inositol, sorbitol, xylitol, glycerol and galactinol were detected. Monoterpene carvacrol as main constituent of oregano aroma was also identified. The representative MS chromatograms of oregano sample are depicted on Fig. 1, and in Supplementary material to this paper.

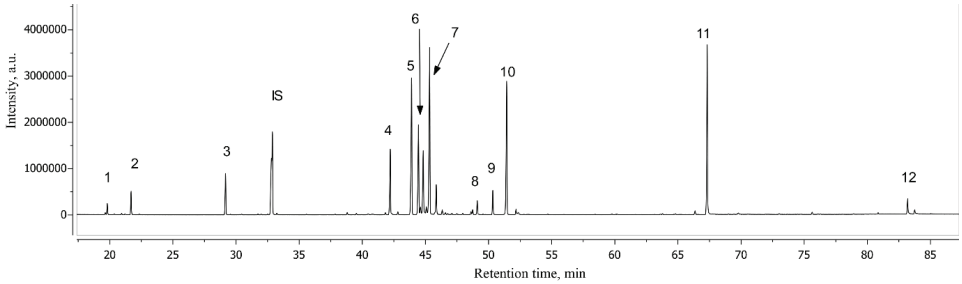

Fig. 1. GC-FID chromatogram of derivatized sample of *Origanum vulgare*. 1 – glycerol, 2 – carvacrol, 3 – malic acid, 4 – citric acid, 5 – quinic acid, 6 – fructose, 7 – glucose, 8 – glucaric acid, 9 – catechollactate, 10 – myo-inositol, 11 – sucrose, 12 – rosmarinic acid and IS – internal standard 2-deoxy-ribose.

Multivariate data analysis

All the 104 samples (8 intact oreganos, 96 samples adulterated with Venetian sumac, myrtle and olive to make binary mixtures in mass ratios 1, 5, 10 and 20) GC–MS metabolomics profiles were subjected to multivariate data analysis. Firstly, PCA as an unsupervised variable reduction technique was performed. On this way, smaller number of novel variables that will account for most of the variation in the observed variables developed. It has resulted in 8 principal components (PCs) model explaining 87.6 % of the total data variance.

Based on PCA score plot of the first two PCs, no grouping was observed according to the adulterations with different plants (Fig. 2a). Interestingly, the

samples were separated in some degree in PC1/PC2 space due to different botanical origin of oregano, regardless of degree of adulteration up to 20 % (Fig. 2b).

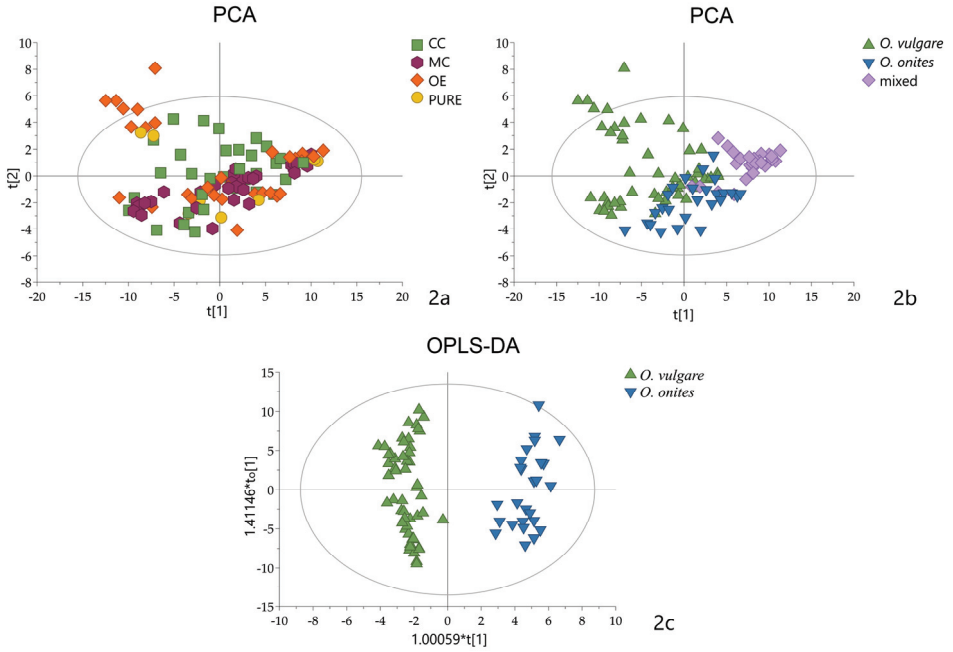

Fig. 2. a) and b) Principal component analysis (PCA) score plot (PC1 *versus* PC2) of all studied samples. The scores are colored according to the (a) adulterants added: OE – olive (*Olea europaea*) leaves, CC – Venetian sumac (*Cotinus coggygria*) leaves, MC – myrtle (*Myrtus communis*) leaves, and PURE – intact oregano samples; b) oregano species: *O. vulgare*, *O. onites*, and mixture of two *O.* species; (**c**) OPLS-DA score plot comprising oregano species *O. vulgare* and *O. onites.*

Next, OPLS-DA as supervised technique was performed. With this technique, novel variables will account for maximum separation between two predefined classes, in this case – *O. vulgare* and *O. onites* as botanical origin of the oregano samples. The samples of mixed origin were set aside. Since in the orthogonal model systematic variation of the variables is divided into two parts: one linearly related to the class information and one orthogonal to it, is the model interpretation is therefore facilitated.[16] Hence, OPLS-DA is suitable for finding variables that have the greatest discriminatory power between two predefined classes.

The quality of the obtained model was assessed by goodness of fit (R^2) indicating how well the variation of variables is explained using the predictive components and predictive ability of the model (Q^2) indicating how well the model predicts new data, as estimated by cross validation. Thus, R^2 and Q^2 values over

0.9 and close to 1 (maximum value) indicated remarkable goodness of fit, and predictive ability (Table I).

The OPLS-DA model was then validated by permutation test and CV-ANOVA. In the permutation test, the R^2 and Q^2 values of the original models were compared with the values of series of models with randomly perturbed class information. The model was considered satisfactory since regressions of Q^2 line intersected the vertical axis at below zero, and all Q^2 and R^2 values of permuted Y vectors were lower than the original ones. Similarly, the model was significant according to CV-ANOVA, with p value less than 0.05 (Table I).

TABLE I. Parameters of the multivariate analysis models

Model name	No. of components	R^2	Q^2	p (CV-ANOVA)	F (CV-ANOVA)
PCA	8	0.876	0.682	–	–
OPLS-DA, oregano species	1+2	0.950	0.926	4×10^{-38}	149
OPLS, adulteration with OE	1+2	0.914	0.830	5×10^{-11}	26
OPLS, adulteration with CC	1+5	0.983	0.910	5×10^{-11}	23
OPLS, adulteration with MC	1+4	0.938	0.753	2×10^{-5}	8

The score plot of the OPLS-DA model indicated good separation between classes along the predictive components (Fig. 2c). The selection of the most influential variables was based on variable influence on projection scores of the predictive components (VIPpred). Variables with the VIPpred score above 1.4 were considered as important for the separation. Thus, catechollactate was found to be discriminating metabolite for *O. onites* samples.

Three OPLS multivariate regression models created corresponding adulteration of oregano with Venetian sumac, myrtle and olive leaves. Five different oregano-to-adulterants mixing ratio levels used in each model. Excellent goodness of fit and predictive ability was obtained according to Q^2 and R^2 values. According to CV-ANOVA, the OPLS models were significant with $p < 0.05$ (Table I). This is also in accordance with the results of the permutation test. According to the score plots of the OPLS models, oregano samples with lower grades of adulteration were clearly separated from those with the higher grades (Fig. 3). The criterion for the selection of the most influential variables in the OPLS models was VIPpred score above 1.4, and loadings scaled as a correlation coefficient above 0.3. Using the above-mentioned criteria sorbitol was found to be the most influential variable in the model of adulteration of oregano with olive leaves. Adulteration of oregano with Venetian sumac resulted in appearance of shikimic and quinic acids as the most influential variable in the model. Similarly, fructose and quinic acid occurred as markers in the model of oregano adulteration with myrtle.

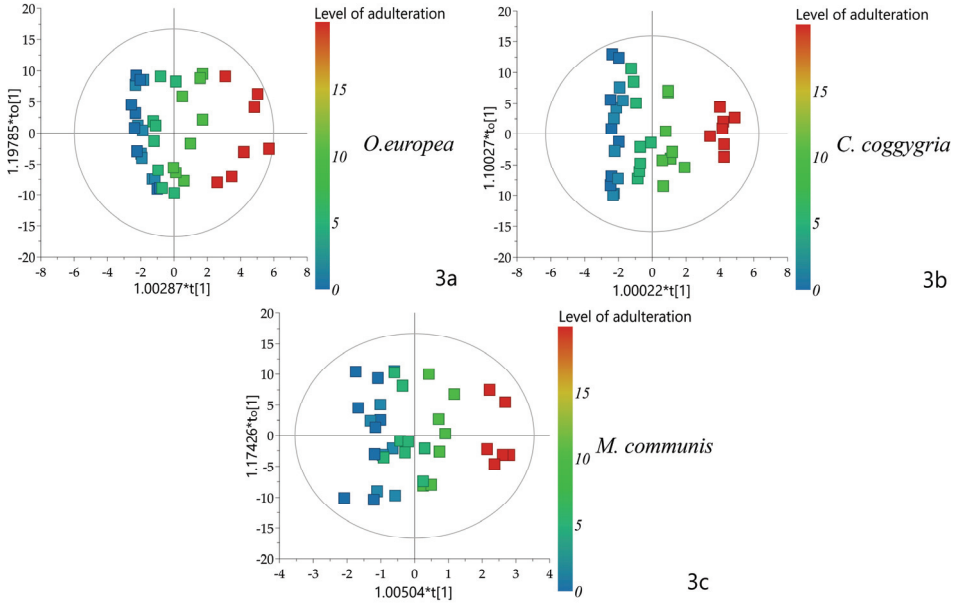

Fig. 3. OPLS score plot comprising oregano adulterated with: a) olive (*Olea europaea*) leaves, b) Venetian sumac (*Cotinus coggygria*) leaves, c) myrtle (*Myrtus communis*) leaves. The scores are colored according to the level of adulteration.

CONCLUSION

The GC–MS-based metabolomics approach has demonstrated to be a very reliable technique for the detection of oregano adulteration. Although the GC–MS technique is primarily intended for nonpolar and volatile samples, the application of the two-step derivatization procedure involving methoxyamination and silanization enabled analysis of more polar metabolites. The utilization of PCA and OPLS multivariate analysis methods on GC–MS profiles, obtained after two-step derivatization of samples, enabled detection of oregano adulterations with olive (*Olea europaea*), Venetian sumac (*Cotinus coggygria*), and myrtle (*Myrtus communis*) leaves.

According to the results, sorbitol levels distinguished oregano samples adulterated with olive leaves, while shikimic and quinic acids were recognized as discrimination factor for adulteration of oregano with Venetian sumac. Fructose and quinic acid levels correlated with oregano adulteration with myrtle.

The proposed workflow confirmed the potential of GC–MS in combination with chemometrics to detect adulteration of food-based products.

SUPPLEMENTARY MATERIAL

Additional data and information are available electronically at the pages of journal website: https://www.shd-pub.org.rs/index.php/JSCS/article/view/11045, or from the corresponding author on request.

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ИЗВОД

МЕТАБОЛОМИКА ЗАСНОВАНА НА ГАСНОЈ ХРОМАТОГРАФИЈИ–МАСЕНОЈ СПЕКТРОМЕТРИЈИ ЗА ДЕТЕКЦИЈУ КРИВОТВОРЕЊА УЗОРАКА ОРИГАНА

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Оригано је једна од најчешће коришћених кулинарских биљака и често се кривотвори јефтинијим биљкама. У овој студији, гасна хроматографија–масена спектрометрија коришћена је за идентификацију и квантификацију метаболита из 104 узорка оригана (*Origanum vulgare* и *O. onites*) кривотвореног маслином (*Olea europea*), венецијанским сумаком (*Cotinus coggygria*) и миртом (*Myrtus communis*), у пет различитих концентрација. Метаболомички профили добијени након двостепене дериватизације, која укључује метоксиаминовање и силанизацију, подвргнути су мултиваријантној анализи података како би се открили маркери кривотворења и направили регресиони модели на основу односа мешања оригана и биљака за кривотворење. Ортогонална делимична анализа најмањих квадрата је омогућила детекцију кривотворења оригана лишћем маслине, венецијанског сумака и мирте. Садржај сорбитола разликовао је узорке оригана кривотворених лишћем маслине, док су шикиминска и кининска киселина препознате као фактор разликовања за кривотворење оригана венецијанским сумаком. Садржај фруктозе и кининске киселине у корелацији су са кривотворењем оригана миртом. Ортогонална делимична анализа најмањих квадрата – дискриминантна анализа је омогућила разликовање узорака *O. vulgare* и *O. onites*, при чему је одређено да је катехоллактат метаболит који разликује ове две биљне врсте.

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REFERENCES

1. P. Galvin-King, S. A. Haughey, C. T. Elliott, *Food Control* **88** (2018) 85 (https://doi.org/10.1016/j.foodcont.2017.12.031)
2. S. Lohumi, S. Lee, H. Lee, B.-K. Cho, *Trends Food Sci. Technol.* **46** (2015) 85 (https://doi.org/10.1016/j.tifs.2015.08.003)
3. C. Black, S. A. Haughey, O. P. Chevallier, P. Galvin-King, C. T. Elliott, *Food Chem.* **210** (2016) 551 (https://doi.org/10.1016/j.foodchem.2016.05.004)
4. L. Drabova, G. Alvarez-Rivera, M. Suchanova, D. Schusterova, J. Pulkrabova, M. Tomaniova, V. Kocourek, O. Chevallier, C. Elliott, J. Hajslova, *Food Chem.* **276** (2019) 726 (https://doi.org/10.1016/j.foodchem.2018.09.143)
5. M. Marieschi, A. Torelli, A. Bianchi, R. Bruni, *Food Control* **22** (2011) 542
6. (https://doi.org/10.1016/j.foodcont.2010.10.001)
7. M. Mandrone, L. Marincich, I. Chiocchio, A. Petroli, D. Gođevac, I. Maresca, F. Poli, *Food Control* **127** (2021) 108141. (https://doi.org/10.1016/j.foodcont.2021.108141)

8. L. E. Rodriguez-Saona, M. E. Allendorf, *Ann. Rev. Food Sci. Technol.* **2** (2011) 467 (https://doi.org/10.1146/annurev-food-022510-133750)
9. M. Bononi, I. Fiordaliso, F. Tateo, *Ital. J. Food Sci.* **22** (2010) 479 (https://www.researchgate.net/publication/288447203_Rapid_GCMS_test_for_identification_of_Olea_Europaea_L_leaves_in_ground_oregano)
10. P. Galvin-King, S. A. Haughey, C. T. Elliott, *Food Control* **88** (2018) 85 (https://doi.org/10.1016/j.foodcont.2017.12.031)
11. European Directorate for the Quality of Medicines & HealthCare, *European Pharmacopoeia*, 9th ed., European Pharmacopoeia, Strasbourg, 2017
12. J. H. Ietswaart, *A Taxonomie Revision of the Genus Origanum (Labiatae)*, Leiden University Press, The Hague, 1982 (https://doi.org/10.1002/fedr.19820930120)
13. J. Lisec, N. Schauer, J. Kopka, L. Willmitzer, A. R. Fernie, *Nat. Protoc.* **1** (2006) 387 (https://doi.org/10.1038/nprot.2006.59)
14. E. Dervishi, G. Zhang, G. Zwierzchowski, R. Mandal, D. S. Wishart, B. N. Ametaj, *J. Proteomics* **213** (2020) 103620 (https://doi.org/10.1016/j.jprot.2019.103620)
15. C. A. Smith, E. J. Want, G. O'Maille, R. Abagyan, G. Siuzdak, *Anal. Chem.* **78** (2006) 779 (https://doi.org/10.1021/ac051437y)
16. R Core Team, *R: A language and environment for statistical computing*, R Foundation for Statistical Computing, Vienna, 2018 (https://www.R-project.org/)
17. S. Wiklund, E. Johansson, L. Sjöström, E. J. Mellerowicz, U. Edlund, J. P. Shockcor, J. Gottfries, T. Moritz, J. Trygg, *Anal. Chem.* **80** (2008) 115 (https://doi.org/10.1021/ac0713510).