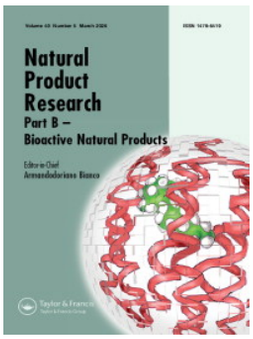

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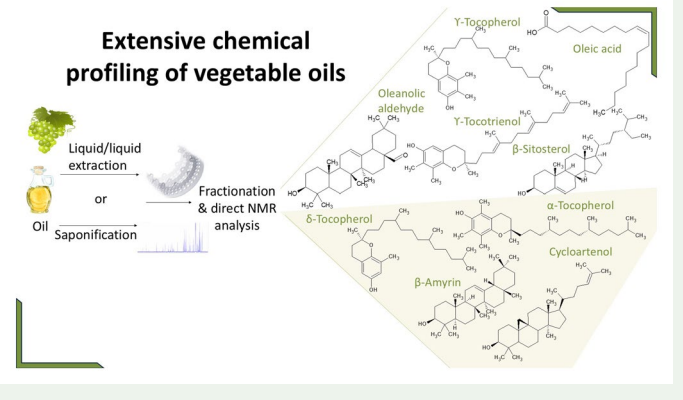
Non-targeted NMR chemical profiling of vegetable oils: a two-path workflow for fast access to a detailed metabolome

Anaïs Cyatwa[a], Joseph Thiery[a], Agathe Martinez[b], Alexis Kotland[a] and Jane Hubert[a]

[a]NatExplore SAS, Prouilly, France; [b]Institut de Chimie Moléculaire de Reims, UMR CNRS 7312, Université Reims-Champagne-Ardenne, UFR Sciences, Reims Cedex 2, France

ABSTRACT

Vegetable oils are increasingly used to formulate bioactive ingredients with pharmaceutical, nutraceutical or cosmeceutical applications. Their biological properties depend directly on their chemical profile, but so far it remains difficult to simultaneously characterise the major long chain lipidic constituents together with the minor but valuable unsaponifiable constituents specific to botanical species. Therefore accurate and comprehensive analytical strategies appear crucial. In this work, a NMR-based dereplication workflow was developed to decipher the metabolome of vegetable oils. The approach involves either a direct liquid-liquid extraction of minor oil constituents or a saponification treatment on the crude starting oil, followed by liquid-liquid fractionation, and direct NMR analysis of all fractions. Oil metabolites are then identified with the help of computer tools and a natural metabolite database. The whole process was applied to a grape seed oil sample, resulting in a rapid and unambiguous identification of 23 metabolites including fatty acid derivatives, triterpenes, sterols, and tocopherols. This NMR-based analytical workflow offers interesting perspectives for the chemical profiling of vegetable oils and oily extracts.



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Vegetables oils; nuclear magnetic resonance; dereplication; grape seed oil; chemical profiling; liquid-liquid extraction; unsaponifiable

CONTACT Jane Hubert jane.hubert75@gmail.com
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1. Introduction

Vegetable oils extracted from seeds, fruits, kernels or nuts play an important role in the human diet due to their nutritional value and health promoting effects. Some obtained with good yields and good stability dominate the world market, for instance palm, soybean, rapeseed, and olive oils. Others, like avocado, almond, argan or grape seed oils are minor in terms of market size but highly interesting regarding their nutritional and sensory characteristics, phytochemical profiles, and biological properties (Tian et al. 2023). Such oils are increasingly used in high value-added sectors including the pharmaceutical, nutraceutical, and cosmetic industries.

From a chemical point of view, vegetable oils represent complex mixtures of compounds from various chemical classes including mono-, di-, and triglycerides, free fatty acids and alcohols, phospholipids, and also a range of polyphenols, sterols, liposoluble vitamins or triterpenes depending on the species. Mono-, di-, and triglycerides together with free fatty acids are by far the major constituents of all vegetable oils. They represent up to 95–99% of the oil biomass and represent the 'saponifiable' fraction. Besides, the unsaponifiable fraction accounts generally for around 2% of the oil biomass and comprises sterols, terpenes, tocopherols, and a range of other specialised metabolites like polyphenols or carotenoids (Chen et al. 2024; Zheng et al. 2024).

The distribution and chemical profiles of both saponifiable and unsaponifiable metabolites govern directly the quality and biological effects of vegetable oils. Their concentration can be strongly affected by environmental factors such as geographical location, climate or harvesting period, as well as by extraction processes, oil treatments, and storage conditions.

Determining the composition of vegetable oils is therefore highly important, not only focusing on the major fatty acid constituents but also on the diversity of minor specialised metabolites that often give to the oil its specificity and added value. In this context, comprehensive and reliable analytical methods are decisive. Various techniques have been explored to chemically profile vegetable oils and more globally oily samples. Near-infrared spectroscopy (NIRS) is rapid, non-destructive, and provides spectral fingerprints of oil composition. It can be useful for the quality control of large sample collections, for instance to assess oxidative level, oil stability, moisture content or fatty acid distribution (Aktas et al. 2023; Srivastava et al. 2024). Gas chromatography or liquid chromatography coupled to mass spectrometry (GC/MS or LC/MS) are also common techniques to analyse oily samples. They enable the detection of individual molecules or chemical signatures that characterise plant species, origin, quality, or even adulteration with a good level of sensitivity. GC/MS is more focused on the separation and detection of volatile constituents while LC/MS enables the analysis of polar and non-volatile constituents of vegetable oils like phospholipids, free and esterified fatty acids, sterols, polyphenols and other minor unsaponifiable compounds (Mota et al. 2021). LC/MS-based lipidomic platforms are today very powerful (Rakusanova and Cajka 2024). Nuclear Magnetic Resonance (NMR) is also an interesting spectroscopic technique for the analysis of vegetable oils. It is non-destructive, rapid, detect all organic molecules without discrimination between chemical classes and does not require prior metabolite separation or derivatization, nor authentic standards for unambiguous structure

elucidation (Siudem et al. 2022). 1D and 2D NMR are commonly used in combination with chemometric tools to assess the nutritional quality of common edible oils or to determine adulterations (Truzzi et al. 2021; Shi et al. 2025).

In this study, a NMR-based dereplication workflow was developed to deeply investigate the chemical composition of vegetable oils and oily extracts, with the aim of getting a detailed metabolome comprising all classes of metabolites, i.e. primary and secondary metabolites, saponifiables and unsaponifiables, phenolic and aliphatic compounds, in a single process. The strategy can be applied in two versions which both combine liquid-liquid fractionation of the starting oil, followed by NMR characterisation of the produced fractions using Hierarchical Clustering Analysis and a predictive NMR database dedicated to natural molecules.

As a case study, grape seed oil was selected to demonstrate the effectiveness of the analytical strategy. Grape seeds represent a valuable source of oil rich in essential fatty acids such as linoleic acid, oleic acid, and linolenic acid, as well as in minor bioactive compounds, such as tocopherols, phytosterols and other specialised metabolites, conferring grape seed oil all its health promoting properties (Di Pietro Fernandes et al. 2023, Milian-Linares et al. 2018).

2. Results and discussion

2.1. First strategy: direct liquid-liquid extraction, centrifugal partition chromatography, NMR dereplication

A first liquid-liquid extraction strategy was applied to separate a maximum of minor specialised constituents of the grape seed oil from the abundant mono-, di-, and triglycerides representing the largest proportion of the oil matrix. For this purpose, a biphasic liquid system was created using an mixture of methanol and water directly in contact with the oil. In this way, a stable biphasic system was obtained and the most polar metabolites of the grape seed oil were extracted in the upper hydroalcoholic phase, with an extraction yield of ≈ 2%. This extract was further fractionated by CPC using a non-polar Arizona solvent system composed of *n*-heptane/ethyl acetate/methanol/water (9/1/9/1, v/v), resulting in six fractions of simplified chemical composition, obtained in 67 min without loss of biomass. CPC conditions are described in details in the experimental section of the Supplementary file. All fractions were then directly analysed by 1D and 2D NMR and a dereplication strategy, based on hierarchical clustering analysis of ^{13}C NMR data, was applied for metabolite identification (Hubert et al. 2014; Kotland et al. 2024). Briefly,^{13}C NMR peak fingerprints detected in adjacent CPC fractions, possibly corresponding to individual molecule skeletons, were aggregated in a heatmap which is presented in Figure 1. The heatmap was made of 6 columns corresponding to the CPC fractions obtained from the grape seed oil and 141 rows corresponding to ^{13}C NMR peaks detected in at least one fraction. The deeper the yellow colour in the map, the higher the intensity of ^{13}C NMR peaks. Each cluster was submitted to our NMR natural product database, and database proposals were then rigorously scrutinised for proton/proton, and proton/carbon correlations from HSQC, HMBC, and COSY spectra of the fraction series. NMR spectra of CPC fractions are illustrated in Figure S1.

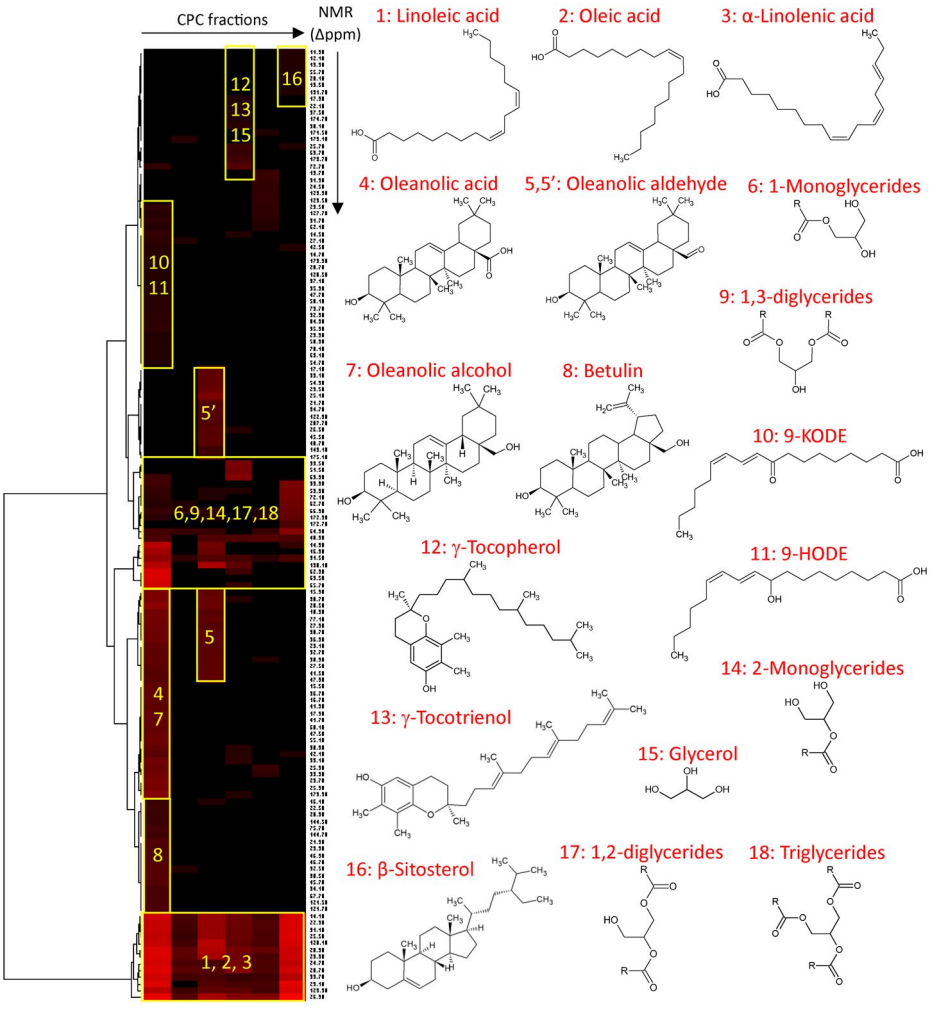

Figure 1. HCA correlation map of ^{13}C NMR signals with the identified compounds. Liquid liquid extraction strategy. R = fatty acid alkyl chain*.

We observed that fatty acids were still by far the major constituents of CPC fractions, despite the preliminary liquid-liquid extraction performed on the crude oil. Clusters 1, 2, and 3 detected in all fractions corresponded to linoleic, oleic, and α-linolenic acids, the main fatty acids of grape seed oil in accordance with literature data (Carmona-Jiménez et al. 2022). Grape seed oil is in fact a rich source of unsaturated fatty acids, mainly linoleic acid which is involved in many biochemical pathways in the human body, such as cell membrane construction or energy supply, and has also a protective role against cardiovascular diseases (Bertoni et al. 2023; Jackson et al. 2024). Fatty acids were identified under their free form or esterified to glycerol as mono-, di-, and triglycerides, as indicated by clusters 6, 9, 14, 17, and 18.

Besides, a range of minor but specialised metabolites were also detected in our grape seed oil sample. Among them, several triterpenes were unambiguously identified, including oleanolic acid (cluster 4), oleanolic alcohol (cluster 7), and betulin

(cluster 8) in the most polar fraction F01, and oleanolic aldehyde (clusters 5+5′) in fraction F03. Triterpenes represent a highly diverse group of plant metabolites with complex polycyclic chemical structures and various biological properties. They are synthesised as defense metabolites to protect against insect pests, diseases, and other external aggressions, and exhibit several important pharmaceutical activities among which a well-recognized anti-inflammatory activity (Sandeep 2020; Mantiniotou et al. 2025). Oleanolic acid and other pentacyclic triterpenes have been reported in grape berries and leaf cuticular waxes (Szakiel et al. 2012; Pensec et al. 2016), but to our knowledge, this is the first time that oleanolic acid, oleanolic alcohol, oleanolic aldehyde, and betulin, are described in the seed oil. Only a few triterpenes like β-amyrin have been previously reported in grape seed oil from different *Vitis vinifera* varieties (Burdziej et al. 2019; Harbeoui et al. 2019).

Two oxylipins including 9-oxo-10,12-octadecadienoic acid (9-KODE) and 9-hydroxy-10,12-octadecadienoic acid (9-HODE), corresponding to clusters 10 and 11, respectively, were also detected in significant amounts in the first CPC fraction F01. Oils with high linoleic acid content are known to produce significant levels of oxilipins, therefore their presence was not surprising (Richardson et al. 2017). Both compounds result from a linoleic acid oxidation, under enzymatic action or under free radical oxidative stress. They contribute to host defense in plants and have been suggested as markers of lipid oxidation in edible oils (Rao and Wu 2025).

Two liposoluble forms of vitamin E including γ-tocopherol and γ-tocotrienol were also identified in fraction F04. Vitamin E is a very effective natural liposoluble antioxidant which protects cytoplasmic membranes from oxidation and low-density lipoproteins from lipid peroxidation (Carmona-Jiménez et al. 2022). And finally β-sitosterol was identified in fraction F06. This metabolite is a bioactive phytosterol naturally present in plant cell membranes with a chemical structure close to mammalian cell cholesterol. It possesses various biological properties such as analgesic, antimicrobial, anti-diabetic, and immunomodulatory, and has a great potential for human health (Babu and Jayaraman 2020). NMR data including ^{13}C and HSQC spectra of the CPC fractions, and ^{1}H and ^{13}C NMR chemical shifts of all identified metabolites are presented in Supplementary Table S1.

2.2. Second strategy: saponification, centrifugal partition chromatography, NMR dereplication

A second version of the workflow starting with a saponification treatment was used as an alternative approach to separate the major fatty acid derivatives from the minor unsaponifiable constituents. Saponification followed by liquid extraction is a well-recognized method to recover unsaponifiable constituents from lipidic raw materials. A saponification process most often starts with an alkali treatment of the crude oil with potassium hydroxide or sodium hydroxide. By this way all fatty acid esters (glycerides) are hydrolysed to form a soap composed of fatty acid salts and free glycerol, in mixture with the unsaponifiable constituents which have not been hydrolysed. By becoming salts and forming a soap, the hydrophilicity of fatty acids is increased, making them more easily separated from the lipophilic unsaponifiable fraction by liquid extraction. In the present work, the grape seed oil was

saponified by an hydroalcoholic solution of KOH. Taking into account the saponification index of grape seed oil, which ranges from 188 to 194 mg KOH/g oil (*Codex Alimentarus*, amended in 2024; Laqui-Estaña et al. 2024), and after optimising KOH concentration, we found that an excess of KOH in a 1/1 (w/w) ratio with the oil in a final reaction volume of 250 mL was the best compromise to ensure efficient saponification of grape seed oil in a short time (20 min) and in a reasonable volume of solvent.

Then a solvent system composed of methyl-*ter*-butyl ether and water was used to recover the unsaponifiable fraction in the upper phase while trapping the soap in the lower phase. Liquid extraction of unsaponifiables is often described in diethyl ether, but we replaced this solvent by methyl-*ter*-butyl ether to avoid any risk related to the use of diethyl ether which is a highly flammable solvent. As a result, an unsaponifiable fraction of 640 mg was obtained, corresponding to 1.3% (w/w) of the grape seed oil biomass.

The unsaponifiable fraction was further fractionated by CPC in the same conditions as described above for the first liquid-liquid strategy, resulting in a total of 9 fractions. The same NMR-based dereplication approach was applied on this fraction series, resulting in the HCA heatmap presented in Figure 2. NMR spectra of CPC fractions are illustrated in Figure S2.

Most metabolites identified in the fraction series obtained after saponification were the same as those identified after the direct liquid-liquid extraction strategy. They include free fatty acids, mainly linoleic acid, oleic acid, and α-linolenic acid, as well as 9-hydroxy-10,12-octadecadienoic acid, 9-Oxo-10,12-octadecadienoic acid, and residual monoglycerides which were not fully hydrolysed during saponification. The sterol β-sitosterol and the triterpenes oleanolic acid, oleanolic alcohol, oleanolic aldehyde, and betulin were also identified. Lupeol and β-Amyrin were additionally identified after saponification. These two triterpenes were not detected using the first strategy, probably because they are much less polar than oleanolic acid derivatives and thus not well separated from fatty acid derivatives during direct liquid-liquid extraction on the crude grape seed oil, a process which was only based on polarity differences.

A range of other additional metabolites were identified after saponification. They include 24-methylenecycloartenol and cycloartenol, two important sterols derived from 2,3(*S*)-oxidosqualene and precursors of almost all sterols in plants, as well as geranylgeraniol and phytol, two structurally related linear diterpenic alcohols occurring in plants and involved in many biological processes (Gutbrod et al. 2019). Such compounds are for instance important intermediates in the synthesis of vitamins E and K.

More tocopherols were also identified after saponification. In addition to γ-tocopherol and γ-tocotrienol, other vitamers including α-tocopherol and α-tocotrienol, as well as δ-tocopherol and δ-tocotrienol, were detected after saponification. These results are in accordance with literature data, as grape seed oil is known to contain significant levels of vitamin E with γ-tocotrienol and α-tocotrienol as the major constituents. As tocopherols exhibit a very low polarity, they were probably retained in the oily phase during liquid-liquid extraction using the first strategy, whereas their separation from glycerides was easier after saponification. NMR data including ^{13}C and HSQC spectra of the CPC fractions, and ^{1}H and ^{13}C NMR chemical shifts of all identified metabolites are also presented in Supplementary Table S1.

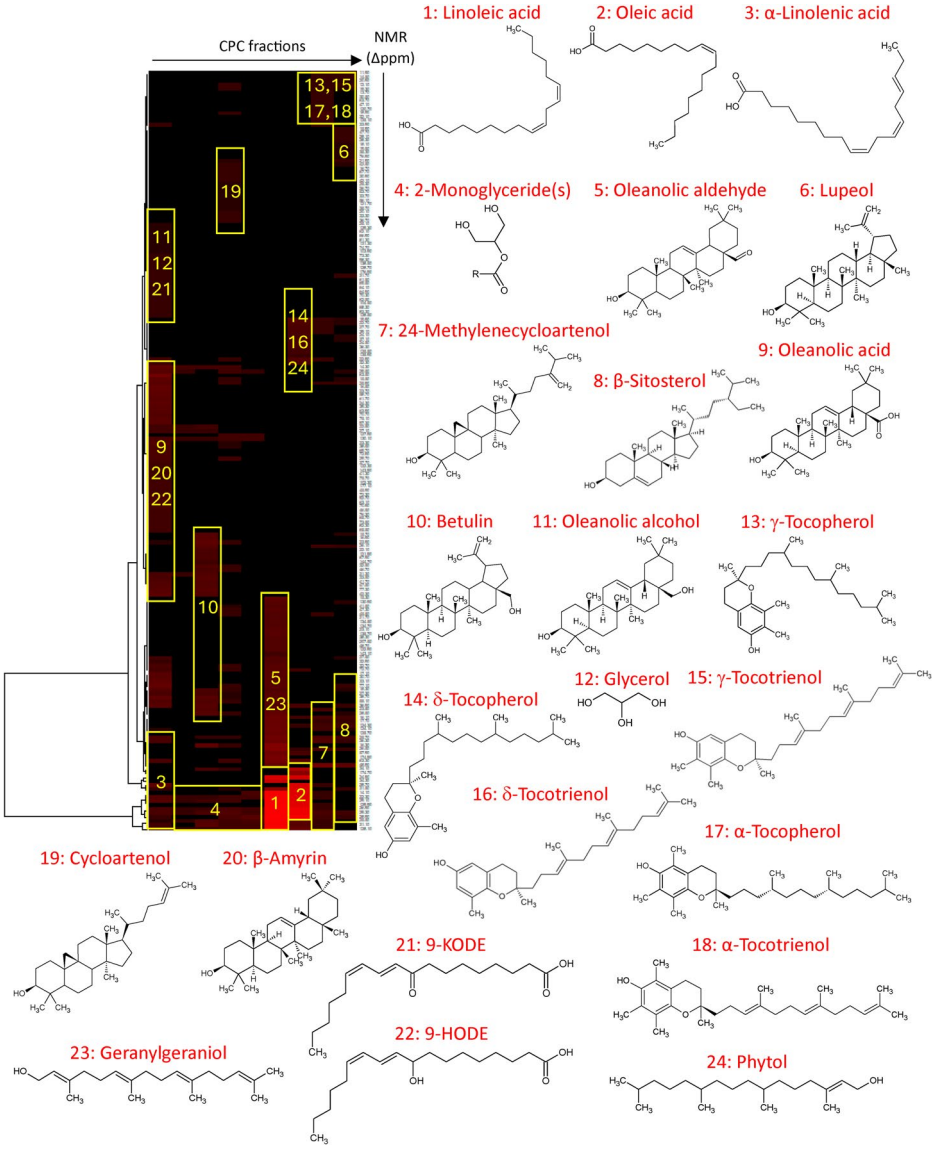

Figure 2. HCA correlation map of ^{13}C NMR signals with the identified compounds. Saponification strategy. R = fatty acid alkyl chain*.

3. Experimental section

See Supplementary Materials.

4. Conclusion

Two alternative NMR-based workflows were developed in parallel for the chemical profiling of vegetable oils. A total of 13 non-glyceride metabolites were accurately identified in a grape seed oil sample using a direct liquid-liquid extraction approach

followed by CPC fractionation and NMR-based dereplication. Direct liquid-liquid extraction enabled to recover minor specialised metabolites from the oil based on polarity differences with the predominant fatty acid glycerol esters. This approach is soft, rapid, and requires low amounts of solvents. The second strategy, introducing a saponification step before CPC fractionation, was much more efficient in terms of metabolite identification, with 23 metabolites accurately identified by NMR, but slightly more fastidious than direct liquid-liquid extraction.

Overall, both alternative can be used separately or in combination to investigate the metabolome of oily samples, such as vegetable oils and oily plant macerates. A detailed chemical profile of grape seed oil was obtained including sterols, triterpenes, and vitamins, without discrimination between chemical classes. Such strategies could be really interesting for the analysis of vegetable oil composition, for the detection of adulterations, for sample classification or for the determination of relevant biomarkers in oily extracts.

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Author contributions

CRediT: **Anaïs Cyatwa**: Conceptualization, Data curation, Methodology, Writing – original draft; **Joseph Thiery**: Data curation, Formal analysis, Methodology; **Agathe Martinez**: Data curation, Formal analysis, Methodology; **Alexis Kotland**: Conceptualization, Data curation, Investigation, Methodology, Project administration; **Jane Hubert**: Conceptualization, Data curation, Investigation, Methodology, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Disclosure statement

No conflict of interest was reported by the authors.

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ORCID

Jane Hubert http://orcid.org/0009-0000-5713-9327

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